PREPARATION OF PURIFIED
HUMAN GROWTH HORMONE AND A CRUDE HUMAN
PITUITARY GONADOTROPHIN

By

Olav Trygstad and Irene Foss

ABSTRACT

The methods described for the preparation of a purified HGH*, a crude HPG, and an adipotrophic fraction are simple, and suitable for the preparation of relatively small batches of pituitary glands. The crude fractions were obtained by alterations in the hydrogen ion concentration of a saline extract of frozen pituitary glands, and a subsequent precipitation with acetone and ammonium sulphate. The crude HGH fraction was purified by Sephadex filtration. The yield of purified HGH was approximately 7 mg per g of fresh pituitary glands. Acrylamide gel electrophoresis demonstrated two bands in the HGH preparation. The bands could be separated by DEAE-cellulose chromatography. The homogeneous HGH preparations, however, had a significantly reduced growth-promoting activity.

The crude HPG was also obtained in a yield of approximately 7 mg per g of pituitary glands, and was used clinically without further purification.

The HGH and HPG preparations have been used therapeutically with success for seven years. The hormones are easily soluble, and well tolerated. No lack of response to HGH because of antibody production has been observed in more than 30 treated hyposomatotrophic children. The purified HGH is suitable for radioimmunoassay. Furthermore an adipotrophic-diabetogenic fractions can be obtained. Modifications of the preparation procedure are discussed.

* Abbreviations: IU = international units
HGH = human growth hormone
HPG = human pituitary gonadotrophins
LH = luteinizing hormone
FSH = follicle-stimulating hormone
STH = somatotrophic hormone.
The human pituitary growth hormone has been isolated by several procedures for more than ten years. A chemically homogeneous preparation with retained biological activity, however, seems hard to obtain. The different procedures have provided HGH preparations with variations both in the physicochemical characteristics and in the biological somatotrophic, prolactin or lipid-mobilizing (diabetogenic) activities. The growth hormone is regarded as a pituitary agent possessing growth-promoting as well as lipolytic and hyperglycaemic activities. It has still not been possible to prepare the somatotrophin with growth-promoting activity only.

For the preparation of HGH at this institute the procedure of Roos et al. (1963) was preferred, since it seemed physiological to use frozen pituitary glands, and aqueous extraction at near neutral pH. Previously we observed that this growth hormone could be separated into a somatotrophic and an adipotrophic-diabetogenic component (Trygstad & Foss 1968). Electrophoresis demonstrated a purified growth hormone with increased growth-promoting activity on a weight basis. This preparation has been used clinically with success for seven years; lack of response because of antibody production has so far not been observed. The somatotrophic component was better tolerated by the patients than the original growth hormone, probably because of its reduced adipokinetic potency. The preparation procedure of this component is simple. Nevertheless it provides a highly purified HGH, a good yield of HPG, and the possibility of isolating a human pituitary lipid-mobilizing (diabetogenic) factor (Trystad 1968), and other metabolically active pituitary fractions (Lindemann et al. 1969).

MATERIALS AND METHODS

Human pituitary glands removed at autopsy were collected in a beaker, and kept frozen below -18°C until the extraction.

Sephadex gel filtration was performed on columns (5.6 X 150 cm) of Sephadex G-100 (Pharmacia, Uppsala) equilibrated with 0.1 M potassium phosphate buffer pH 7.0 (i.e. 5.3 g KH₂PO₄ and 10.6 g K₂HPO₄ per litre of buffer) containing 0.3 M sodium chloride (i.e. 17.5 g per litre). The column was run with a flow rate of 40-50 ml per hour, and the effluent was collected in fractions of 20 ml.

The determination of molecular weight was performed on a Sephadex G-50 gel column (1.6 X 110 cm) according to the method described previously (Trygstad 1968; Trygstad & Foss 1968).

DEAE-cellulose chromatography (cellulose N,N-diethyl-aminoethyl ether, Eastman, Rochester) was performed on a column (1.2 X 25 cm) equilibrated with 0.01 M phosphate buffer pH 5.4. The flow rate was 20 ml per hour, and the eluate was collected in fractions of 5 ml. The starting buffer was replaced by a 0.067 M phosphate buffer pH 5.4, and a 0.667 M phosphate buffer pH 5.4 in succession when the absorbancy of the eluate disappeared at 280 nm.
The protein content was determined spectrophotometrically at 280 nm assuming that an absorbancy of 1 in 1 cm quartz cells corresponds to 1 mg per ml solution.

