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PREPARATION OF HUMAN GROWTH HORMONE BY GEL FILTRATION

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SUMMARY

(1) A simple method for preparation of human pituitary growth hormone has been described. It involves extraction of homogenized glands, ammonium sulfate fractionation and gel filtration on Sephadex G-100. All procedures were performed in solutions of near neutral pH.

(2) The active product yielded a single peak when refiltrated in columns packed with Sephadex G-100, G-75 and G-50, respectively, and a single sedimentation boundary in the ultracentrifuge.

(3) The yield of human pituitary growth hormone was about 5 mg/g frozen whole pituitaries and the material contained negligible amounts of follicle-stimulating hormone, luteinizing hormone, adrenocorticotropic hormone and thyrotropic hormone.

(4) Subsequent zone electrophoresis in agarose suspensions revealed the presence in the active material of more than one molecular species with growth-stimulating activity. No significant difference in specific activity in these components has been established.

INTRODUCTION

Purified HGH has been prepared by several different procedures[1-6]. We wish to report a very easy and rapid procedure for the preparation of HGH in which the material is subjected only to aqueous solutions of near neutral pH.

MATERIALS AND METHODS

Extraction and ammonium sulfate fractionation

A crude HGH-containing concentrate was prepared according to a procedure developed by one of us (P.R.) in connection with the purification of human FSH. All steps were performed at 0–4°.

Frozen human pituitary glands collected at autopsy were homogenized for 2 min in 0.03 M sodium phosphate buffer (pH 6.2) by means of a Turmix blender. 5 ml buffer

Abbreviations: HGH, human pituitary growth hormone; FSH, follicle-stimulating hormone; LH, luteinizing hormone; TSH, thyroid-stimulating hormone; ACTH, adrenocorticotropic hormone.

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were used per gram of tissue. After 1 h of continuous stirring the homogenate was centrifuged for 10 min at 16000 × *g* in a MSE "High Speed 17" refrigerated centrifuge, and the supernatant decanted. The residue was reextracted in 1/4 of the original volume and centrifuged. The supernatants were combined. The residue was further washed two times with about 1/8 of the original volume of buffer and the washings added to the combined extract. The pH was adjusted to 7.0 with 0.1 N NaOH. The total volume was measured and an equal volume of a solution of saturated ammonium sulfate prepared at 4° was slowly added under continuous stirring. Neglecting the small contraction in volume, this solution is considered to be 50% saturated in ammonium sulfate. The stirring was continued for 1 h after the addition was finished, and the precipitate was collected by centrifugation as before, dialyzed against distilled water, and lyophilized. Normally about 300 glands (about 200 g) were used in each preparation.

Gel filtration

The freeze-dried material was extracted three times with a 1:5 dilution of 0.1 M potassium phosphate buffer (pH 6.6), containing 0.5 M NaCl. In each extraction 5 ml of diluted buffer were used per gram of protein. The combined extracts were concentrated by ultrafiltration[7] and applied to a column packed with Sephadex G-100 equilibrated with the undiluted buffer. The column was run at 4°, and the absorbancy of eluant fractions at 280 mμ was determined by use of a Zeiss model PMQ-II spectrophotometer. The material showing high growth hormone activity was rechromatographed under identical conditions to assure its chromatographic homogeneity.

The eluant was concentrated, divided in half, and filtered through columns of Sephadex G-75 and G-50 which had been prepared under conditions described for the original G-100 filtration.

Ultracentrifugation

The active fraction from the original filtration on Sephadex G-100 was also investigated for sedimentation homogeneity in a Spinco model-E ultracentrifuge. The sample was concentrated by ultrafiltration and dialyzed against a sodium phosphate buffer (pH 7.0, *I* 0.02) containing 0.10 M sodium chloride. The centrifuge was operated at 59780 rev./min and 20.0°.

