A Naturally Occurring Structural Variant of Human Growth Hormone*

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A structural variant of human growth hormone was isolated from pituitary extracts. The newly recognized modification had a molecular weight of 20,000 when analyzed by electrophoresis in sodium dodecyl sulfate, whereas human growth hormone gave a value near 22,000. By isoelectric focusing the variant had a pI of 5.85; human growth hormone had a value of 5.8. All extracts of 30 individual fresh frozen pituitary glands, analyzed by electrophoresis in sodium dodecyl sulfate, contained the 20,000-dalton component. The amount was estimated to be between 5 and 10% of the growth hormone in the extract.

Amino acid composition of the 20,000-dalton variant showed about 10 fewer amino acids than in growth hormone. Structure studies indicated that the difference between the variant and human growth hormone was probably in the region of residues 39 to 64. There could also be differences in the region of 24 to 38 and 159 to 167, but the results were inconclusive. The first 23 residues of the 20,000-dalton form were identical with those found in human growth hormone and 17 corresponding tryptic peptides of the two forms had identical amino acid compositions. Phospholylalanine was the COOH-terminal amino acid and glycine was in the penultimate position in both forms. The 20,000-dalton form remained intact after reduction with 2-mercaptoethanol. We conclude that the 20,000-dalton form is a structural variant of growth hormone.

An unusual property of the 20,000-dalton variant was that it reacted rather poorly with antibody to human growth hormone that may not be accurately detected by current radioimmunoassays.
Structural Variant of Human Growth Hormone

Preparative isoelectric focusing was done with the model 8101 apparatus of LKB using a sucrose gradient and ampholytes with a pH range of 4 to 6. It was necessary to incorporate 4 M urea in the sucrose gradient in order to minimize precipitation of the protein. The 20K and hGH were located by SDS electrophoretic analyses of the fractions. The pH of the individual fractions was measured at room temperature by a pH meter with no correction made for these concentrations of sucrose and urea.

Isolation Procedure

1. Extraction - The first steps in the purification of the 20,000-dalton protein were the same as those used in an isolation procedure for human growth hormone (1). The starting material used in this analysis, tubes containing hGH, was obtained from a prior preparation. The material represented the "dimer fraction" resulting from chromatography of an impure preparation of hormone on Sephadex G-100 in 0.01 M NH₄HCO₃.

2. DEAE-cellulose Chromatography (Fig. 1A) - The dimer fraction was dialyzed against 0.01 M NH₄HCO₃ and then chromatographed on DEAE-cellulose (Whatman D32). The column was packed with exchanger, previously equilibrated with 0.01 M NH₄HCO₃, and washed with a solution of 6 M urea adjusted to a conductivity of 1.1 mmho with solid NH₄CO₃. All the urea/bicarbonate buffers used for the chromatography were made in this manner. They had to be prepared fresh just before use because the conductivities dropped significantly upon standing. A flow rate of 1 ml/min for a column (2.5 x 20 cm) was used. For this size column, 1.5 g of dimer fraction dissolved in 60 ml of 6 M urea/NH₄HCO₃ (conductivity = 1.1 mmho) were applied. Once the sample had been introduced, the column was washed with urea/bicarbonate with a conductivity of 2.2 mmho until the optical density of the effluent, measured at 280 nm, returned to a plateau value. The elution buffer was then changed to a 6 M urea/NH₄HCO₃ solution with a conductivity of 5.1 mmho, which eluted some 20K, but a conductivity of 4.1 mmho was needed to remove all the 20K. The hGH was eluted immediately after the 20K. Some quite acidic material was finally removed by elution with a buffer with a conductivity of 7.5 mmho.

The 20K was located in the elution pattern by SDS electrophoretic analysis. Dialysis of the hGH against the pool of 20K (shaded portion, Fig. 1A). The 20K-rich fraction was dialyzed against 0.01 M NH₄HCO₃ and lyophilized. Yield was about 150 mg from 1.5 g of dimer fraction.

