Structural Studies on Bovine Growth Hormone

I. ISOLATION AND CHARACTERIZATION OF CYANOCEN BROMIDE FRAGMENTS*

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SUMMARY

Purified bovine growth hormone, consisting of a single polypeptide chain, has been found to contain NH2-terminal alanine, phenylalanine, and methionine in nearly equal amounts. The minimum molecular weight calculated from its amino acid composition is 20,846. Five unique fragments have been prepared from bovine growth hormone by cleavage at 4 methionyl residues with cyanogen bromide. In the order of their elution from Sephadex G-50 and G-75, these have been designated Fragment A, with 109 amino acids; Fragment B, 31 amino acids; Fragment C, 25 amino acids; Fragment D, 12 amino acids; and Fragment E, a pentapeptide. Partial characterization of these fragments indicates a high degree of similarity between this molecule and human growth hormone. Isolation of tryptic peptides derived from Fragment C lends additional support to the apparent homology.

In contrast to early reports that bovine growth hormone was significantly larger than the growth hormones of man and several other species, recent evidence (1, 2) indicates that, with a molecular weight of about 21,000, it is similar in size to human growth hormone. Unlike the latter, however, it readily aggregates under a variety of conditions of temperature, pH, solvent composition, and ionic strength (3).

Amino end group analysis has consistently shown the presence of two and occasionally three NH2-terminal amino acids in the molecule (4). This finding was originally thought to be evidence for a branched polypeptide structure. However, recalculation of these data on the basis of a molecular weight of 20,800 suggests that BGH consists of a single polypeptide chain with NH2-terminal heterogeneity. Although the origin of this heterogeneity has been attributed to the action of proteolytic enzymes during isolation of the hormone, the possibility of genetic variants within the bovine species must also be considered.

While previous investigations of the structure of bovine growth hormone have failed to clarify the nature of the NH2-terminal heterogeneity, they have lent support to the hypothesis that the primary structure of this molecule is homologous with growth hormones from other species. The amino acid sequence of the carboxyl-terminal 13 residues of bovine growth hormone, determined by Santone et al. (5) and confirmed in part by Wallis (6) is similar to the corresponding sequences proposed by Li, Liu, and Dixon (7) for human growth hormone and reported by Mills (8) for pig growth hormone. In addition, a number of peptides have been isolated after digestion of BGH with trypsin and many of these have been placed in a tentative order by comparison with the amino acid sequence of HGH (3). Although confirmation of these alignments will await complete sequence analysis of the bovine hormone, the apparent ability to align BGH peptides by reference to the structure of HGH testifies to the similarity between the 2 molecules.

An aim of this investigation has been to cleave BGH into smaller unique fragments which would be more amenable to sequence analysis than the intact molecule. Use has been made of the reaction of cyanogen bromide with proteins at low pH (9) to produce cleavage of the polypeptide chain at the carboxyl peptide bond of methionine with conversion of this residue to homoserine. Cleavage at the 4 methionyl residues of BGH has led to the isolation and characterization of five unique fragments which are being studied with respect to primary structure. Biological activities of these fragments have been investigated and will be reported elsewhere.

EXPERIMENTAL PROCEDURE

Bovine Growth Hormone—NIH-GH-B11 and NIH-GH-B12, provided by the Endocrinology Study Section of the National Institutes of Health, were used in these experiments.

Purification of BGH—Crude hormone was dissolved directly
in 1% formic acid at a concentration of 10 to 50 mg per ml and trace amounts of insoluble material were removed by centrifugation. Samples were chromatographed on Sephadex G-100 (Pharmacia) equilibrated with 1% formic acid, and elution was carried out with the same solvent at room temperature or at 4°. In analytical experiments, columns, 1.5 x 85 cm, were used at flow rates of approximately 5 ml per hour while, in preparative experiments, the column sizes were 2.5 x 90 cm or 5.0 x 90 cm, with flow rates of 15 and 45 ml per hour, respectively. Elution patterns from different columns were almost identical. Peak I and Peak II (Fig. 1A) were lyophilized and rechromatographed under the same conditions used in the initial separation. After lyophilization, rechromatographed Fraction II (designated II-2) was used for production of cyanogen bromide fragments and related studies.

In a single experiment Fraction I was dissolved in dilute sodium hydroxide (final pH 11.5) and chromatographed on a column, 2.5 x 90 cm, of Sephadex G-100 equilibrated and eluted with 1% triethylamine carbonate, pH 9.5.

**Preparation and Isolation of Cyanogen Bromide Fragments**—Reaction with cyanogen bromide was performed essentially as described by Steers et al. (10). In preliminary experiments it was found that the conversion of methionine to homoserine was incomplete at 12 to 18 hours, but complete at 24 hours. In a typical experiment, 660 mg of BGH Fraction II-2 were dissolved in 35 ml of 70% formic acid. After addition of 1.32 g of cyanogen bromide (Eastman), the solution was stirred magnetically at 25° for 24 hours. The reaction mixture was diluted with 15 volumes (495 ml) of deionized water and lyophilized twice to remove volatile products of the reaction.

The mixture of fragments was initially separated by chromatography on Sephadex G-50 (fine) equilibrated with 10% acetic acid and eluted with the same solvent (Fig. 2). Because of low yields of Fragment E resulting from its limited solubility in this solvent, 20% formic acid was used for elution in repeated experiments. Gel columns, 1.5 x 85 cm and 2.5 x 90 cm, were eluted at flow rates of 5 and 15 ml per hour, respectively, at room temperature.

After lyophilization, Fragments C and D were purified by rechromatography on Sephadex G-50, under the same conditions used for the initial separation. The fraction containing Fragment E was taken to dryness by rotary evaporation under reduced pressure, dissolved in 1 ml of 70% formic acid, and chromatographed at room temperature on a column, 1.5 x 85 cm, of Sephadex G-100 equilibrated and eluted with the same solvent. Fragment E was detected in column fractions by colorimetric reaction with ninhydrin after alkaline hydrolysis, as previously described (11).

