

Preparation of Growth Hormone from a Rat Mammosomatotropic Pituitary Tumor¹

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SUMMARY

Rat growth hormone was extracted from an acetone powder of transplantable mammosomatotropic pituitary tumors using buffered ammonium sulphate. Active material, precipitated by increasing the ammonium sulphate concentration to 1.5 M, was fractionated first by diethylaminoethyl cellulose chromatography and then by preparative polyacrylamide gel electrophoresis. The yield of material in the purest fraction was 44 $\mu\text{g/gm}$ wet weight of starting material. Tibia assays showed that the crude extract gave a 60-micron stimulation in cartilage width at a 2.0-mg dose level, whereas the purest growth hormone fraction gave a 53-micron stimulation at a 0.15-mg dose level. Analytical polyacrylamide gel electrophoresis at pH 9.5 indicated that the tumor growth hormone migrated more rapidly than growth hormone isolated from pituitaries; a single, but rather broad band was observed. The overall purification obtained was 12-fold.

INTRODUCTION

At the time this study was initiated, rat growth hormone (GH)³ was not available in ready supply, in part due to the difficulty of reported purification procedures and to the large number of pituitaries required for an average preparation. To resolve this problem, we decided to attempt to isolate GH from rat pituitary tumors, which are easily transplanted and which provide relatively large quantities of starting material. These tumors have been shown to synthesize large quantities of GH both *in vivo* (7) and *in vitro* (8). In the present study an effort was made to purify rat growth hormone from mammo-

somatotropic tumors through the use of DEAE-cellulose chromatography and preparative polyacrylamide gel electrophoresis. The results indicate that GH activity can be extracted from the tumor and purified 12-fold by these procedures. However, the final yield of material is low, and the hormonal activity of the most potent fraction is about 70% that of standard bovine growth hormone (NIH-BGH-B11). Some of the techniques employed in this study more recently have been employed in an efficient method for the purification of GH from rat pituitaries (5).

MATERIALS AND METHODS

Reagents. Microgranular DEAE-cellulose (DE52) was obtained from Reeve Angel, Clifton, New Jersey. Acrylamide, *N,N'*-methylenebisacrylamide (Bis), *N,N,N',N'*-tetramethylethylenediamine (TMED), and glycine were purchased from Eastman, tris(hydroxymethyl)aminomethane (Tris) from Sigma (Trizma Grade), and Coomassie Brilliant Blue from Colabs; all other materials were reagent grade. Acrylamide was recrystallized from redistilled acetone and Bis from redistilled methanol; TMED was purified by vacuum distillation. Bovine growth hormone (NIH-BGH-B11) was a gift from the Endocrinology Study Section of the National Institutes of Health.

Tumor. The rat mammosomatotropic pituitary tumor used in this study, designated MtT-W15, has been shown to secrete substantial amounts of growth hormone, modest amounts of prolactin, and little or no adrenocorticotrophic hormone (U. Kim, personal communication, 1964). It was propagated by subcutaneous implantation of sterile, whole-cell homogenates into 100-gm Wistar-Furth female rats. The tumors initially employed were in their eighth passage, whereas those ultimately employed were in their twenty-fifth passage; good growth-promoting activity was observed throughout.

Bioassay of Growth Hormone Activity. Growth hormone activity was assayed by the tibial cartilage method of Greenspan *et al.* (3). Hypophysectomized Sprague-Dawley rats, obtained from Hormone Assay Laboratories, Chicago, were not used until two weeks after pituitary removal, and only those rats with weight changes of 5 gm or less were selected. Four rats (average weight, 100 gm) were used per group, and each received 0.25 ml of the sample a day for 4 days. All samples were dialyzed against 0.9% NaCl before injection. Rats were killed 24 hr after the last injection and the epiphyseal cartilage width determined at $\times 60$ magnification by making 20 readings across each bone.

¹Supported in part by USPHS Research Grant No. AM-07375 from the Institute of Arthritic and Metabolic Diseases (USPHS), the National Science Foundation Grant No. GB-5743, the Damon Runyon Memorial Fund for Cancer Research Grant No. DRG-743, and the American Lebanese Syrian Associated Charities (ALSAC).

²W. E. G. acknowledges support by USPHS Training Grant No. 5-T01-CA-05176 from the National Cancer Institute.

³Abbreviations: MtT-W15, rat mammosomatotropic pituitary tumor, strain W15; GH, growth hormone; DEAE-cellulose, diethylaminoethyl-cellulose; Bis, *N,N'*-methylenebisacrylamide; TMED, *N,N,N',N'*-tetramethylethylenediamine; Tris, tris(hydroxymethyl)aminomethane; NIH-BGH-B11, standard NIH bovine growth hormone, preparation B11.