3. Gel Filtration in 6 M Urea - The material from the DEAE-cellulose chromatography (Fig. 1A) was then chromatographed on Sephadex G-75 in 6 M urea/0.1 M NH₄HCO₃ (conductivity = 1.1 mmho) at a flow rate of 1 ml/min. A volume of 60 ml (150 mg) of the material was applied to a column (90 cm) packed with microcrystalline cellulose. Chromatography was done in the first direction with n-butyl alcohol/glacial acetic acid/pyridine/water (15:8:12:10). Electrophoresis was carried out in the solvent described above for mapping on paper sheets (17) for 1.5 h at 25 V/cm. A Desagn-Braminkchromatography apparatus was used. Staining for detection only was done with a cadmium-ninhydrin spray (18). The thin layer procedure could also be used for quantitation. Here the plate was sprayed with dilute ninhydrin (17) and when the spots appeared they were scraped from the plate, eluted with 6 M HCl on a small sintered glass funnel, and hydrolyzed (17). The amino acid composition was done as with peptides eluted from the paper (17).

Total amino acid analyses were performed on untreated and Cys(O-methyl)-20K. Hydrolysis was done in evacuated tubes with 6 M HCl containing 0.1% thioglycolic acid for 20, 36, and 48 h. The procedure used to reduce and alkylate 20K was as described for hGH (19). Tryptophan was measured by the procedure of Beaven and Holiday (20).

4. Second DEAE-cellulose Chromatography - The 20K from the gel filtration (Step 3, above) still contained some material that migrated behind 20K during SDS electrophoresis. To remove this material the 20K was chromatographed on DEAE-cellulose (Whatman D32) in 6 M urea with gradient elution instead of the stepwise process used in Step 2 above. For 50 to 60 mg of sample, a column (0.9 x 15 cm) was used. The elution buffer was equilibrated with 6 M urea/NH₄HCO₃ and then elution was done as described above for DEAE-cellulose chromatography. The sample was adjusted to a conductivity of 1.1 mmho and when applied was complete, the column was washed with more of the 3 mmho buffer until the optical density at 280 nm returned to base-line. A gradient made by mixing equal portions (150-ml each) of two 6 M urea/NH₄HCO₃ buffers was then begun. The conductivities of the two buffers were 3 and 2 mmho, respectively. The elution pattern is shown in Fig. 1C. The most homogeneous 20K (shaded portion, Fig. 1C) was located by SDS electrophoresis, dialyzed against 0.01 M NH₄HCO₃, and lyophilized. The yield was usually between 30 and 40 mg. This type of material was used for bioassays, radioimmunoassays, and structure work.

Sephadex Chromatography in Dissociating Media

In an effort to dissociate any noncovalently bound peptides that might be associated with 20K, 5 to 10 mg samples of the most homogeneous material were chromatographed on Sephadex G-50. Both 50% formic acid and 0.1% SDS in 0.01 M NH₄HCO₃ were used as eluants and in each case a column (1.5 x 90 cm) was used. The 20K, which eluted as a single peak, was dialyzed first against water, then 0.01 M NH₄HCO₃, and lyophilized. This material was used for sequencing, peptide mapping, and total amino acid composition.

Structure Analyses

NH₂-terminal residues were determined by the 5-dimethylaminonaphthalene-1-sulfonyl chloride method as described by Percy and Buchwald (11). Carboxypeptidase A was used for the COOH-terminal analyses (12).

Automated sequencing was done with a Beckman model 890C Sequencer. The dimethylallylamine program 102974 reported by Beckman Laboratorieg was used. Thermal conversion of the thiazolines to the phenylthiohydantoin derivatives was used (13) and identification was made by gas chromatography with a column of 10% SP4000 on Chromosorb W and by thin layer chromatography (14, 15). Amino acid analysis after hydrolysis of the thiazolines with HI (16) was also used for identification.