**Reduction and Carboxymethylation of Fragment AB**—Reduction and alkylation of the single disulfide bond connecting Fragments A and B was performed in a manner similar to that described by Crestfield, Moore, and Stein (12). After lyophilization, Fragment AB was rechromatographed at room temperature on a column, 1.5 x 85 cm, of Sephadex G-100 equilibrated and eluted with 10% acetic acid at a flow rate of 5 ml per hour to remove partially cleaved and aggregated material. Purified Fragment AB was dissolved in 0.5 M Tris hydrochloride buffer, pH 8.6, containing 8 M urea and 0.001 M EDTA, at a concentration of 20 mg per ml. The solution was made 0.1 M in β-mercaptoethanol and stirred magnetically at room temperature for 3 hours. It was then cooled to 0° and iodoacetic acid (Eastman, recrystallized from petroleum ether, b.p. 60-75°) was added to a concentration of 0.00 M. The reaction was terminated after 15 min at 0° by the addition of excess β-mercaptoethanol. Fragments A and B were separated on a column, 1.5 x 80 cm, of Sephadex G-75 equilibrated and eluted at room temperature with 50% acetic acid at a flow rate of 5 ml per hour (Fig. 4).

**Preparation and Isolation of Tryptic Peptides from Fragment C**—Five micromoles (14 mg) of Fragment C were suspended in 3 ml of deionized water and adjusted to pH 8.5 with 0.1 N sodium hydroxide. The sample was incubated at 40° and 0.14 mg of L-1-lysylamino-2-phenylethyl chloromethyl ketone-treated trypsin (Worthington), dissolved in 14 µl of 0.001 N HCl was added. A second addition of the same amount of trypsin was made after 30 min. The solution was kept at pH 8.5 for 1 hour by frequent addition of 0.01 N sodium hydroxide and then adjusted to pH 2.0 with 0.01 N HCl. All peptides remained soluble through this pH range.

After addition of 2.0 µM of cysteic acid as a marker, the peptides, in 3.5 ml, were applied to a column, 1.9 x 17 cm, of Dowex 50-X8 (Bio-Rad amino acid analyzer resin, Q15S) which had been cycled with HCl, water, and NaOH before equilibration with 0.2 N pyridine acetate buffer, pH 3.1. The peptides were eluted with 60 ml of 0.2 N pyridine acetate buffer, pH 3.1, followed by a linear gradient between 500 ml of this buffer and 500 ml of 2.0 N pyridine acetate buffer, pH 5.0. Elution was carried out at 50°, with a flow rate of 80 ml per hour. Five per cent of the column effluent was diverted for automatic detection of peptides after alkaline hydrolysis and ninhydrin reaction as previously described (11). Fractions containing peptides were taken to dryness by rotary evaporation under reduced pressure, dissolved in a known amount of water or 10% acetic acid, and stored at -20°.

Purity of peptides and fragments was determined by descending chromatography on Whatman No. 3MM paper, with pyridine-1-butanol-acetic acid-water (100:150:30:120) solvent. Peptides were detected by spraying the paper with ninhydrin.

**Amino-terminal Analysis of Peptides and Fragments**—Subtractive Edman degradation of peptides and fragments was carried out in a manner similar to that described by Königsgberg and Hill (13), except that the sample (0.2 to 0.5 µM) was dissolved in 2 ml of 5% N-ethyl morpholine acetate buffer, pH 0.5, containing 50% pyridine. After flushing the solution with nitrogen, 1 drop of phenylisothiocyanate (Eastman, redistilled at 4.5 mm Hg, b.p. 68-70°) was added and the mixture was incubated at 40° for 2 hours. Cyclization was carried out for 1 hour with 1 ml of anhydrous trifluoroacetic acid (Baker Chemical Company) at room temperature. Phenylthiohydantoins were extracted three times with 6 ml of benzene and the sample was taken to dryness by rotary evaporation before aliquots were taken for amino acid analysis.

Amino-terminal analysis of bovine growth hormone was performed exactly as described by Stark and Smyth (14) and Stark (15). After carboxymylation with potassium cyanate in 8 M urea, the protein was desalted by dialysis at 4° against four 2-liter volumes of deionized water.

**Amino Acid Analysis**—Samples (0.05 to 0.1 µM) for amino acid analysis were hydrolyzed with 6 N HCl under reduced pressure at 110° for 24 hours. Duplicate samples of Fragments A and B and BGH Fraction II-2 were hydrolyzed for 24, 48, and 72 hours. The samples were then taken to dryness by rotary evaporation and redissolved in 2.2 ml of 0.01 N HCl. Amino acid analyses were made on a Spinco model 120 amino acid analyzer.
modified for accelerated analysis. Methionine in the cyanogen bromide fragments was estimated from the sum of homoserine and homoserine lactone. Cystine was determined as cysteic acid by the method of Moore (16). Tryptophan was detected by a colorimetric spot test on paper with Ehrlich reagent (17) and quantitatively determined by the method of Edelhoch (18) and Barman and Koshland (19).

Starch Gel Electrophoresis—Vertical starch gel electrophoresis was performed by the method of Smithies (20), with 0.05 M Tris borate (pH 8.9) gel buffer and 0.5 M Tris borate (pH 8.6) bridge buffer. Runs were carried out at 250 volts and 10 mA for 18 hours at 4°C. Protein was located by staining the sliced gel with 0.5% Amido black 10B in 50% methanol, 10% acetic acid solvent.

Materials—All chemicals were of reagent grade unless otherwise stated. Pyridine and N-ethyl morpholine were redistilled from ninyhydrin prior to use. Urea (9.5 M) was deionized by slow passage through columns of Mallinckrodt MB-3 mixed bed resin and used immediately.