Somatotrophic activity was evaluated by the tibia test (Greenspan et al. 1949) and was compared with the U. S. P. standard of bovine growth hormone (1 mg = 1 IU). Hypophysectomized female albino Wistar rats weighing about 80 g were obtained from one supplier.

The gonadotrophic effect was tested by an ovarian assay in infantile albino Wistar rats weighing about 40 g (Albert 1956). The «Second International Reference Preparation for Human Menopausal Gonadotrophins» (kindly provided by the National Institute for Medical Research, Mill Hill, London) was used as standard (1 mg = 40 IU FSH and 40 IU LH).

Polyacrylamide gel disc electrophoresis was run according to the method of Ornstein (1964) and Davis (1964).

**PREPARATION PROCEDURE**

All steps were performed at 0–4°C, the centrifugation was done in a Lourde «Beta-Fuge» at 16,000 × g for 10 minutes. Usually a batch of 200 g of frozen human pituitary glands (i.e. ca. 300 glands) was used for each preparation, Fig. 1. The glands were homogenized in a Turmix blender for two minutes, and extracted in distilled water (3 ml/g) for two hours. The residue obtained by centrifugation was re-extracted for two hours in the same volume of 0.1 M phosphate buffer pH 8.5, and the residue washed in the Turmix blender with the same buffer (1 ml/g). The combined supernatant 1 was filtered through glass-wool to remove lipids. Residue 1 was kept for further extraction at pH 3.

To supernatant 1 was added 1 N hydrochloric acid to pH 4.8, the precipitate was denominated 2 a; to supernatant 2 a cold acetone up to 55% concentration was added under continuous stirring, and centrifuged. These initial steps have previously been given in detail (Trygstad 1968). Supernatant 2 b was kept for the preparation of the lipid-mobilizing factor. The combined precipitates 2 a and 2 b were extracted overnight with 0.05 M phosphate buffer at pH 7.0, 1000 ml per batch of 200 g. After centrifugation the residue was re-extracted for one hour in half the original volume. Residue 3 was combined with residue 1 for further extraction.

An equal volume of saturated ammonium sulphate (AS) was added dropwise to supernatant 3 with continuous stirring, which was continued for another hour. The final concentration of AS was 2.7 M. The precipitate 4 produced is a crude HGH (ca. 15 g per 200 g pituitary glands). Solid AS was stirred into the supernatant 4 until 75% saturation (i.e. 4 M). The stirring, after the AS was dissolved, was continued for one hour. Supernatant 5 was discarded.

Precipitate 5 is crude HPG. It was dissolved in 500 ml of distilled water, and dialyzed against water. The residue after centrifugation was discarded. The supernatant contains HPG suitable for clinical use. It was filled into ampoules, and lyophilized. The yield of HPG from 200 g pituitaries was approximately 1.5 g.

The combined residues 1 and 3 were homogenized in the Turmix blender in 0.1 M glycine buffer at pH 3.0 for 90 seconds, and extracted overnight in the same buffer, 800 ml per batch of 200 g pituitaries. The residue formed by centrifugation was washed in 200 ml of the glycine buffer. To the combined supernatant 3 b was added, drop by drop, an equal volume of saturated AS, and this was treated as was super-
Fig. 1.

Summary of the extraction procedure of human pituitary glands.

Frozen human pituitary glands (200 g) homogenized, extracted in distilled water, and re-extracted in 0.1 M phosphate buffer pH 8.5.