Peptide mapping of tryptic digests of Cys(O-methyl)-20K (6 mg) on filter paper was done as described by Seavey et al. (17) except that the electrophoresis buffer used was glacial acetic acid/pyridine/acetic acid/water (2:1:8:40). pH 4.4. Analytical mapping of 0.2 mg of Cys(Omethyl)hGH was done on thin layer plates coated with Avicel 400 microcrystalline cellulose. Chromatography was done in the first direction with n-butyl alcohol/glacial acetic acid/pyridine/water (15:8:12:10) Electrophoresis was carried out in the solvent described above for mapping on paper sheets (17) for 1.5 h at 25 V/cm. A Desagn-Braminkchromatography apparatus was used. Staining for detection only was done with a cadmium-ninhydrin spray (18).

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Dissociation with Pyruvylalanate Aminopeptidase

The enzyme, a calf liver protein, was purchased from Boehringer Mannheim; Cys(O-methyl)-20K was used for the substrate. The reaction was carried out at 30°C for 30 h in 0.05 M sodium phosphate buffer, pH 8.0, made 0.01 M in 3-mercaptoethanol and 0.001 M in EDTA. A 0.5 mg sample of 20K, dissolved in 0.25 ml of buffer, was treated with 0.025 mg of enzyme protein. The commercial preparation was stated* to be about 4% enzyme protein with an activity of 0.1 unit/mg (enzyme protein) when tested against pyroglutamyl-fl-naphthylamide.

The reaction was stopped by danylation as described above and NH₂-terminal residues were examined. As a control, an identical sample of 20K was incubated at 37°C for 30 h and enzyme added just before the addition of 5-dimethylaminonaphthalene-1-sulfonyl chloride. This was to correct for NH₂-terminal residues from the enzyme. We found them to be negligible.

Elution of Protein from SDS Gels

To determine the NH₂-terminal residue of 20K in the form that it migrates in SDS gels, the band was eluted and danylated as described by Weiner et al. (21).

Radioimmunoassays

The double antibody procedure was used (22). Guinea pig antiserum to hGH and the hormone preparation HS-2002F used for

* See In Sequence (April 1975) No. 7, Beckman Instruments, Inc., Palo Alto, Cal.

See In Sequence (March 1971) p. 31-7, Beckman Instruments, Inc., Palo Alto, Cal.

Distribution Program of the National Institute of Arthritis, Metabolic and Digestive Diseases of the National Institutes for Health.

...were fewer tyrosine residues in 20K than in hGH, a separate assay was done according to the method of Nicoll (25). In both assays a 2 x 2 design was used (26), that is, two dosage levels of the reference material and two levels of test sample were tested. For the weight gain assay 50 μg and 10 μg/day were used for both reference and test sample; 10 μg and 2 μg (total dose) of ovine prolactin and 40 μg and 8 μg of test sample were used in the crop sac assay.

**Determination of Protein**

The method of Hartree (27) was used for determination of the amount of protein used in bioassays and radioimmunoassays. Since there were fewer tyrosine residues in 20K than in hGH, a separate standard curve had to be used for each protein. We found that a value for 20K when read from a standard curve for hGH had to be multiplied by 1.2 to give a correct dry weight figure.

**Analysis of Individual Pituitary Glands for 20K**

Individual human pituitary glands were homogenized in 0.05 M Na₂CO₃/NaHCO₃, pH 10 (5 ml/g of tissue), and centrifuged to remove insoluble material. The solution was made 2 M in (NH₄)₂SO₄ and the resulting precipitate collected by centrifugation. This precipitate was then dissolved in 0.05 M NaHCO₃, at a volume equal to that used for the extraction and 25 μl were analyzed by SDS electrophoresis. The relative concentrations of hGH and 20K were determined by scanning densitometry of a stained gel.

**RESULTS**

**Isolation Procedure**—There was some batch to batch variation in the DEAE-cellulose which altered slightly the ionic strength needed to elute the 20K. The conditions shown in Fig. 1A proved satisfactory in most cases. Actually, no serious problem arose even when 20K eluted a little early or late because SDS electrophoresis accurately indicated the location of 20K. Without urea we found it impossible to separate 20K and hGH by DEAE-cellulose chromatography. With urea the more basic 20K eluted before hGH and the separation was quite good.