RESULTS
Chromatographic Preparation of Bovine Growth Hormone—Bovine growth hormone was initially purified by chromatography on Sephadex G-100 in 1% formic acid (pH 2.2) at room temperature. This solvent was selected to avoid both the pH range of 3.0 to 9.5 at which several proteinases and peptidases in anterior pituitary extracts (21–25) and BGH preparations (24) exhibit significant activity and the possibility of disulfide interchange during chromatography at high pH. As shown in Fig. 1A, the NIH hormone preparations were resolved into two major components, designated Fraction I and Fraction II, and a minor component, Fraction III. Since the absorbance at 280 nm of the column effluent did not fall to base line between the largely excluded Fraction I and Fraction II, an equilibrium distribution between BGH aggregate and monomer was suspected. However, on rechromatography under the same conditions used in the original separation (Fig. 1B), Fraction II was eluted as a single symmetrical peak, designated Fraction II-2, and there was no significant regeneration of Fraction I material. Fraction II-2 accounted for approximately 60% of the absorbance at 280 nm of NIH-GH-B11 and approximately 50% of the absorbance of NIH-GH-B12. When the chromatography was repeated with NIH-GH-B12 at 4°C, the same ratio of Fraction I to Fraction II was observed. After lyophilization, Fraction II-2 yielded a white powder which was insoluble at neutral pH, but soluble in dilute NaOH (pH 9.5 to 10.5) or 1% formic acid (pH 2.2). At both acid and alkaline pH, Fraction II-2 was less readily soluble than the starting material, but more soluble than Fraction I. Although rechromatography of Fraction I under the original conditions of separation suggested that it contained at least three poorly resolved protein peaks, there was no evidence of formation of Fraction II-like material. An attempt was made to dissociate BGH dimer or aggregate in Fraction I, a possibility suggested by the studies of Edelhoch et al. (29), who showed an apparent dissociation and a fall in the molecular weight of bovine growth hormone at pH 11.5. When Fraction I was dissolved by the addition of 0.1 N NaOH to pH 11.5 and then rechromatographed on Sephadex G-100 in 1% triethylamine carbonate, pH 9.5, no material was observed at the elution volume of Fraction II.

Fraction III was rechromatographed on a column, 1.5 × 85 cm, of Sephadex G-50 in 1% formic acid at room temperature, yielding several incompletely resolved peptide fractions. At least one of these is notable for having relatively large amounts of glutamic acid, glycine, and half-cystine by amino acid analysis, and studies are in progress to determine the relationship between this component and other peptides isolated from pituitary extracts.

Additional evidence for the heterogeneity of bovine growth hormone was found by vertical starch gel electrophoresis at pH 8.9. The starting material, NIH-GH-B11, was found to have one component which migrates slowly toward the anode as a discrete band and another which moves more rapidly as a broad zone. Fraction II-2 has an electrophoretic mobility identical with that component of NIH-GH-B11 which forms the band, while Fraction I is similar to the more diffuse component. Judging from the electrophoretic patterns, there is no significant cross-contamination of Fraction I and Fraction II-2 isolated by gel filtration. Electrophoresis of NIH-GH-B12 revealed a similar pattern, but with increased amounts of the slowly moving band and less of the more rapidly migrating component, consistent with the differences in relative amounts of Fractions I and II observed on gel filtration. Fraction III was not observed in stained starch gels and is therefore presumed to contain small polypeptide material which either did not stain with Amido black or was eluted by the acetic acid-methanol staining solvent.

Amino Acid Composition of Fraction II-2—The amino acid composition of Fraction II-2 is given in Table I. Results are expressed as residues per molecule assuming a molecular weight of 20,300 g (1). Data for threonine and serine were derived by linear extrapolation to zero time and, in separate experiments, values for tyrosine were determined after the addition of 0.1 ml of 0.1 M phenol to the hydrolysis mixture to inhibit oxidation. Findings from this study have been compared with those of other investigators who have isolated highly purified BGH under dif-
Structural Studies on Bovine Growth Hormone. I

Amino acid composition of bovine growth hormone

Values for amino acids are expressed as residues per molecule, assuming a molecular weight of 20,800 g. Nearest integral residues of Fraction II-2 are given in parentheses. Compositions of BGH reported by Free and Sonenberg (26) and Mills and Wilhelmi (27) have been recalculated from the original data based on a molecular weight of 20,800. In addition, only glutamic acid of Fraction II-2 is shown in Table II. Three significant NH₂-terminal residues were found in similar amounts. There were 0.37 residue of alanine, 0.32 residue of phenylalanine, and 0.26 residue of methionine per molecule of hormone, based on a molecular weight of 20,800. In addition, only glutamic acid and valine were observed in trace amounts. The total recovery of end groups in this study was 0.99 residue per molecule of hormone.

Isolation of Cyanogen Bromide Fragments—Analysis of the lyophilized products of the reaction of Fraction II-2 with cyanogen bromide indicated that 96% of the original content of methionine had disappeared with the concomitant appearance of homoserine and homoserine lactone. The mixture of fragments was resolved into five fractions, designated AB, C, D, E, and F by gel filtration on Sephadex G-50 in 10% acetic acid (Fig. 2). Fraction AB, which includes the major excluded peak and a small trailing shoulder, was rechromatographed on Sephadex G-100 with 10% acetic acid solvent (Fig. 2). Fraction AB, which includes the major excluded peak and a small trailing shoulder, was rechromatographed on Sephadex G-100 with 10% acetic acid solvent (Fig. 2). Fraction AB, which includes the major excluded peak and a small trailing shoulder, was rechromatographed on Sephadex G-100 with 10% acetic acid solvent (Fig. 2). Fraction AB, which includes the major excluded peak and a small trailing shoulder, was rechromatographed on Sephadex G-100 with 10% acetic acid solvent (Fig. 2). Fraction AB, which includes the major excluded peak and a small trailing shoulder, was rechromatographed on Sephadex G-100 with 10% acetic acid solvent (Fig. 2). Fraction AB, which includes the major excluded peak and a small trailing shoulder, was rechromatographed on Sephadex G-100 with 10% acetic acid solvent (Fig. 2). Fraction AB, which includes the major excluded peak and a small trailing shoulder, was rechromatographed on Sephadex G-100 with 10% acetic acid solvent (Fig. 2). Fraction AB, which includes the major excluded peak and a small trailing shoulder, was rechromatographed on Sephadex G-100 with 10% acetic acid solvent (Fig. 2). Fraction AB, which includes the major excluded peak and a small trailing shoulder, was rechromatographed on Sephadex G-100 with 10% acetic acid solvent (Fig. 2). Fraction AB, which includes the major excluded peak and a small trailing shoulder, was rechromatographed on Sephadex G-100 with 10% acetic acid solvent (Fig. 2). Fraction AB, which includes the major excluded peak and a small trailing shoulder, was rechromatographed on Sephadex G-100 with 10% acetic acid solvent (Fig. 2). Fraction AB, which includes the major excluded peak and a small trailing shoulder, was rechromatographed on Sephadex G-100 with 10% acetic acid solvent (Fig. 2).