Received June 20, 1968; accepted October 23, 1968.

Preparative Procedures

Preparation of Crude Extract. All isolation steps were performed at 0–5°C unless noted otherwise. Initially an acetone powder of the tumor material was prepared at –20°C; for each 110 gm of fresh tissue, 15 gm of freeze-dried powder were obtained. A crude extract was prepared by stirring this dry material for 1 hr in 350 ml of 0.25 M ammonium sulfate adjusted to pH 7.5 with 0.10 M sodium phosphate buffer (2). Insoluble material was removed by centrifugation for 15 min at 30,000 X g and reextracted with 175 ml of buffer.

Ammonium Sulfate Fractionation. A solution of 3.50 M ammonium sulfate in 0.10 M sodium phosphate buffer, pH 7.5, was added to the combined supernatant fluid to a final concentration of 1.50 M. After stirring for 2 hr, the precipitate was collected by centrifugation for 20 min at 12,000 X g, resuspended in 50 ml 0.01 M Tris-HCl buffer, pH 8.5, and then dialyzed for 16 hr on a rocker against 1 liter of buffer.

DEAE-Cellulose Chromatography. After centrifugation for 15 min at 30,000 X g, the dialyzed sample was pumped into a DEAE-cellulose column previously equilibrated with 0.01 M Tris-HCl buffer, pH 8.5. The column was washed with 250 ml of Tris buffer before starting linear-gradient elution using NaCl.

Polyacrylamide Gel Electrophoresis. Material eluting from the DEAE-cellulose column between 30 and 70 mM NaCl was dialyzed exhaustively against distilled water and freeze-dried. The resultant powder was dissolved in 10 ml of 8 mM Tris-glycine, pH 8.90, containing 5% sucrose and dialyzed for 16 hr on a rocker against 1 liter of the same buffer. The preparative polyacrylamide gel electrophoresis apparatus employed was the one designed by Jovin *et al.* (6) and manufactured by Buchler Instruments, Fort Lee, New Jersey. Only a resolving gel was used, and it was prepared by mixing equal parts of the following aqueous solutions: (a) acrylamide 30.0 gm/100 ml; (b) Bis, 0.80 gm/100 ml; (c) 0.172 M Tris-0.184 M glycine, pH 8.90, containing 0.30 ml TMED/100 ml; and (d) freshly prepared ammonium persulfate, 0.80 gm/100 ml. To form the gel, 120 ml of this mixture were introduced into the apparatus giving a gel height of 7 cm. The upper buffer was 0.043 M Tris-0.046 M glycine, pH 8.98 (conductivity, 340 μmho), whereas the lower and elution buffers were both 0.120 M Tris-0.060 M HCl, pH 8.25 (conductivity, 4950 μmho). Before introducing the sample, the gel was cleaned by electrophoresis for 17 hr at 40 milliamperes to remove unidentified, ultraviolet-absorbing material. The dialyzed sample was pumped onto the top of the gel and concentrated for 30 min at 15 milliamperes (130 volts), at which time the current was increased to and maintained at 50 milliamperes (400 volts initially). Fractions of interest were combined, dialyzed exhaustively against distilled water, and freeze-dried.

Analytical Procedures

pH, Conductivity, Protein and Absorbance Measurements. The pH and conductivity measurements indicated above were made at 24–25°C. Protein concentration was determined spectrophotometrically according to the method of Groves *et al.* (4). Absorbance measurements were made on a Zeiss PMQII spectrophotometer.

Polyacrylamide Gel Electrophoresis. Analytical polyacrylamide gel electrophoresis was performed using a resolving gel only and the system employed was the same as described for preparative electrophoresis.

RESULTS

After removal of lipid, GH activity was completely extracted using buffered ammonium sulfate which allowed maximum extraction of growth promoting activity but minimum protease activity (2). To check for completeness of extraction, the residue remaining after ammonium sulfate treatment was extracted further with saturated calcium hydroxide, since GH is extremely soluble in this solution. As shown in Table 1, no further GH activity was extracted by this procedure. A total injected dose of 2000 μg of this crude extract gave a 60-micron increase in cartilage width over the controls.

To determine whether salt fractionation could be used to purify GH, the crude extract was treated with increasing ammonium sulfate in increments of 0.25 M. GH activity was precipitated over a relatively broad range, i.e., between 0.75 M and 1.50 M, with no purification. All activity was precipitated by 1.50 M ammonium sulfate. When assayed at a total injected dose of 1000 μg, the tibia-width stimulation resulting from the 1.50 M ammonium sulfate precipitate was 26 microns.