After chromatography on Sephadex G-75 in 6 M urea (Fig. 1B), the 20K was not always homogeneous by SDS electrophoresis. The contaminants usually had molecular weights around 30,000. The second DEAE-cellulose chromatography with gradient elution (Fig. 1C) eliminated most of these higher molecular weight components, but a faint trace of the slowest migrating contaminant can still be seen just behind the 20K (Fig. 2). That this was not hGH was shown by disc electrophoresis at pH 7.8 (see below). The identities of these slower migrating components are still unknown.

**Chromatography in Dissociating Media**—To determine if some lower molecular weight peptides could be dissociated from the 20K isolated as described above, the hormone was chromatographed on Sephadex G-50 in 0.1% SDS, 0.01 M NH₄HCO₃, and also in 50% formic acid. In each case only one peak was observed, with no indication of a lower molecular weight peptide.

This type of purified 20K was analyzed by end group determinations, peptide mapping, and automatic sequencing. The results were identical with those obtained with 20K that had not been chromatographed in the dissociating media. Details of the structure studies are given below.

**Electrophoretic Analyses**—Fig. 2 shows the SDS electrophoretic patterns for 20K and hGH. Note that when the two proteins were mixed they separated well and each was easily distinguished. No component with a mobility of hGH was seen in the purified 20K preparation, and 20K remained intact after reduction with 2 mercaptoethanol. Also, no component with a mobility greater than that of 20K was seen on these gels. This was also true for samples heated 3 min at 100°C in 1% SDS before analysis in the presence of 2-mercaptoethanol.

**FIG. 1.** Elution patterns for purification of 20K. The sample load, flow rate, and column size are given in the text. The 20K was located by SDS electrophoresis in the shaded portion of each diagram. A, chromatography on DEAE-cellulose of a dimer fraction obtained from crude hGH. The column affects the separation of 20K from hGH. The conductivities are of the NH₄HCO₃/6 M urea buffers used for the stepwise elution. Each tube represents 10 ml. B, chromatography on Sephadex G-75 of the crude 20K obtained from A. The elution buffer was 6 M urea/0.1 M NH₄HCO₃ and each tube contained 10 ml of effluent. C, gradient elution of 20K from a column of DEAE-cellulose. Starting sample was the 20K obtained from B. Elution was carried out with 6 M urea buffers containing NH₄HCO₃. The limits of the conductivities of the buffers were 3 and 5 mmho. Each tube contained 0.5 ml of effluent.
The disc electrophoresis at pH 10 was done without urea in 8% acrylamide. The pH 7.8 analyses were carried out with 8 M urea in a gel of 6.5% acrylamide. The pH 4 gel was of 6.5% acrylamide. Analysis in SDS was done in 10% acrylamide. The sample was 50 μg for the disc electrophoresis and 25 μg for the SDS gels.

At pH 4 the 20K and hGH did not separate, nor was the heterogeneity seen at the alkaline pH observed. This system does indicate that there is no significant amount of prolactin in the 20K preparation, since prolactin separates well from growth hormone at pH 4 (29). As indicated below, this was confirmed by radioimmunoassay for human prolactin.

At pH 10, 20K had only a slightly lower mobility than hGH, whereas at pH 7.8, the separation was considerably better. Urea incorporated into the gel improved the separation in both systems. Note that in the pH 7.8 system contamination of 20K by hGH could have been detected easily if present. The lower Rf value for 20K is in agreement with the more basic pI noted by isoelectric focusing.

Electrophoretic Estimation of Molecular Weight—From the SDS electrophoresis data, 20K was calculated to have a molecular weight of 20,000. In the same experiments hGH gave a value near 22,000, a value close to theoretical as calculated from the primary structure (30). The SDS electrophoresis values were based on migration data of the reduced proteins. Of interest was the observation that when 2-mercaptoethanol was omitted, the increase in mobility was greater for 20K than for hGH. Although small, these differences were consistently noted in the six determinations. From the amino acid composition a molecular weight of 20,940 was calculated.