Zone electrophoresis

In order to study the electrophoretic homogeneity of the HGH preparation the refiltered material from the different gel columns was submitted to zone electrophoresis in columns with a 0.2% agarose suspension as supporting medium[8]. The type of column was that introduced by PORATH AND HJERTÉN[9]. The columns were graded in cm and emptied from the top by sucking up the suspension (1 cm at a time) by means of a small plastic tubing[10]. The runs were performed in veronal buffer (pH 8.6, *I* 0.02) and prior to electrophoresis the concentrated eluates were dialyzed against this buffer diluted with distilled water (2:1, v/v). The protein distribution obtained was determined by the LOWRY modificiaton of the Folin reaction[11], and on this basis the fractions were pooled into larger units from which the agarose was nearly quantitatively removed by centrifugation[9].

Biological assay

Growth hormone assays were performed according to the method of GREENSPAN *et al.*[12], based on the increment of the width of uncalcified tibial cartilage in hypophysectomized rats. All doses injected were founded on absorbancy measurements, assuming that an absorbancy of 1.0 in 1-cm quartz cells at 280 mμ corresponds to 1 mg protein per ml solution.

RESULTS

An average yield of the crude HGH concentrate was 33 mg per gram frozen pituitaries. (All yields and also the weights of the different samples given in the legends to the figures are based on absorbancy measurements and on the same assumptions as mentioned under *Biological assay*.)

Figs. 1–4 show the results of the various gel filtrations. It is apparent that the purified active material obtained on Sephadex G-100 filtration gives a single peak on subsequent G-100, G-75 and G-50 filtrations. The material is retarded when filtered through the columns with Sephadex G-100 and G-75 but is unretarded in the column packed with Sephadex G-50.

The material recovered from peak III of the original gel filtration (Fig. 1) accounted for 25–30 % of the protein applied to the column with a total recovery of between 90 and 95 %. The sedimentation pattern of peak-III material in the ultracentrifuge is shown in Fig. 5. The pattern shows only one boundary with a corrected sedimentation constant ($s_{20,w}$) of 2.6 S.

Agarose electrophoresis demonstrated the presence of three major and several minor components in the material obtained from the gel filtration. A typical pattern is illustrated in Fig. 6. These components all appeared to have similar biological activity. Table I contains the biological activity values of material at various stages of the purification.

TABLE I

ASSAYS OF HGH FRACTIONS OBTAINED IN THE PURIFICATION PROCEDURE FROM HUMAN PITUITARIES

Material	*Total dose (μg)*	*Tibial width (μ)*	± *S.E.*	*Number of rats*
50% $(NH_4)_2SO_4$ ppt.	20	216	10	4
1st gel filtration*	20	255	14	4
3rd gel filtration**	20	257	8	4
3rd gel filtration***	20	248	8	4
Zone electrophoresis				
Peak I	10	243	15	6
Peak II	10	209	12	3
Peak III	10	246	7	4
Peak IV	10	237	10	3
Blank	—	174	4	11

* On Sephadex G-100.
** On Sephadex G-50 following two G-100 filtrations.
*** On Sephadex G-75 following two G-100 filtrations.

The active growth hormone material from the gel filtration contained negligible amounts of FSH, LH, ACTH, and TSH activities as determined by the assays of ovarian weight augmentation of STEELMAN AND POHLEY[13], the haemagglutination inhibition test of WIDE *et al.*[14], the production *in vitro* of adrenal corticoid test of