Amino-terminal analysis of NIH-GH-B12 Fraction II-2

Values were obtained by the hydantoin method of Stark and Smyth (14). The data of Free and Sonenberg (26) and Mills and Wilhelmi (27) have been recalculated from the original data based on a molecular weight of 20,800. There are 11 lysyl and 12 arginyl residues so that 24 unique peptides would be expected after complete digestion of the molecule with trypsin. The predominant amino acids are glutamic acid (including glutamine) and leucine, while histidine, half-cystine, and tryptophan occur in sufficiently limited amounts to be of importance for chemical modification and convenient markers in structural studies. Cleavage at four methionines with cyanogen bromide would be expected to produce five unique fragments. The molecular weight of Fraction II-2 calculated on the basis of the amino acid composition adjusted to nearest integral values is 20,546.

Table I

<table>
<thead>
<tr>
<th>Amino acid</th>
<th>NIH-GH-B12 Fraction II-2</th>
<th>Free and Sonenberg Component A</th>
<th>Mills and Wilhelmi (27)</th>
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</thead>
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<tr>
<td>Lysine</td>
<td>11.1 (11)</td>
<td>10.5</td>
<td>10.7</td>
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<tr>
<td>Histidine</td>
<td>2.55 (3)</td>
<td>2.96</td>
<td>2.81</td>
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<tr>
<td>Arginine</td>
<td>11.9 (12)</td>
<td>11.9</td>
<td>11.9</td>
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<tr>
<td>Aspartic acid</td>
<td>10.2 (10)</td>
<td>15.2</td>
<td>15.0</td>
</tr>
<tr>
<td>Threonine</td>
<td>11.9 (12)</td>
<td>11.7</td>
<td>11.5</td>
</tr>
<tr>
<td>Serine</td>
<td>11.6 (12)</td>
<td>11.7</td>
<td>11.5</td>
</tr>
<tr>
<td>Glutamic acid</td>
<td>23.9 (24)</td>
<td>21.7</td>
<td>23.4</td>
</tr>
<tr>
<td>Proline</td>
<td>6.30 (6)</td>
<td>6.10</td>
<td>6.88</td>
</tr>
<tr>
<td>Glycine</td>
<td>9.52 (10)</td>
<td>9.50</td>
<td>9.84</td>
</tr>
<tr>
<td>Alanine</td>
<td>12.8 (13)</td>
<td>13.5</td>
<td>13.3</td>
</tr>
<tr>
<td>Half-cystine</td>
<td>3.99 (4)</td>
<td>4.60</td>
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</tr>
<tr>
<td>Valine</td>
<td>5.87 (6)</td>
<td>6.37</td>
<td>5.13</td>
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<tr>
<td>Methionine</td>
<td>3.98 (4)</td>
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<td>Isoleucine</td>
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<td>6.50</td>
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<td>Leucine</td>
<td>24.4 (24)</td>
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<td>23.7</td>
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<tr>
<td>Tyrosine</td>
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<td>5.59</td>
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<td>Phenylalanine</td>
<td>11.7 (12)</td>
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<td>11.6</td>
</tr>
<tr>
<td>Tryptophan</td>
<td>1.09 (1)</td>
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No. of residues. 182

* Extrapolated to zero time of hydrolysis.
* Determined by the method of Edelhoch (18).
* Determined as cysteic acid.
* Determined as methionine sulfone.

Table II

Amino-terminal analysis of NIH-GH-B12 Fraction II-2

Values were obtained by the hydantoin method of Stark and Smyth (14) and are based on a molecular weight of 20,800.

<table>
<thead>
<tr>
<th>Amino acid</th>
<th>Observed</th>
<th>Blank</th>
<th>Net</th>
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</thead>
<tbody>
<tr>
<td>Alanine</td>
<td>0.46</td>
<td>0.09</td>
<td>0.37</td>
</tr>
<tr>
<td>Phenylalanine</td>
<td>0.32</td>
<td>0.00</td>
<td>0.32</td>
</tr>
<tr>
<td>Methionine</td>
<td>0.26</td>
<td>0.00</td>
<td>0.26</td>
</tr>
<tr>
<td>Glutamic acid</td>
<td>0.27</td>
<td>0.01</td>
<td>0.06</td>
</tr>
<tr>
<td>Valine</td>
<td>0.03</td>
<td>0.02</td>
<td>0.01</td>
</tr>
<tr>
<td>Total</td>
<td>1.34</td>
<td>0.35</td>
<td>0.99</td>
</tr>
</tbody>
</table>

* Corrected for operational losses with the factors of Stark and Smyth (14).

Fig. 2. The chromatographic separation of cyanogen bromide fragments of Fraction II-2. Fragments produced from 34 mg of Fraction II-2 were applied in 1 ml to a column, 1.5 × 85 cm, of Sephadex G-50 (fine) and eluted with 10% acetic acid. Effluent fractions of 1.5 ml were monitored at 253 mp and pooled into five fractions as indicated by brackets.
was developed with 10% acetic acid and effluent fractions of 1.5 ml were monitored at 280 m\(\mu\).