Bioassays—Table I tabulates the growth-promoting activities of three different preparations of 20K. These values are quite close to the potency of most preparations of hGH (between 1 and 2 IU/mg) and if the 95% confidence limits are considered, they are indistinguishable. For example, we found (32) purified hGH to have a potency of 1.1 IU/mg with 95% confidence limits of 0.6 to 2.1 IU/mg.

By the pigeon crop sac assay, a potency of 1.5 IU/mg was measured for 20K (Table I). This value is similar to that found for hGH (32), which in that case was 1.6 IU/mg with 95% confidence limits of 1.1 to 2.3 IU/mg. This indicated that 20K, like hGH, is a rather poor lactogen in the pigeon. Prolactin usually has a potency between 15 and 30 IU/mg in the test.

Radioimmunoassays—Fig. 3 shows the dilution curves for three preparations of 20K when tested in a radioimmunoassay for hGH. There was some variation between preparations, but when averaged the results indicated that using antibody and antiserum distributed by the National Institutes of Health, 20K was about one-third as effective as hGH in displacing labeled hGH from the antibody. The 50% displacement values (B/B0 × 100) were 5.9, 7.0, and 8.4 ng (mean = 7.1 ng) for the three preparations of 20K. The corresponding value for hGH was 2.4 ng. A total of three separate assays all indicated this lower immunological reactivity for 20K.

Preparations of 20K were tested for contamination with human prolactin. None was detected when 20K was analyzed at a concentration of 1 μg/assay tube.

Amino Acid Composition—Table II gives the amino acid compositions of 20K and hGH. The results are the average of six analyses for each protein. The 20K had about 10 fewer amino acids than did hGH. Fewer residues of tyrosine, phenylalanine, glutamic acid, leucine, proline, and lysine were found in 20K. Glycine and valine were higher in the 20K variant. Even though rather small, these differences were consistently noted in the six determinations. From the amino acid composition a molecular weight of 20,940 was calculated. Similar results were obtained with material carried through the standard isolation procedure described above and with 20K that had been passed over a Sephadex G-50 column developed with 0.1% SDS.

End Group Analyses—Phenylalanine was the NH2-terminal residue of 20K before and after passage over a column of Sephadex G-50 developed either with 0.1% SDS or 0.5% formic acid. No other residues were detected in an amount estimated to be greater than 5%. Likewise, 20K eluted from SDS electrophoresis gels also had principally (>90%) phenylala-
nine as the NH₂ terminus, the remaining fluorescent spots being traces of aspartic acid, glycine, and leucine.

Phenylalanine was the COOH-terminal residue and glycine was in the penultimate position in the 20K variant. These are the same residues found in hGH (30).

**Treatment with Pyroglutamate Aminopeptidase** — After treatment of Cys(Cm)-20K with pyroglutamate aminopeptidase, no new NH₂-terminal amino acid was detected by the dansylation procedure. Phenylalanine was still the only major residue observed.

**Sequence Analysis** — By automated Edman sequence analysis the first 23 residues of 20K were the same as for hGH (30). The identity may extend farther, but sequencing results became uninterpretable after residue 23. For this analysis, 200 nmol of 20K were used. There was no evidence of two chains being sequenced. Identification of the sequence by amino acid analysis of HI hydrolysates of phenylthiohydantoin derivatives indicated an average yield for 24 cycles to be about 25%. When 200 nmol of hGH were sequenced almost identical recoveries of amino acids were obtained. The HI hydrolysis procedure permitted identification of a greater number of residues than was possible by gas chromatography and the latter technique was used to confirm the residues that were altered during HI hydrolysis. Those were methionine, ascorine, and threonine.

The sequencing was also carried out with 150 nmol of 20K that had been passed over a column of Sephadex G-50 developed with 0.1% SDS. The sequencing was carried through 15 cycles and the sequence was identical with that of hGH. No indication of two-chain heterogeneity was seen.