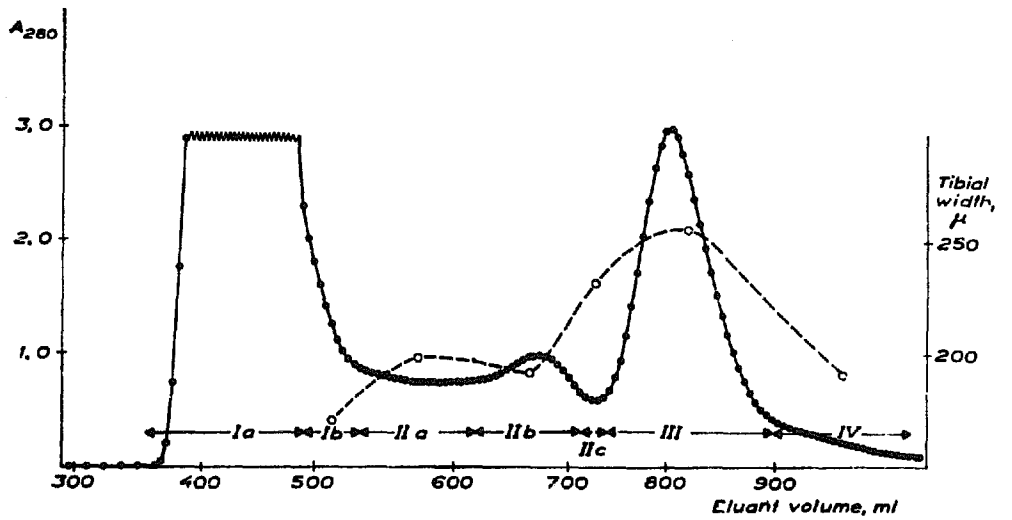

Fig. 1. Gel filtration of 50% ammonium sulfate precipitate from human pituitary extract. Column packed with Sephadex G-100. Column size 4.8 × 120 cm. Flow rate 50 ml/h. Fraction size 5–6 ml. 1.7-g sample applied in 15 ml. ●—●, absorbancy at 280 mμ; O--O, HGH activity.

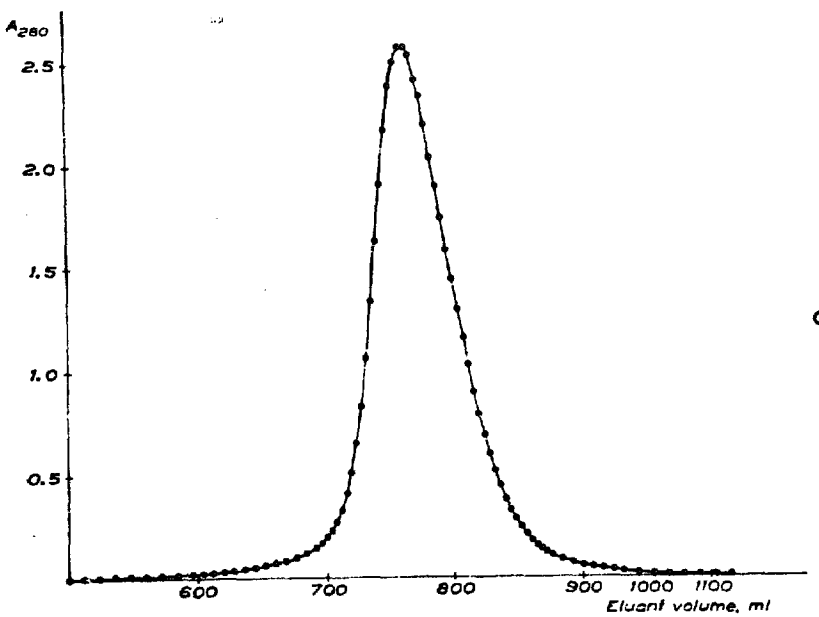

Fig. 2. Refiltration of peak III in Fig. 1. Column packed with Sephadex G-100. Column size 4.8 × 120 cm. Flow rate 45 ml/h. Fraction size 4 ml. 300-mg sample applied in 6.4 ml.

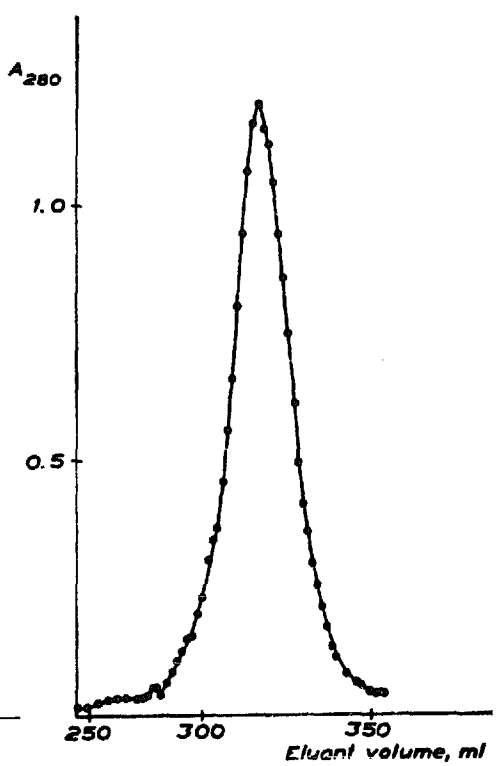

Fig. 3. Gel filtration of HGH preparation after two filtrations on Sephadex G-100. Column packed with Sephadex G-75. Column size 4 × 64 cm. Void volume 192 ml. Flow rate 1.5 ml/h. Fraction size 1.5 ml. 59 mg applied in 3.1 ml.