Centrifuged

Residue 1 ———————————— Supernatant 1
pH adjusted to 4.8
Centrifuged

Precipitate 2a ———————————— Supernatant 2a
acetone added to
55% concentration
Centrifuged

Precipitate 2b ———————————— Supernatant 2b
Combined precipitates 2a + 2b
for preparation of the
lipid-mobilizing factor
extracted in 0.05 M phosphate
buffer pH 7.0

Centrifuged

Residue 3 ———————————— Supernatant 3
Combined residues 1 and 3 are re-homogenized
and extracted in 0.1 M glycine buffer pH 3.0
Centrifuged

Residue 3b ———————————— Supernatant 3b
discarded
an equal volume of saturated
ammonium sulphate solution added
Centrifuged

Precipitate 4b ———————————— Supernatant 4b
crude HGH (ca. 8 g)
Centrifuged

Supernatant 4b
an equal volume of saturated
ammonium sulphate solution added
discarded

Precipitate 4 ———————————— Supernatant 4
crude HGH (ca. 15 g)
ammonium sulphate added
to 75% saturation
Centrifuged

Precipitate 5 ———————————— Supernatant 5
crude HPG (ca. 1.5 g) discarded
natant 3. Precipitate 4 b is crude HGH, ca. 8 g per 200 g pituitaries. Supernatant 4 b was discarded.

In order to ensure that the HGH present in the original batch was well extracted, the residue 3 b was re-extracted at pH 11.0, and the supernatant formed by centrifugation was treated in the same manner as supernatant 3.

Precipitates 4 and 4 b were separately dissolved in distilled water, dialyzed against water, and lyophilized. For Sephadex gel filtration these precipitates were dissolved in 100 ml and 50 ml, respectively, of the buffer used. After centrifugation, the lyophilizing flasks and the residues were washed twice in half the volumes of the buffer. The residues, ca. 5 g from precipitate 4 and 2 g from precipitate 4 b, were discarded. The total volumes to be applied on the Sephadex G-100 gel columns were about 180 ml with an extract from precipitate 4, and about 90 ml from precipitate 4 b.

Typical Sephadex gel filtrations are given in Figs. 2 and 3. The somatotrophic activity tested by the tibia test was located in the last protein peak (Table 1). The elution volume corresponding to these peaks was dialyzed against distilled water, filled into ampoules and lyophilized. The yield of purified somatotrophic hormone was in the range of 900–1200 mg from precipitate 4, and 200–400 mg from precipitate 4 b. The extraction at pH 11 of residue 3 b gave 50 mg of protein with somatotrophic activity. Physicochemically the HGH-preparations from precipitates 4 and 4 b seemed to be identical, as they had the same pattern on Sephadex gel filtration, and a similar picture on polycrylamide gel disc electrophoresis (Fig. 4). The HGH extracted at pH 11 had one additional band on disc electrophoresis.

The elution pattern for the DEAE-cellulose chromatography of 25 mg purified HGH is shown in Fig. 5.

![Graph](https://via.placeholder.com/150)

**Fig. 2.**

Sephadex G-100 gel filtration (column 5.6 \times 150 cm) of precipitate 4 (crude human growth hormone, Fig. 1) in 0.1 M phosphate buffer pH 7.0 containing 0.3 M sodium chloride.
Polyacrylamide gel electrophoresis of the different peaks demonstrated one band in fractions 11 through 27 in the starting buffer (the slow moving component of HGH), and in fractions 11 through 18 in the 0.667 M phosphate buffer (the fast moving component, Fig. 4). The first peak with the least acidic components had four slow moving bands. The protein eluted in the second buffer had the typical electrophoretical pattern of the starting material, and tubes 3 through 10 with the last buffer had an additional fast moving band. The yields of the slow and fast moving components were approximately 12 mg and 3 mg. The molecular weights of the homogeneous components determined by Sephadex G-50 gel filtration were in the same range as for the original HGH preparation.

**BIOLOGICAL ACTIVITIES**

*Somatotrophic activity* (Table 1)

The HGH prepared from precipitate 4 and precipitate 4b has consistently been found to be more potent than the USP standard. The growth-promoting activity was located in the last protein peak eluted from the Sephadex column, with an equivocal somatotrophic effect in the previous peak. The HGH extracted at near neutral pH had invariably a higher somatotrophic potency than that extracted at pH 3. The somatotrophic component obtained by extraction at pH 11 had a good growth-promoting effect as evaluated by the tibia test.
Polyacrylamide gel disc electrophoresis (column 0.7 × 8 cm) of approximately 0.5 mg human growth hormone (HGH) extracted at pH 7, pH 3, and pH 11, and of purified HGH separated by DEAE-cellulose chromatography into a slow and fast moving component (HGH 1 and HGH 2, respectively, cf. Fig. 5), run anodally in glycine-tris buffer at pH 8.7 for 2 hours at 4 mA.