**Peptide Mapping** — The most obvious difference between 20K and hGH, when the Cys(Cm) modifications were analyzed by peptide mapping of tryptic digest, was the absence of the tripeptide Gln-Gln-Lys (residues 39 to 41) from 20K. As shown in Figs. 4 and 5, this difference was easily seen on both the thin layer plates and the larger filter paper maps. A photograph of a map of 20K made on filter paper is not included because the small amount of material available did not permit making a map for photography. The peptides had to be eluted from the paper as soon as ninhydrin treatment indicated their locations. Prolonged contact with ninhydrin significantly decreased the yield of peptides. The amino acid compositions of the peptides detected on the maps are given in Table III. Those shown as **dashed lines** on the map stained poorly with ninhydrin and do not show up well on the photograph. Although faint, the spots could be analyzed and gave values equal to those obtained with dark-staining peptides. Less obvious from visual observation was the finding that peptide 6 (residues 42 to 64) could not be located on the 20K maps. A cysteine-containing peptide with a composition that suggested it could be a portion of peptide 6 was noted just to the left of peptide 19, but the peptide was not well enough separated from peptide 19 to give a conclusive amino acid composition. The peptide composed of residues 20 to 38 was not found on maps of either hGH or 20K. Since this peptide contains 2 residues of tyrosine, specific detection sprays were used to try to locate the peptide. However, no well-defined spot on maps of either protein was ever seen that could be identified as the peptide. On maps of hGH there was a light smear of tyrosine-positive materials just above peptides 19 and 20.

A spot with the amino acid composition of the peptide composed of residues 159 to 169 (peptides 16 and 16a) was usually found in two locations, which probably indicates that 16a is a deamidated form of 16. Since residue 159 is asparagine, this is not unlikely. On the 20K maps the recovery of amino acids after hydrolysis of peptides 16 and 16a was always very low, so their occurrence in 20K is tentative.

The cleavage at Lys 168 was not quantitative, since peptides corresponding to both 168 to 178 and 169 to 178 were found. The single lysine peptide (No. 19), however, was always the most prevalent form. There was no evidence that the Lys-Asp linkage at 171 and 172 was attacked by trypsin.

Two additional comments should be made on the amino acid compositions given in Table III. Frequently, but not consistently, aspartic acid or asparagine was noted in peptide 9 (residues 78 to 94) with both 20K and hGH. In peptide 10,
FIG. 4 (upper). Maps of tryptic digests of Cys(Cm)-hGH. Experimental conditions are given in the text. The photograph of the map on the left was made on thin layer plates of cellulose; that on the right was done on filter paper. The numbers in the corresponding drawings refer to the peptide numbers given in Table III. The dotted circles indicate weakly staining peptides.

FIG. 5 (lower). Maps of tryptic digests of Cys(Cm)-20K. Experimental conditions are given in the text. The map on the left was made on thin layer plates of cellulose; the map on the right is a representation of a pattern when the mapping was done on filter paper. The numbers refer to peptides in Table III. Spots staining weakly with ninhydrin are shown as dotted circles.
### Table III