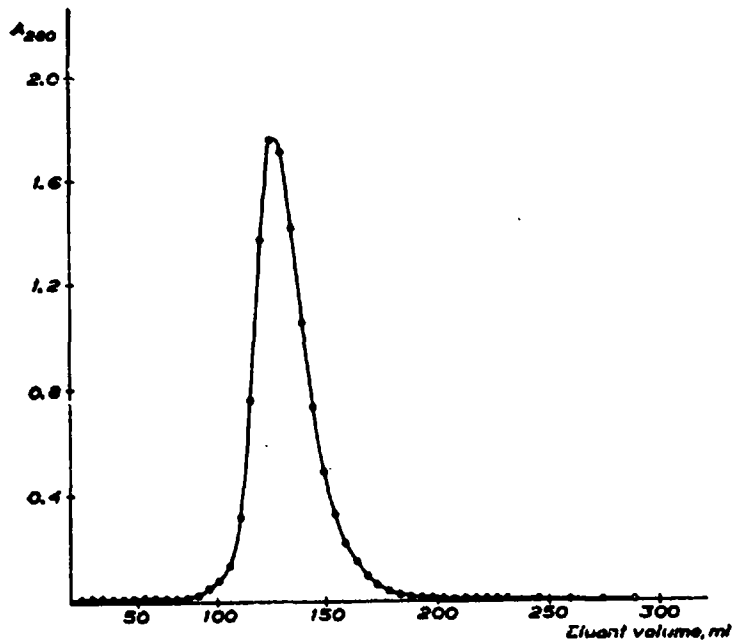

Fig. 4. Gel filtration of HGH preparation after two previous filtrations on Sephadex G-100. Column packed with Sephadex G-50. Column size 3.2 × 51 cm. Void volume 118 ml. Flow rate 18–20 ml/h. Fraction size 4.5–5.0 ml. 57-mg sample applied in 3.0 ml.

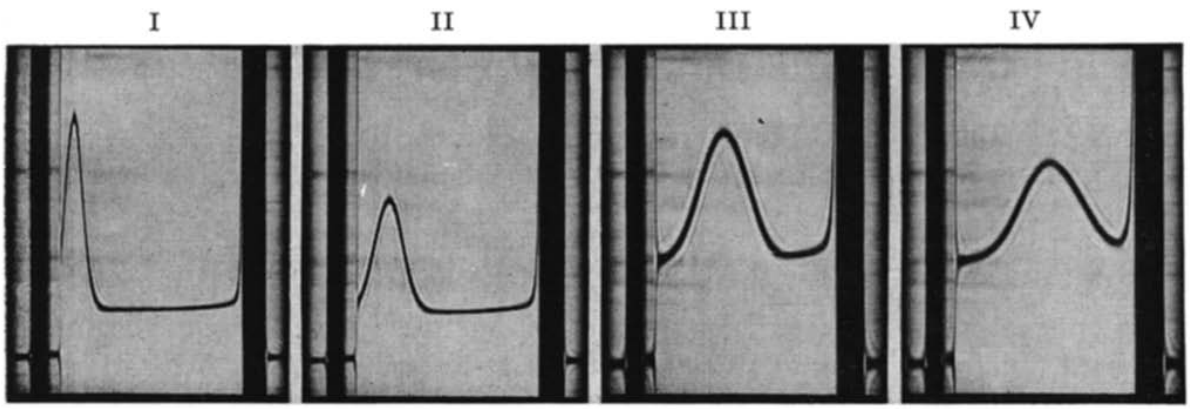

Fig. 5. Sedimentation patterns of HGH from the first gel filtration on Sephadex G-100 after 25, 73, 121 and 169 min at 59780 rev./min. Buffer, sodium phosphate (pH 7.0, *I* 0.02) containing also 0.10 M NaCl. In I and II the angle of the schlieren diaphragm was 60°, in III and IV 40°.