Eluates from the middle and trailing edge of the major peak were pooled to obtain Fragment AB.

This peak contained a single component which because of limited solubility or unusual conformation exhibited nonideal behavior on gel filtration.

Material from the center and trailing edge of the major peak, designated Fragment AB, was lyophilized and found to contain 2 half-cystine residues and 2 residues of homoserine plus homoserine lactone per 3 residues of histidine. Since this implied that it consisted of two fragments connected by an intact disulfide bond, Fragment AB was reduced with 0.1 \(M\) \(\beta\)-mercaptoethanol and alkylated with iodoacetic acid under conditions which led to the production of 2 residues of carboxymethylcysteine but no detectable amounts of other carboxymethyl amino acid derivatives. The reduced and alkylated Fragment AB was separated into Fragment A and Fragment B by chromatography on Sephadex G-75 equilibrated and eluted with 50% acetic acid (Fig. 4). Although Fragment B is soluble in 10% acetic acid, Fragment A is not. This, along with the significant size difference between the two fragments, may account for the asymmetrical peak obtained when Fragment AB was subjected to gel filtration in 10% acetic acid.

The small trailing shoulder of Fraction AB (Fig. 3) was found to have an amino acid composition similar to that of purified Fragment B and is thought to consist of a small amount of free Fragment B produced by nonspecific cleavage of the disulfide bond connecting it to Fragment A during the reaction with cyanogen bromide in 70% formic acid.

When Fragments C and D (Fig. 2) were rechromatographed separately on the original column of Sephadex G-50 under the conditions used in the initial separation, each yielded a single symmetrical peak.

Fragment E was initially found to contain only small amounts of peptide material. Since the recovered material was poorly soluble in 10% acetic acid, subsequent preparations of cyanogen bromide fragments of Fraction II-2 were separated on Sephadex G-50 with 20% formic acid as the eluting solvent. Fraction E was then subjected to gel filtration on Sephadex G-10 in 70% formic acid, to yield Fragment E in a single symmetrical major peak identified by alkaline hydrolysis and ninhydrin color development of aliquots of the effluent fractions.

The absorbance of Fraction F (Fig. 2) was found to be primarily due to reagents and nonprotein products of the cyanogen bromide reaction. This peak could be largely eliminated by repeated lyophilization of the mixture of fragments before gel filtration. However, Fraction F was also found to contain a small but significant amount of free methionine (homoserine plus homoserine lactone).

In repeated experiments the yields of Fragments A, B, C, and D, uncorrected for operational losses, were 70 to 85%, while the yield of Fragment E was 25 to 30% when chromatography was carried out in formic acid solvents. Fragments B, C, D, and E were subjected to descending paper chromatography in a pyridine-butanol-acetic acid-water (100:150:30:120) solvent. After spraying with ninhydrin, one spot was observed for Fragment D, while Fragments B and C showed two spots, probably indicating the separation of homoserine and homoserine lactone forms of each fragment. Fragment E was found to contain several ninhydrin-positive spots and was judged to contain a minor impurity.

Amino Acid Composition of Cyanogen Bromide Fragments—The amino acid composition of each fragment and that of the starting material, Fraction II-2, are given in Table III. In repeated experiments, 0.1 ml of 0.1 M phenol was added to samples of Fragments A, B, and C prior to hydrolysis to inhibit oxidation of tyrosine.

Fragment A, with 109 residues, constitutes approximately 60% of the molecule. It contains 5 lysyl and 6 arginyl residues; therefore, it would be expected to yield 12 unique peptides after hydrolysis with trypsin. In addition, it contains 2 of the 3 histidyl residues of bovine growth hormone, 1 residue of carboxymethylcysteine, and 1 residue of methionine as estimated from the sum of homoserine and homoserine lactone.

Bovine growth hormone Fraction II-2 and each fragment were examined for the presence of tryptophan by a colorimetric spot test on paper with Ehrlich reagent. Only Fraction II-2 and Fragment A were found to have definite evidence of tryptophan, which was determined quantitatively by the method of Edelhoch (18) or Barman and Koeshland (19). With the former method, 1.03 residues of tryptophan were observed per mole of Fraction II-2 obtained from NIH-GH-B12. By the latter method, it was possible to determine the tryptophan content of each fragment.

Fig. 3. The chromatographic purification of Fragment AB. Fraction AB (Fig. 2) was applied to a column, 1.5 \(\times\) 85 cm, of Sephadex G-100 equilibrated with 10% acetic acid. The column was developed with 10% acetic acid and effluent fractions of 1.5 ml were monitored at 280 m\(\mu\). Eluates from the middle and trailing edge of the major peak were pooled to obtain Fragment AB.

Fig. 4. The chromatographic separation of Fragment A and Fragment B after reduction and alkylation of a single disulfide bond. A 5-mg sample of Fragment AB (Fig. 3) was reduced and alkylated as described in the text and applied in 1.0 ml to a column, 1.5 \(\times\) 80 cm, of Sephadex G-75. The column was developed with 50% acetic acid and effluent fractions of 1.5 ml were monitored at 280 m\(\mu\). Fragment A and Fragment B were pooled from the eluates as indicated by brackets.
Histidine 1.88 (2)

Valine

Amino-terminal residues of all fragments were determined by the subtractive Edman procedure (13)

Tyrosine

Leucine

Methionine

Half-cystine

Alanine

Glycine

Proline

Glutamic acid

Serine

Threonine

Aspartic

Lysine

Arginine

Isoleucine

Values are expressed as residues per molecule. Sums of the assumed number of residues, in parentheses, are compared with integral values for the whole molecule. Amino-terminal residues of all fragments were determined by the subtractive Edman procedure (13) and for Fragment A and Fraction II-2 by the method of Stark and Smyth (14).

Fragment A. Related structurally to Fragment E.