| Amino Acid | Peptide No. as denoted on peptide maps of Figs. 4 & 5. | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 | 15 | 16 | 17 | 18 | 19 | 20 | 21 |
| Asp        | 1.9(1)                                             | (1) | (1) | (1) | (1) | (1) | (1) | (1) | (1) | (1) | (1) | (1) | (1) | (1) | (1) | (1) | (1) | (1) | (1) | (1) | (1) | (1) | (1) |
| Thr        | 0.8(1)                                             | (1) | (1) | (1) | (1) | (1) | (1) | (1) | (1) | (1) | (1) | (1) | (1) | (1) | (1) | (1) | (1) | (1) | (1) | (1) | (1) | (1) | (1) |
| Ser        | 0.7(1)                                             | (1) | (1) | (1) | (1) | (1) | (1) | (1) | (1) | (1) | (1) | (1) | (1) | (1) | (1) | (1) | (1) | (1) | (1) | (1) | (1) | (1) | (1) |
| Pro        | 2.0(2)                                             | (2) | (2) | (2) | (2) | (2) | (2) | (2) | (2) | (2) | (2) | (2) | (2) | (2) | (2) | (2) | (2) | (2) | (2) | (2) | (2) | (2) | (2) |
| Ala        | 0.8(1)                                             | (2) | (2) | (2) | (2) | (2) | (2) | (2) | (2) | (2) | (2) | (2) | (2) | (2) | (2) | (2) | (2) | (2) | (2) | (2) | (2) | (2) | (2) |
| Cys-Cys    |                                                   |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |
| Val        | 0.9(1)                                             | (1) | (1) | (1) | (1) | (1) | (1) | (1) | (1) | (1) | (1) | (1) | (1) | (1) | (1) | (1) | (1) | (1) | (1) | (1) | (1) | (1) | (1) |
| Met        | 0.9(1)                                             | (1) | (1) | (1) | (1) | (1) | (1) | (1) | (1) | (1) | (1) | (1) | (1) | (1) | (1) | (1) | (1) | (1) | (1) | (1) | (1) | (1) | (1) |
| Leu        | 0.9(1)                                             | (1) | (1) | (1) | (1) | (1) | (1) | (1) | (1) | (1) | (1) | (1) | (1) | (1) | (1) | (1) | (1) | (1) | (1) | (1) | (1) | (1) | (1) |
| Tyr        | 0.9(1)                                             | (1) | (1) | (1) | (1) | (1) | (1) | (1) | (1) | (1) | (1) | (1) | (1) | (1) | (1) | (1) | (1) | (1) | (1) | (1) | (1) | (1) | (1) |
| Phe        | 0.9(1)                                             | (1) | (1) | (1) | (1) | (1) | (1) | (1) | (1) | (1) | (1) | (1) | (1) | (1) | (1) | (1) | (1) | (1) | (1) | (1) | (1) | (1) | (1) |
| Lys        | 0.9(1)                                             | (1) | (1) | (1) | (1) | (1) | (1) | (1) | (1) | (1) | (1) | (1) | (1) | (1) | (1) | (1) | (1) | (1) | (1) | (1) | (1) | (1) | (1) |
| His        | 0.9(1)                                             | (1) | (1) | (1) | (1) | (1) | (1) | (1) | (1) | (1) | (1) | (1) | (1) | (1) | (1) | (1) | (1) | (1) | (1) | (1) | (1) | (1) | (1) |
| Arg        | 0.9(1)                                             | (1) | (1) | (1) | (1) | (1) | (1) | (1) | (1) | (1) | (1) | (1) | (1) | (1) | (1) | (1) | (1) | (1) | (1) | (1) | (1) | (1) | (1) |
| Trp        |                                                   |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |

Results indicate molar ratios of amino acids in the peptide. The number in parentheses is the number of residues based on the sequence (30).

* Accessing or undirected on a map of 20K. Molar ratios were obtained with peptide eluted from a map of N2H.

† Sequence numbers are those reported for NGH (30).

‡ Amino terminus. Value may be low because of partial destruction during detection by ninhydrin.

UV-absorbing spot on the paper.

QR = Off scale. The value was estimated to be at least 5 residues but an exact number could not be calculated because of high O.D.

Fig. 6. SDS electrophoresis patterns of 2 M (NH₄)₂SO₄ precipitates of extracts of individual pituitary extracts. Source of the glands: a, 30-year-old female; b, 76-year-old female; c, 24-year-old male, acromegalic; d, 64-year-old male (gland had been stored frozen at -20°C for 2 months). The first two glands had not been frozen and were analyzed immediately after removal at autopsy. The gland from the acromegalic was examined within 4 h after surgical removal.

However, we always found what appeared to be 2 residues of phenylalanine instead of 1 as has been reported (30). This was observed with all preparations of both hGH and 20K.