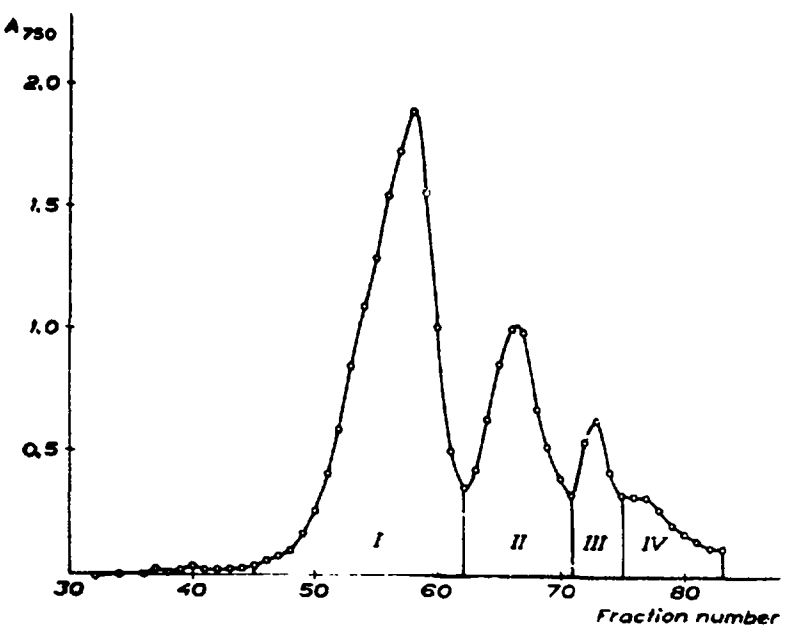

Fig. 6. Protein distribution from column electrophoresis at pH 8.6 of HGH obtained through gel filtration on Sephadex G-100. Veronal buffer (*I* 0.02); column dimensions, 0.6 × 80 cm; current, 5 mA; time, 28 h.

SAFFRAN AND SCHALLY[15], and the modified chick ^{131}I-uptake test of BATES AND CORNFIELD[16], respectively. Small amounts of HGH, FSH, and LH activities were found in areas IIa and IIb of the gel filtration pattern in Fig. 1.

DISCUSSION

It appears that HGH activity can be separated from most of the other pituitary hormones by a simple Sephadex G-100 gel filtration of the material precipitated from the original extract by 50% saturation with ammonium sulfate. The yield from this procedure compares well with those from other methods[1-6].

If the original extraction is done with $Ca(OH)_2$ (see ref. 1), no difference in the gel filtration or electrophoretic patterns is noted. The gel columns have also been operated in Tris buffer (pH 8.6), with and without NaCl, and in phosphate buffer (pH 6.6 or 7.0) with and without NaCl, and no difference in distribution of protein or growth hormone activity has been observed. In addition, for preparation of material for clinical use the procedure can be simplified by dissolving the 50% ammonium sulfate precipitate in buffer without prior lyophilization and applying this solution directly to the gel column. We have applied up to 50 ml containing 1 g protein on a 4.8 × 120-cm column and still maintained adequate separation of peaks I, II, and III (Fig. 1).

Retardation of growth hormone activity in columns packed with Sephadex G-50 has been reported for the hormones from porcine[17] (mol. wt., 41600) and from human[6,18] (mol. wt., 27100) pituitaries. The runs were performed in 0.1 M acetic acid and the low pH is presumably the cause of these unexpected findings.

From the agarose electrophoresis it is evident that our preparation contains more than one molecular species with growth hormone activity. Similar results have been reported from other laboratories using starch gel electrophoresis[19,20]. Whether these different components are artifacts of the preparation procedure[21] or native products of the pituitary gland is not known. Investigations to establish the chemical relationships between these components are in progress.

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