No. of residues 109

Tryptophan

Phenylalanine

Amino-terminal residue

fragment containing 2 lysyl and 2 arginyl residues.

contains the remaining residue of histidine in bovine growth hormone and is devoid of proline and isoleucine. Fragment C is a 25-residue fragment containing 2 lysyl and 2 arginyl residues. Fragment D contains 12 amino acids, including 2 residues of half-cystine which form the second disulfide bond of bovine growth hormone. After reduction of this fragment with 0.1 M β-mercaptoethanol and alkylation with iodoacetic acid under conditions similar to those used for Fragment AB, 2 residues of carboxymethylcysteine were obtained. Since Fragment D does not contain methionine, it is derived from the carboxyl terminus of bovine growth hormone, a conclusion reached by Santone et al. (5) who isolated Fragment D from bovine growth hormone under different conditions and reported an amino acid sequence for this part of the molecule. Fragment E is a pentapeptide containing phenylalanine, proline, homoserine, and 2 residues of alanine. In addition, there is evidence of contamination of Fragment E with a tetrapeptide containing alanine, phenylalanine, proline, and homoserine. Sequence analysis of the tetrapeptide suggests that it is not an unique cyanogen bromide fragment but is related structurally to Fragment E.

found that all of this (1.04 residues per mole) was located in Fragment A.

Fragment B is a 31-residue peptide which, in the intact molecule, is connected to Fragment A by a disulfide bond. It contains the remaining residue of histidine in bovine growth hormone and is devoid of proline and isoleucine. Fragment C is a 25-residue fragment containing 2 lysyl and 2 arginyl residues. Fragment D contains 12 amino acids, including 2 residues of half-cystine which form the second disulfide bond of bovine growth hormone. After reduction of this fragment with 0.1 M β-mercaptoethanol and alkylation with iodoacetic acid under conditions similar to those used for Fragment AB, 2 residues of carboxymethylcysteine were obtained. Since Fragment D does not contain methionine, it is derived from the carboxyl terminus of bovine growth hormone, a conclusion reached by Santone et al. (5) who isolated Fragment D from bovine growth hormone under different conditions and reported an amino acid sequence for this part of the molecule. Fragment E is a pentapeptide containing phenylalanine, proline, homoserine, and 2 residues of alanine. In addition, there is evidence of contamination of Fragment E with a tetrapeptide containing alanine, phenylalanine, proline, and homoserine. Sequence analysis of the tetrapeptide suggests that it is not an unique cyanogen bromide fragment but is related structurally to Fragment E.

The sum of amino acid residues found in each fragment closely approximates that expected on the basis of the amino acid composition of Fraction II-2. There are single residue differences in arginine, serine, alanine, and valine. The significant difference in arginine has not been accounted for since there are 7 arginyl residues per 2 of histidine and 5 of lysine have been repeatedly observed in Fragment A. The variation in serine may reflect inaccuracies in extrapolation to zero time of hydrolysis.

The NH₂-terminal residues of the cyanogen bromide fragments have been determined by the carboxymethylation method of Stark and Smyth (14) for Fragment A and by the subtractive Edman procedure for all fragments (Table III). The first residue of Fragment E is alanine, a residue also found at the NH₂ terminus of BGH Fraction II-2. The possibility that only 37% of Fraction II-2 molecules have this residue could account for the 1-residue difference between the total alanine content of the fragments and Fraction II-2. With the exception of Fragment A, for which no NH₂-terminal residue was detected, the rest of the fragments begin either with arginine (Fragments B and C) or lysine (Fragment D), so that three of the four methionines in the
Intact hormone molecule appear to be followed immediately by a basic amino acid.

Isolation of Tryptic Peptides of Fragment C—After digestion with trypsin, the Fragment C peptides were adjusted to pH 2.5 with 0.1 N HCl and separated by ion exchange chromatography on Dowex 50-X8 with a linear gradient of pyridine acetate buffers (Fig. 5). Effluent fractions containing peptides were pooled and taken to dryness by rotary evaporation under reduced pressure. Fraction 1 consists of cysteic acid which was added to the sample as a chromatographic marker. Fraction 2 contains a peptide in a yield (1.6%) too low to account for the ninhydrin color of this peak, which is due to a nonprotein ninhydrin-positive contaminant. Together, Fractions 2 and 3 contain a decapeptide in 4.4% yield which has an amino acid composition identical with that of the major peptide found in Fraction 4 and which may represent deamidated forms of the original peptide.

Fraction 4 and also Fraction 5 contain the decapeptide Lys, Asp, Thr, Glu, Tyr, Phe, Met. The distribution of this peptide between Pools 4 and 5 may result either from amide differences or from separation of homoserine and homoserine lactone forms of the peptide. The total yield of this peptide, which since it contains methionine must be derived from the carboxyl end of Fragment C, is 22.8% (Table IV). Repeated attempts to degrade the peptide by the Edman reaction were unsuccessful, suggesting that it could contain NH₂-terminal glutamine. Failure to cleave at an internal lysyl bond with trypsin may result from an asparyl-lysine sequence, since there is no proline in the peptide.

Fraction 6 contains the hexapeptide Lys, Glu, Gly, Ala, Ile, Leu in a yield of 26.4%. A single stage Edman degradation (Table IV) resulted in a 98% loss of alanine. In view of the finding that intact Fragment C has arginine at the NH₂ terminus, this peptide can be assigned to a middle position in the fragment.