Except for the protein in the second buffer (Fig. 5) all the fractions eluted from the DEAE-cellulose column had a reduced somatotrophic activity as compared with the original material. The observation was confirmed by a radioimmunological HGH assay*, which demonstrated an activity of the homogeneous components of one third of that of the original HGH on a weight basis.

Gonadotrophic activity (Fig. 6)

Evaluated by the rat ovary test the HPG activity of 100 µg of precipitate 5

* Dr. N. Norman, Hormone and Isotope Laboratory, Aker Hospital, Oslo, has most kindly done the radioimmunological determinations of HGH and LH.
DEAE-cellulose chromatography of 25 mg HGH

DEAE-cellulose chromatography (column 1.2 × 25 cm) of 25 mg purified human growth hormone (HGH) which yielded a homogeneous slow (HGH 1) and fast (HGH 2) moving component, cf. Fig. 4.

Dose-effect curves assayed by the rat ovary test for crude human pituitary gonadotrophin (HPG – precipitate 5, Fig. 1, solid line), and for a standard preparation of human menopausal gonadotrophin (H. M. G., broken line). The marks indicate the mean ovarian weight of 10 animals and the vertical bars ± 1 standard error of mean.
Table 1.
Somatotrophic activity of different HGH fractions tested biologically by the rat tibia test with a U.S.P. standard of bovine growth hormone, and radioimmunologically with a purified HGH preparation as standard (kindly performed by Dr. N. Norman, Hormone and Isotope Laboratory, Aker Hospital, Oslo).

<table>
<thead>
<tr>
<th>Material and dose (µg)</th>
<th>Number of rats</th>
<th>Mean tibial width (µm) ± se*</th>
<th>Radioimmunological activity (ng/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Physiological saline</td>
<td>10</td>
<td>163 ± 4</td>
<td></td>
</tr>
<tr>
<td>U.S.P. standard</td>
<td>20</td>
<td>5</td>
<td>209 ± 9</td>
</tr>
<tr>
<td></td>
<td>40</td>
<td>5</td>
<td>229 ± 11</td>
</tr>
<tr>
<td></td>
<td>80</td>
<td>5</td>
<td>233 ± 2</td>
</tr>
<tr>
<td>HGH prepared from</td>
<td>10</td>
<td>9</td>
<td>225 ± 3</td>
</tr>
<tr>
<td>precipitate 4</td>
<td>20</td>
<td>9</td>
<td>251 ± 3</td>
</tr>
<tr>
<td></td>
<td>40</td>
<td>5</td>
<td>287 ± 6</td>
</tr>
<tr>
<td></td>
<td>80</td>
<td>5</td>
<td>317 ± 8</td>
</tr>
<tr>
<td>Fig. 2 tube 52</td>
<td>40</td>
<td>10</td>
<td>195 ± 10</td>
</tr>
<tr>
<td></td>
<td>40</td>
<td>10</td>
<td>172 ± 9</td>
</tr>
<tr>
<td></td>
<td>20</td>
<td>14</td>
<td>285 ± 6</td>
</tr>
<tr>
<td></td>
<td>40</td>
<td>10</td>
<td>368 ± 8</td>
</tr>
<tr>
<td></td>
<td>80</td>
<td>5</td>
<td>267 ± 5</td>
</tr>
<tr>
<td>HGH prepared from</td>
<td>20</td>
<td>8</td>
<td>204 ± 2</td>
</tr>
<tr>
<td>precipitate 4b</td>
<td>40</td>
<td>15</td>
<td>246 ± 3</td>
</tr>
<tr>
<td></td>
<td>80</td>
<td>8</td>
<td>267 ± 3</td>
</tr>
<tr>
<td>Fig. 3 tube 48</td>
<td>40</td>
<td>10</td>
<td>195 ± 10</td>
</tr>
<tr>
<td></td>
<td>40</td>
<td>10</td>
<td>201 ± 7</td>
</tr>
<tr>
<td></td>
<td>20</td>
<td>10</td>
<td>254 ± 3</td>
</tr>
<tr>
<td></td>
<td>40</td>
<td>10</td>
<td>301 ± 5</td>
</tr>
<tr>
<td></td>
<td>20</td>
<td>10</td>
<td>258 ± 2</td>
</tr>
<tr>
<td></td>
<td>40</td>
<td>10</td>
<td>293 ± 9</td>
</tr>
<tr>
<td>HGH prepared at pH 11</td>
<td>20</td>
<td>10</td>
<td>278 ± 3</td>
</tr>
<tr>
<td></td>
<td>40</td>
<td>10</td>
<td>318 ± 6</td>
</tr>
<tr>
<td>HGH fractionated on</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DEAE-cellulose. Fig. 5</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Buffer 0.01 M</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>tubes 4-8</td>
<td>40</td>
<td>5</td>
<td>173 ± 15</td>
</tr>
<tr>
<td></td>
<td>40</td>
<td>5</td>
<td>198 ± 8</td>
</tr>
<tr>
<td>Buffer 0.067 M</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>tubes 3-9</td>
<td>40</td>
<td>5</td>
<td>298 ± 9</td>
</tr>
<tr>
<td></td>
<td>40</td>
<td>5</td>
<td>218 ± 6</td>
</tr>
</tbody>
</table>