Table 1

Sample assayed	Total dose in 4 days (μg)	Tibia assay	
		Width ^a (μ)	Δ ^b (μ)
Experiment 1, initial extract			
Saline	0	144 ± 4	
0.25 M ammonium sulfate in PO ₄ , pH 7.5	2000	204 ± 3	60
Saturated Ca(OH) ₂ , pH 11.0	2000	146 ± 5	2
Experiment 2, ammonium sulfate fractionation			
Saline	0	152 ± 4	
1.50 M ammonium sulfate precipitate	1000	178 ± 5	26
1.50 M ammonium sulfate supernatant	1000	152 ± 2	0
Experiment 3, DEAE-cellulose chromatography			
Saline	0	131 ± 3	
Before column ^c	1000	157 ± 7	26
Fraction I	1000	145 ± 3	14
Fraction II	1000	144 ± 2	13
Fraction III	1000	145 ± 3	14
Fraction IV	1000	155 ± 4	24
Fraction V	1000	193 ± 2	62
Fraction VI	1000	255 ± 11	124
Fraction VII	1000	245 ± 15	114
Fraction VIII	1000	191 ± 5	60
Experiment 4, preparative polyacrylamide gel electrophoresis			
Saline	0	150 ± 5	
Before electrophoresis ^c	150	180 ± 2	30
Fraction I	150	177 ± 5	27
Fraction II	150	203 ± 6	53
Fraction III	150	172 ± 4	22
Fraction IV	150	178 ± 4	28
Fraction V	150	151 ± 4	1

Tibia assay data on various fractionated samples.
^aMean width of uncalcified portion of epiphyseal cartilage ± standard error.
^bDifference from mean width of saline group.
^cSee Footnote 4.

DEAE-cellulose chromatography results in additional purification of the hormone. A gradient up to 1000 mM NaCl showed all GH activity was eluted between 30 and 100 mM salt concentration. Since GH activity was eluted over this broad concentration range, an attempt was made to obtain greater resolution by employing a shallower salt gradient in the elution procedure. The results (Chart 1; Table 1) showed that maximum GH activity was eluted between 45 and 50 mM NaCl (Fraction VI), but significant activity also was distributed over a salt concentration range from 35 to 60 mM NaCl. To recover as much GH activity as possible, all material eluting between 30 and 70 mM NaCl was retained.

Additional purification was obtained using preparative polyacrylamide gel electrophoresis. From the absorbance profile (Chart 2) the eluate was divided into 5 fractions for assay. As shown in Table 1, Experiment 4, Fraction II had the highest

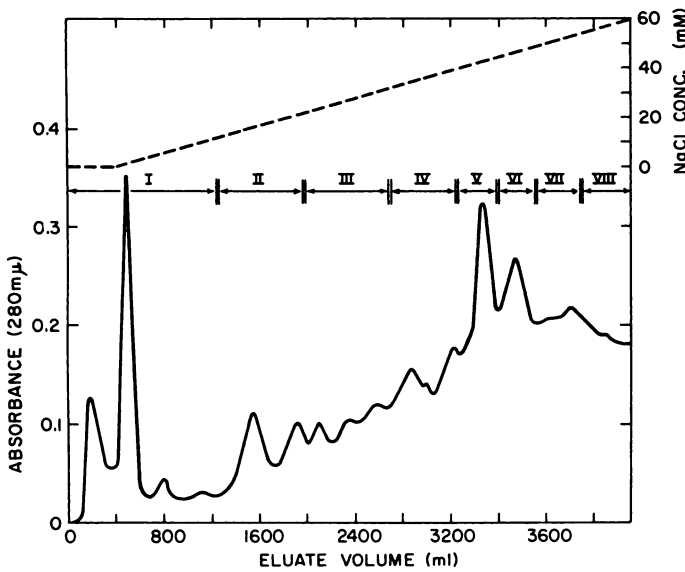


Chart 1. Resolution of the 1.50 M ammonium sulfate fraction on a DEAE-cellulose column using a 0 to 0.10 M NaCl gradient. Total A²⁸⁰ units applied was 35. (One A²⁸⁰ unit is the amount of light absorbed at 280 mμ using a cuvet with a 1.00-cm light path.) Column size was 3 x 70 cm; flow rate was 50 ml/hr. Dashed line represents NaCl concentration and solid line absorbance at 280 mμ. Tubes combined for bioassay are indicated.