<table>
<thead>
<tr>
<th>Amino acid</th>
<th>TP 1, 2</th>
<th>TP 3</th>
<th>TP 4, 5</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lysine</td>
<td>1.92 (2)</td>
<td>1.11 (1)</td>
<td>0.96 (1)</td>
</tr>
<tr>
<td>Arginine</td>
<td>1.02 (1)</td>
<td>0.99</td>
<td>0.70</td>
</tr>
<tr>
<td>Aspartic acid</td>
<td>0.97 (1)</td>
<td>1.07</td>
<td>3.05 (3)</td>
</tr>
<tr>
<td>Threonine</td>
<td>0.91 (1)</td>
<td>1.03</td>
<td>1.98 (2)</td>
</tr>
<tr>
<td>Glutamic acid</td>
<td>0.91 (1)</td>
<td>0.92</td>
<td>1.06</td>
</tr>
<tr>
<td>Proline</td>
<td>0.97 (1)</td>
<td>0.98 (1)</td>
<td>1.88</td>
</tr>
<tr>
<td>Glycine</td>
<td>0.98 (1)</td>
<td>1.02</td>
<td>1.94</td>
</tr>
<tr>
<td>Alanine</td>
<td>0.96 (1)</td>
<td>1.02</td>
<td>0.37</td>
</tr>
<tr>
<td>Valine</td>
<td>0.97 (1)</td>
<td>0.98 (1)</td>
<td>0.71</td>
</tr>
<tr>
<td>Methionine</td>
<td>0.96 (1)</td>
<td>0.99</td>
<td>0.97</td>
</tr>
<tr>
<td>Isoleucine</td>
<td>0.98 (1)</td>
<td>1.17 (1)</td>
<td>1.17 (1)</td>
</tr>
<tr>
<td>Leucine</td>
<td>0.99 (1)</td>
<td>0.97</td>
<td>0.98</td>
</tr>
<tr>
<td>Tyrosine</td>
<td>0.98 (1)</td>
<td>0.98</td>
<td></td>
</tr>
<tr>
<td>Phenylalanine</td>
<td>0.67 (1)</td>
<td>0.98</td>
<td></td>
</tr>
<tr>
<td>No. of Residues</td>
<td>9</td>
<td>6</td>
<td>10</td>
</tr>
<tr>
<td>Amino-terminal residue</td>
<td>Arg</td>
<td>Ala</td>
<td>None detected</td>
</tr>
<tr>
<td>Dowex 50-X8 fraction</td>
<td>8, 9</td>
<td>6</td>
<td>4, 5 (2, 3)</td>
</tr>
<tr>
<td>Yield</td>
<td>22.0%</td>
<td>26.4%</td>
<td>22.8%</td>
</tr>
</tbody>
</table>

* Sum of homoserine and homoserine lactone.

* Detected as homoserine and homoserine lactone; not determined quantitatively.
Fractions 8 and 9 both contain a nonpeptide with the composition of Arg, Asp, Thr, Glu, Pro, Gly, Leu in a combined yield of 22.9%. A single Edman degradation resulted in a 0.8 residue fall in arginine (Table IV), indicating that this peptide is derived from the NH₂ terminus of Fragment C. Of interest is the observation that the peptide, whether isolated in Fragment 8 or Fraction 9, contains only 0.6 residue of leucine per residue of glycine, and in addition has 0.3 residue of valine as an apparent contaminant. This might result from the presence in a 2:1 ratio in these pools of two inseparable peptides which are identical except for the replacement of valine for leucine at a single position. This possibility is also reflected in the amino acid composition of Fragment C, in which 0.3 to 0.4 residues of valine have been consistently noted.

**DISCUSSION**

Several studies have shown that standard preparations of bovine growth hormone contain multiple components which can be separated by gel filtration in appropriate solvents into fractions containing aggregated BGH and high molecular weight contaminants, monomeric BGH, and low molecular weight contaminants. Although the contaminants have not been studied in detail, there is reason to suspect (23, 24) that pituitary proteases are included in the high molecular weight fraction, and evidence obtained in the course of this investigation suggests that several pituitary peptides may be included in the low molecular weight fraction.

The factors which result in the isolation of material with the amino acid composition of bovine growth hormone as an aggregate in Fraction I and as a "monomer" in Fraction II are not well defined. The tendency for BGH to aggregate readily in 0.1 M sodium acetate buffer at pH 4.0 (28) but not in phosphate buffer at pH 2.2 (29) has been attributed to an acetate effect. In addition, the sedimentation coefficient of purified BGH similar to Fraction II-2 has been shown to increase from 2.3 to 3.0 in glycine hydrochloride buffer, pH 2.2, after the addition of small amounts of sodium chloride (3). In the present investigation, however, it does not appear that either an acetate effect or an ionic strength effect is primarily responsible for the separation of BGH into Fractions I and II.

The observation that relative yields of Fraction I and II are constant when chromatography is carried out at two different temperatures with varying sample concentrations and that additional aggregate is not formed when Fraction II is chromatographed under the conditions of the original separation suggests that two relatively stable forms of the hormone are being isolated. Although it is reasonable to suspect that changes in conformation brought about by pituitary proteases may be responsible for the differences in chromatographic behavior, the finding that Fraction II-2 behaves as a monomer fraction in spite of marked amino-terminal heterogeneity indicates otherwise. Since disulfide interchange is unlikely in material that has not been exposed to alkaline conditions in the course of preparation, an attractive explanation is related to the finding of Burger, Edelhofer, and Condiffe (30) that a major change occurs in the conformation of BGH without significant change in helical structure as the pH is reduced from 5.0 to 2.0. The molecular species produced by this transition appears to aggregate readily in the presence of low concentrations of salt.

Thus it is possible that the two fractions of BGH observed in this study differ primarily with respect to changes in conformation induced in the course of their preparation. At the same time, consideration must be given to the possibility that BGH isolated from pooled slaughterhouse material may include genetic variants with minor differences in amino acid sequence which predispose them to molecular transitions favoring aggregation. An adequate precedent for this speculation is observed in the case of hemoglobin S, in which the replacement of a single amino acid residue leads to molecular aggregation under reduced oxygen tension (31).