* = standard error of the mean.
was more potent than 10 IU of the HMG standard preparation. The radio-
immunological LH-activity in the protein peak corresponding to tubes 52–62
on Sephadex gel filtration of precipitate 4 (Fig. 2) was observed to be up to
980 IU per mg protein. There was no LH activity in the corresponding peak
obtained from precipitate 4 b.

**DISCUSSION**

A method for the preparation of a purified HGH with preservation of crude
HPG and an adipotrophic fraction is described. The fractions were obtained
by alterations in the pH of a saline pituitary extract, and subsequent addition
of acetone or ammonium sulphate. The somatotrophic fraction was purified by
Sephadex gel filtration. The procedure has been used in this institute for
seven years with very reproducible results. This method is simple, and con-
venient for a hospital intending to prepare HGH and HPG for clinical use
from relatively small batches (100–400 g) of frozen human pituitary glands.

The original method described by Roos et al. (1963) has been completely
modified, only the precipitation with AS and the Sephadex gel filtration has
been kept. The initial extraction in distilled water gave an increased yield of
HPG, possibly because of rupture of the hormone producing cells by osmosis
or hydrolysis of macromolecules by pituitary proteo-lytic enzymes. Further ex-
traction in phosphate buffer at pH 8.5 is important for obtaining the lipid-
mobilizing fraction, which also made the introduction of acetone precipitation
necessary.

In order to get a good precipitate an adequate concentration of inorganic
salts in the first extract was essential. Furthermore the acetone precipitation
removed impurities from the HPG preparation by denaturation of haemo-
globin. This denaturing effect of acetone made it important to centrifuge off
precipitate 2 a before the addition of acetone; if not, the somatotrophic hor-
mone would have been denatured with reduced yield of growth-promoting
activity. The acidification of supernatant 1 to pH 4.8 was observed to pre-
cipitate 90 % of the growth-promoting activity, and only 20 % of the HPG
activity. The addition of acetone to 55 % concentration precipitated all the
somatotrophic and practically all the HPG activity from supernatant 2 b. In
the method described above precipitates 2 a and 2 b were combined in order
to get the best yield of growth-promoting and gonadotrophic activities for
clinical use. The precipitation with AS can be omitted. HGH and HPG can
be prepared directly from precipitate 2 a and 2 b, respectively. However, the
extraction of precipitate 2 a gave a supernatant that is difficult to filter
through the Sephadex gel column because of plugging with marked decrease
of flow rate. In this case filtration on a column of Sephadex G-75 gel gave a
better flow rate than on Sephadex G-100. The FSH:LH ratio is higher for precipitate 5 than for precipitate 2 b, and the latter should be used for the preparation of LH. However, the addition of AS to 30 % saturation in supernatant 3 for the precipitation of HGH increases the LH content in precipitate 5. Sephadex gel filtration of the crude HPG precipitates provides a more purified HPG preparation by the removal of not retarded material and small molecular fractions. Ion-exchange chromatography should be employed for the preparation of a purified LH or FSH. The crude HPG fraction (precipitate 5) has been used clinically with success for more than seven years, and a daily dose of approximately 2.5 mg is usually recommended.