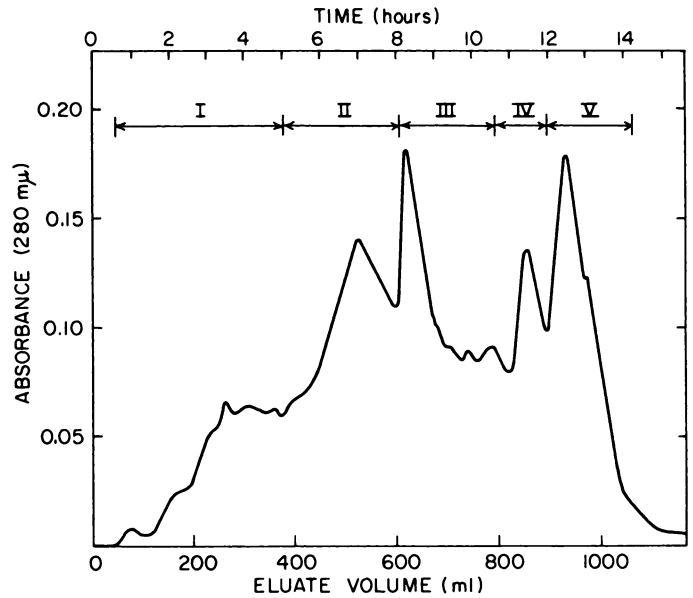


Chart 2. Fractionation by preparative polyacrylamide gel electrophoresis of material eluting from DEAE-cellulose column between 30 and 70 mM NaCl. Total A²⁸⁰ units applied was 101. Gel height was 7 cm and flow rate 75 ml/hr. Tubes combined for bioassay and time required for the fractionation are indicated. For other details see text.

GH activity (53 micron stimulation), although Fractions I, III, and IV also exhibited some activity (22 to 28 micron stimulation). Similar results were observed in duplicate experiments, i.e., maximum GH activity was found in Fraction II.

Table 2 summarizes the purification procedure. To facilitate understanding of the procedure, an arbitrary unit has been introduced and defined as a tibial width increase over control of one micron in 5 days. From these data it can be estimated that the combination of DEAE-cellulose chromatography and polyacrylamide gel electrophoresis gave a 12-fold purification, while the ammonium sulfate precipitation resulted in no increase in activity and a large loss of material.

To estimate the maximum potency of the purified tumor GH, hypophysectomized rats were injected with three different doses of standard bovine GH and Fraction II from an electrophoretic fractionation which yielded material of increased potency. The stimulation resulting from 10-, 22.5-,

Table 2

Description of fraction	Total protein (mg)	Total units recovered ^a	% units recovered	Specific activity ^b	Fold purification
Crude extract	2970	88,800	100	0.030	
1.50 M (NH ₄) ₂ SO ₄ ppt.	557	14,480	16.3	0.026	0
DEAE eluate: 30-70 mM NaCl fraction	70.8	14,160	16.0	0.20	6.7
Preparative polyacrylamide: Fraction II	4.84	17,070	17.3	0.35	11.7

Summary of purification procedure.

^aOne unit is defined here as a tibial width increase over control of one micron in five days.

^bSpecific activity is defined here as units per microgram protein injected.

and 50- μ g doses of NIH-BGH-B11 and 50- μ g of polyacrylamide-Fraction II are shown in Table 3. From the stimulation observed, the potency of 50 μ g of tumor GH was equivalent to that of 36 μ g of NIH-BGH-B11 (as estimated from a plot of log-dose data). This indicated that polyacrylamide-Fraction II was 70% as potent as standard bovine GH.

Table 3

Sample assayed	Total dose in 4 days (μ g)	Tibia assay	
		Width ^a (μ)	Δ^b (μ)
Saline	0	169 \pm 7	
NIH-BGH-B11	10	186 \pm 5	17
NIH-BGH-B11	22.5	201 \pm 15	32
NIH-BGH-B11	50	236 \pm 8	67
Polyacrylamide-Fraction II	50	221 \pm 9	52

Comparative potency of purest tumor GH fraction.

^aMean width of uncalcified portion of epiphyseal cartilage \pm standard error.

^bDifference from mean width of control group.