The results of the peptide mapping indicated that 20K differed from hGH in the region of residues 39 to 64. There could be differences in the region of 24 to 39 and 159 to 167, but mapping data to support that possibility were inconclusive since peptide 28 to 38 was not found in either hGH or 20K and the recovery of peptide 159 to 167 was extremely low.

Detection of 20K in Individual Pituitary Glands — As shown in Fig. 6, a component with the electrophoretic mobility of 20K was seen in the 2 M (NH₄)₂SO₄ precipitates made from extracts of individual pituitary glands. In some cases the Rₓ values do not match from gel to gel because of variation in reagents and the casting of the gels. In all cases, however, authentic samples of hGH and 20K were used as reference standards and there was always excellent agreement between the components of the extract and these reference proteins. For example only are shown in Fig. 6, but this same observation was made with a total of 30 glands. Scanning densitometry of the SDS electrophoresis gels indicated that 20K accounted for between 5 and 10% of the total growth hormone in the extract. The first two glands (a and b) were analyzed immediately after autopsy. Pattern c was obtained with a pituitary gland removed by surgery and analyzed within 3 h after removal. The 20K component was also seen in a gland that had been stored frozen at -20°C for 2 months (pattern d). The amount of 20K seen in these patterns is...
similar to the concentrations observed in a number of purified preparations of hGH (1).

**DISCUSSION**

With its tendency to form a dimer, 20K is similar to a number of non-primate growth hormones (33, 34). Human growth hormone is isolated principally as the monomer during chromatography on Sephadex G-100 (1) and should be relatively free of 20K since aggregate forms are resolved. In fact, contamination of preparations of hGH with 20K can be correlated to a certain extent with the incomplete removal of the dimer fraction during chromatography on Sephadex. For example, when the SDS electrophoresis patterns of various preparations of hGH are examined (1), it can be seen that the samples containing considerable 20K also contain significant amounts of the disulfide dimer of hGH (19). The 20,000-dalton variant has been overlooked during fractionation of pituitary extracts because the major portion of the substance is in a dimeric form and to a large extent is removed from human growth hormone by purification techniques.

A comment should be made on the purification procedure. There was deamidated 20K in the dimer fraction and this form did not separate well from hGH during chromatography on DEAE-cellulose. The deamidation, by increasing the acidity of 20K, decreased the charge difference between hGH and 20K and prevented a good separation. As yet we have not been able to retrieve this material in a form that is free of hGH.

Since 20K was observed as one of the major hormone constituents of the human pituitary gland, its radioimmuno-logical behavior was of special interest. We were surprised to find that 20K was only about one-third as active as hGH in our radioimmunoassay. This suggests that if 20K is secreted in amounts that are lower than those for hGH, this variant could go undetected. Incidentally, the rather low reactivity in the radioimmunoassay supports to some degree the work of Ellis et al. (35) that provided evidence for a biologically active, but immunologically unreactive form of hGH. We are in the process of developing a radioimmunoassay for 20K and this will be reported at a later date.

The results of the analyses of the 30 individual pituitary glands gave no support to the idea that 20K is an allelic modification of hGH. All glands contained both forms and the concentrations never came near to a 1:1 ratio. A Leu-Val allelism for bovine growth hormone is known (36) and in that case the growth hormone of individual glands was of three types: one or the other of the allelic forms by itself or a 1:1 mixture of similar forms, and the area where these differences occur is in the region of 20 to 38. This peptide would then be a mixture and this would explain the spreading observed during mapping. That most preparations of hGH are quite heterogeneous has been shown by a number of electrophoretic techniques (2) and this heterogeneity may prove to be a result of the amino acid deletions in 20K.

The reason for our inability to locate the tryptic peptide made up of residues 20 to 38 on maps of hGH is unexplained. A speculative answer is that hGH as usually isolated is a mixture of similar forms, and the area where these differences occur is in the region of 20 to 38. This peptide would then be a mixture and this would explain the spreading observed during mapping. That most preparations of hGH are quite heterogeneous has been shown by a number of electrophoretic techniques (2) and this heterogeneity may prove to be a result of the amino acid variation in the area of residues 20 to 38.

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