For Sephadex gel filtration the crude HGH precipitates (4 and 4 b) can be extracted in 0.1–0.2 M ammonium bicarbonate solution with the same solution as the running buffer. The separation of the HGH peak is satisfactory, but less distinct than with the buffer used. However, the ammonium bicarbonate solution will inhibit the growth of bacteria on the column, and the fractions can be lyophilized directly without the time-consuming dialyzing procedure.

For therapeutic use all the fractions in the somatotrophic peak have been combined. The administration of 4 mg once a week for patients weighing less than 25 kg and twice a week for the heavier patients has been found to be sufficient. For research purposes (immunization, radioiodination, standard preparations, and metabolic studies) only the central fractions of the HGH peak from precipitate 4 have been used. Initially the fractions were combined, dialyzed, lyophilized, and refiltered on a Sephadex G-100 gel column. One single peak always appeared without any fast moving material suggestive of aggregation or slow moving polypeptides indicating proteolysis of the HGH; refiltration was therefore omitted. We have observed no change in the electrophoretical pattern following repeated lyophilizations of the growth hormone fractions.

Extraction of residue 3 b at pH 11 gave a small yield of HGH activity, this fraction has not been used clinically because of the danger of denaturation of the protein molecule (cf. Fig. 4). Except for an equivocal FSH activity in supernatant 4 b, no somatotrophic, adipotrophic, or gonadotrophic activity was demonstrated in the discarded supernatants 4 b and 5. In addition to the homogenization of the pituitary glands in the Turmix blender, further homogenization with ultrasound has been tried to some extent, but so far with decreased yield of somatotrophic as well as of gonadotrophic activity.

Compared with the method of Roos et al. (1963) the method used by us gave a purer and better tolerated somatotrophic hormone, which is more easily soluble in water, and a HPG fraction with increased potency on a weight basis. The additional extraction at pH 3 gave a further yield of growth-promoting activity. So far the residues 4 and 4 b have been treated separately because of the risk of denaturation of the somatotrophic hormone.
at a low pH, and hence the production of antibodies on repeated injections. In Norway these HGH preparations have been administered up to seven years to more than 30 hyposomatotrophic children, and with successful growth responses (Trygstad 1969).

Ultracentrifugation studies (Foss et al. 1967) gave a molecular weight in the range of 18,900 for the somatotrophic hormone, 10% less than that given by Li et al. (1966) in accordance with the amino acid sequence. Studied by ultracentrifugation and Sephadex gel filtration the HGH prepared was homogeneous, although it had two bands on polyacrylamide gel disc electrophoresis (Fig. 4). The electrophoretically homogeneous preparation obtained by DEAE-cellulose chromatography had a significantly reduced somatotrophic potency and no fraction had an increased activity as compared with the original preparation. Leaver (1966) made the same observation, but got a restored biological activity after reunion of the fractions. Peckham (1967), however, separated the two HGH bands by DEAE-sephadex chromatography, and observed no loss of somatotrophic activity as evaluated by the tibia test; he too observed the same molecular weights of the homogeneous fractions as for the original HGH.

Starch gel electrophoresis (own observations) confirmed the heterogeneity of our HGH, and also that it was as pure as a purified HGH preparation intended for radioimmunoassay (kindly supported by the National Institute of Health, Bethesda, NIH-GH, H5 1216C).

The advantages of the described preparation procedure are (1) a better tolerated HGH preparation up to the present without any therapeutic lack of response because of antibody production, (2) a physicochemically and metabolically more homogeneous growth hormone, suitable both for radioimmunoassay and for metabolic studies, and (3) a good yield of HPG for clinical purposes, and an adipotrophic-diabetogenic fraction for further investigation.

The loss of biological and radioimmunological HGH activity in the electrophoretically homogeneous preparations obtained by DEAE-cellulose chromatography is of interest, and needs further study.

REFERENCES


Received on August 4th, 1970.