Aliquots of various fractions were examined by analytical polyacrylamide gel electrophoresis (see Chart 3). The "before electrophoresis" ⁴ sample and Fractions I through V refer to preparative polyacrylamide gel electrophoresis of tumor material (see Table 1, Experiment 4), whereas the unfractionated rat anterior pituitary extract and purified rat GH, included for comparison, refer to material from normal rats (5). The "before electrophoresis" sample was heterogeneous and contained material which migrated with R_F values ranging from 0.13 to 0.40. As indicated in Chart 2, Fraction I contained the most rapidly migrating material and Fraction V the least; the mean R_F value for each fraction was: I, 0.37; II, 0.28; III, 0.21; IV, 0.18; and V, 0.17. By comparison, the R_F value of purified rat GH was 0.19. The most potent growth-promoting activity of the tumor material was in Fraction II (see Table 1, Experiment 4), which migrated more rapidly than normal GH under these conditions. However, Fraction II exhibited a broad band, suggesting heterogeneity still existed in this fraction.

DISCUSSION

The results indicate relatively potent GH can be isolated from rat mammosomatotropic pituitary tumors grown *in vivo*, but the yield of purified material is low. Ammonium sulfate removed about 81% of the crude extract protein but gave no increase in potency. DEAE-cellulose chromatography, which was the most effective purification step, removed an additional 16% protein and gave a 6.7-fold purification. And finally, preparative polyacrylamide gel electrophoresis removed 2% more protein while giving approximately a 2-fold purification. The

⁴"Before column" refers to 1.50 M ammonium sulfate precipitate and "before electrophoresis" refers to material eluting from DEAE-cellulose column between 30 and 70 mM NaCl.

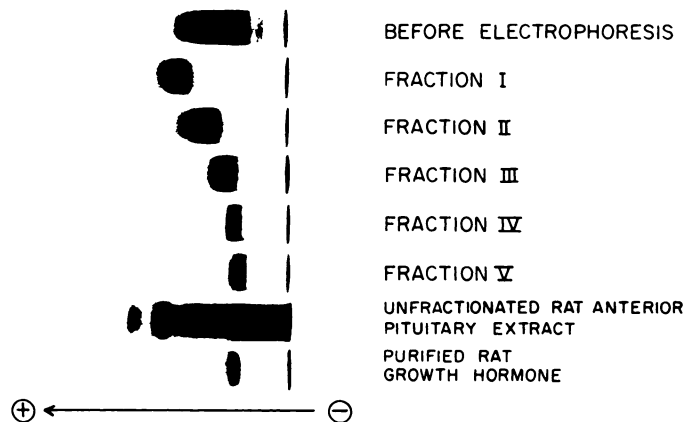


Chart 3. Analytical electrophoresis of preparative polyacrylamide gel electrophoresis fractions. Gels were composed of 7.5% acrylamide and 0.20% Bis. Electrophoresis was at pH 9.5, 0–1°C, and 1.0 ma per gel for 70 min. Micrograms of protein applied per gel were: before electrophoresis, 25; Fractions I through V, 7.5; unfractionated rat anterior pituitary extract, 25; purified rat growth hormone, 7.5. Protein bands were stained with 0.025% Coomassie brilliant blue in 7% acetic acid. For other details, see text.

total purification obtained thus was estimated to be 11.7-fold and the recovery of protein 0.2% of that in the crude extract. Bates *et al.* (1) reported the GH content in rat pituitary tumors was 14% of that in the normal. Since they used a different tumor strain, F4, and earlier generations, 1 through 9 sub-passages, the hormone content they observed may have been greater than in the tumors used in this study. Our best preparation was about 70% as potent as standard bovine GH. These data indicate GH can be purified from pituitary tumors using DEAE cellulose chromatography and preparative polyacrylamide gel electrophoresis, but ammonium sulfate fractionation results in severe loss of activity.

Analytical polyacrylamide data showed the most potent material migrated more rapidly than purified rat GH. Two explanations can be advanced. First the tumor extract may contain protease activity which causes slow degradation during purification. If so, the use of a protease inhibitor might be useful to improve the potency of the final product. And second, the diethylstilbesterol used to induce the pituitary tumor might have caused changes in deoxynucleoprotein structure which resulted in formation of a modified GH molecule. Whatever the reason, present evidence indicates isolated tumor GH is different from purified rat GH (5).

Although the present work emphasizes the difficulty in obtaining good yields of pure rat GH from pituitary tumors grown *in vivo*, it may define a procedure for purifying the hormone from the media of pituitary tumor cells grown *in vitro* (8).

ACKNOWLEDGMENTS

We thank Mrs. Susan S. Dodson for expert technical assistance, the Endocrinology Study Section of NIH for the gift of standard bovine growth hormone, and Dr. U. Kim for the transplantable tumor used in this study.

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