Five cyanogen bromide fragments have been prepared from purified BGH monomer as expected on the basis of methionine content. These fragments account for the amino acid composition of the entire molecule within the limits of experimental error. Nonspecific cleavage of the polypeptide chain in 70% formic acid was not observed and differences in size among the fragments permitted them to be adequately purified by gel filtration alone. In general, the amino acid composition of individual fragments has been determined with little apparent ambiguity. Although none of the cyanogen bromide fragments isolated from BGH is of the same size as the four cyanogen bromide fragments of HGH, there is strong evidence that the 2 molecules are similar in general structure and are genetically related proteins (3). The high yields of Fragments A, B, C, and D indicate that almost all of the BGH molecule has a common structure which makes up a single polypeptide chain approximately six amino acids shorter than that of HGH. Santome et al. (5) have shown that the 12-residue carboxyl-terminal cyanogen bromide fragment of BGH has an amino acid sequence which is homologous with the

**Table V**

Comparison of peptides of bovine growth hormone Fragment C with human growth hormone and human placental lactogen

A continuous sequence from human growth hormone, residues 122 to 147 (7), is shown along the top line of each group. Peptides from Fragment C of bovine growth hormone and human placental lactogen (33) are shown in the second and third lines, with unsequenced residues placed to indicate maximum possible homology.

<table>
<thead>
<tr>
<th></th>
<th>HGH</th>
<th>BGH C-</th>
<th>HPL T</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>122</td>
<td>Met-Gly-Arg-</td>
<td>Arg- (Glu, Leu, Glu, Asp, Pro, Thr, Gly, Arg)</td>
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<tr>
<td></td>
<td>125</td>
<td>Leu-Glu-Asp-Pro-Ser-Gly-Arg-</td>
<td>(Leu, Glu, Asp, Ser, Gly, Arg)</td>
</tr>
<tr>
<td></td>
<td>130</td>
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<td>135</td>
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<tr>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>BGH</td>
<td>HGH</td>
</tr>
<tr>
<td></td>
<td></td>
<td>C-</td>
<td>Thr-Gly-Glu-Ile-Phe-Lys-</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Tp 1.2</td>
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<tr>
<td></td>
<td></td>
<td>HPL a</td>
<td>Ala- (Gly, Glu, Ile, Leu, Lys)</td>
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<tr>
<td></td>
<td></td>
<td>9b</td>
<td></td>
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<tr>
<td></td>
<td></td>
<td>HPL T</td>
<td>(Thr, Gly, Glu, Ile, Leu, Lys)</td>
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<td>16e</td>
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<td></td>
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<td>Glu-Thr-Tyr-Ser-Lys-Phe-Asp-Thr-Asp-Ser</td>
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<tr>
<td></td>
<td></td>
<td>C-</td>
<td>(Glu, Thr, Tyr, Asp, Lys, Phe, Asp, Thr, Asp) Met</td>
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<td></td>
<td></td>
<td>Tp 4.5</td>
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<td></td>
<td>7a</td>
<td></td>
</tr>
</tbody>
</table>

* Human placental lactogen.
corresponding region of HGH and includes a small disulfide loop in both molecules. From the present study, it is clear that the half-cystine residues in Fragment A and B of BGGH participate in a second disulfide bridge which is potentially homologous with the first disulfide bond of HGH.

Similarities between internal amino acid sequences of BGGH and HGH are suggested by comparison of the partially characterized tryptic peptide derived from Fragment C of BGGH with the known linear sequence of HGH. In addition, peptides from Fragment C of BGGH are comparable to tryptic peptides isolated by Sherwood (32) from human placental lactogen, which has also been shown to have amino terminal sequence homology with HGH (33). In Table V, a continuous sequence of BGGH from residues 122 to 147 is shown at the top line of each group of data. A single gap has been inserted in the sequence to accommodate, in the second line, the tryptic peptides of Fragment C from BGGH in the order suggested by amine-terminal analysis. The unsequenced amino acid residues of peptides from BGGH Fragment C and from human placental lactogen, in the third line of each group, have been arranged to show maximal potential homology with the corresponding sequence of HGH. It is of interest that, by this process, an aspartyl-lysine sequence is present at the NH2 terminus of an unbranched polypeptide.

Although the finding of NH2-terminal phenylalanine and alanine occur at the NH2 terminus of an unbranched polypeptide. The finding of 0.68 residue of leucine and 0.33 residue of valine in the peptide Tp 1,2 suggests that such variations may determine to what extent these differences may reflect genetic alignment of tryptic peptides proposed by Dellacha, Santome, and Paladini (3). At present sufficient data are not available to show whether these differences may reflect genetic alignment of tryptic peptides from Fragment C presented in Table V differ from the cleavage by trypsin. The order and composition of the peptides from the amino terminus of bovine growth hormone. The present study has again shown the presence of NH2-terminal homogeneity in a highly purified preparation of BGGH. Although the finding of NH2-terminal phenylalanine and alanine originally led to a proposal of a branched polypeptide chain structure for this hormone, revision of molecular weight estimates of BGGH from 45,000 to 20,000 suggested that these residues both occur at the NH2 terminus of an unbranched polypeptide. In addition to phenylalanine and alanine, a significant amount of NH2-terminal methionine has been observed in this investigation. Methionine had been noted by Ellis (4) to appear at the NH2-terminal of the BGGH under certain conditions of isolation of the hormone and apparently at the expense of NH2-terminal phenylalanine. The structural basis of this heterogeneity has been obscure, although the action of putative aminopeptidase and genetically determined variation at the NH2 terminus have both been postulated to account for this observation.

Of the unique cyanogen bromide fragments, only Fragment E has been shown to have an NH2-terminal residue identical with one of those found in the intact molecule and yields of this fragment are comparable to the yield of NH2-terminal alanine from Fraction I-2. The additional 2 NH2-terminal residues of BGGH, alanine, and methionine could potentially be accounted for by the tetrapeptide contaminant of Fragment E containing NH2-terminal alanine and by the free homoserine isolated in cyanogen bromide Fraction F. Although these are probably derived from Fragment E by the action of specific aminopeptidase, clarification of this relationship will require sequence analysis of larger peptides from the amino terminus of bovine growth hormone.

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