Peptides of a Biologically Active Tryptic Digest of Bovine Growth Hormone*

Nobuyuki Yamasaki,† Motosuke Kikutani,‡ and Martin Sonenberg§

ABSTRACT: From tryptic digests of bovine growth hormone, which had metabolic activity in man similar to human growth hormone was isolated a fraction which appeared as a single component on gel filtration, disc electrophoresis, and sedimentation equilibrium, and had the same molecular weight, 21,000, and amino acid composition as undigested bovine growth hormone. In addition to phenylalanine and alanine of undigested bovine growth hormone, this component had serine and valine as amino-terminal amino acids. Undigested bovine growth hormone contained only one carboxyl-terminal amino acid, phenylalanine, while the component from the tryptic digest of bovine growth hormone contained two additional carboxyl-terminal amino acids, both arginine. The fraction of the tryptic digest of bovine growth hormone was separated into two components by Sephadex G-75 gel filtration in 50% acetic acid. The approximate molecular weight of each component was found to be 16,000 and 5,000. Amino acid analysis of the two components account for the total amino acids in the parent fraction. Two disulfide bonds were found in the larger component and none in the smaller component. The amino-terminal amino acids of the larger component were phenylalanine, alanine, and serine and of the smaller component, valine. Carboxyl-terminal amino acids of the larger component were phenylalanine and arginine and of the smaller component, arginine. Significant ability to increase weight and tibial width of hypophysectomized rats remained in the smaller component and to a much lesser extent in the larger component. Reduction and carboxymethylation of the larger component (molecular weight 16,000) gave two peptides by gel filtration on Sephadex G-75. The larger reduced and carboxymethylated peptide had a molecular weight of approximately 11,000 and contained phenylalanine and alanine as amino-terminal residues and arginine as a carboxyl-terminal amino acid. The molecular weight of the second reduced and carboxymethylated peptide was found to be approximately 5000. Serine as amino-terminal amino acid and phenylalanine as carboxyl-terminal amino acid were found in this second peptide. The data indicate that the hormonally active fraction of a tryptic digest of bovine growth hormone consists of two components formed by the cleavage of two peptide bonds at arginine residues within one of the disulfide loops and that these peptide chains are associated under mild conditions. The biological activity is largely associated with the 5000 molecular weight component.

We previously have presented (Sonenberg *et al.*, 1965; Nadler *et al.*, 1967; Sonenberg *et al.*, 1968) evidence that limited tryptic digests of bovine growth hormone have metabolic activity in humans similar to human growth hormone. We (Sonenberg *et al.*, 1965) and others (Raben, 1962a,b; Li and Liu, 1964) have found undigested bovine growth hormone to be without growth hormone activity in humans.

From tryptic digests of bovine growth hormone we have isolated a fraction which is a single component by disc electrophoresis and sedimentation equilibrium (Sonenberg *et al.*, 1968). This component has essentially the same molecular weight as undigested bovine growth hormone (Sonenberg *et al.*, 1968). To account for this observation, we proposed (Sonenberg *et al.*, 1968) that proteolysis occurred within the disulfide loop(s) of bovine growth hormone. In this paper we present additional data in support of this proposition.

Experimental Section

Materials. Bovine growth hormone was prepared by the method of Dellacha and Sonenberg (1964). Trypsin and soybean trypsin inhibitor employed for the digestions were obtained from Worthington Biochemical Corp., Freehold, N. J. DEAE-cellulose (Cellex-D) and ninhydrin were obtained from Calbiochem, Los Angeles, Calif. Purified DNP-amino acids were obtained from Mann Research Laboratories, New York, N. Y. Anhydrous hydrazine was prepared by the method of Kusama (1958). Hydrazine sulfate was recrystallized three times from hot water and dried at 140°. Eastman chromatogram sheet (Type K301R2), silica gel without fluorescent indicator, and the Eastman chromagram developing apparatus were used for thin-layer chromatography. Sephadex was obtained from Pharmacia Fine Chemicals, Inc., Piscataway, N. J. 2-Mercaptoethanol was obtained from Eastman Organic Chemicals, Rochester, N. Y. Other chemicals used in this study were reagent grade.

Methods

Preparation of Tryptic Digests of Bovine Growth Hormone. Solutions of bovine growth hormone (5 mg/ml), trypsin

* From the Division of Endocrinology, Sloan-Kettering Institute for Cancer Research, New York, New York 10021. *Received July 18, 1969.* A preliminary report of some of the data in this paper was presented at the 51st Meeting of The Endocrine Society, June 29, 1969, New York, N. Y. Supported in part by Grant CA-08748 from the National Cancer Institute and Grant P-437 from the American Cancer Society.

† Present address: Faculty of Agriculture, Ehime University, Matsuyama City, Japan.

‡ Present address: Biochemistry Laboratory, Faculty of Pharmacy, University of Nagasaki, Nagasaki, Japan.

§ Please address inquiries to: Martin Sonenberg, Sloan-Kettering Institute, New York, N. Y. 10021.

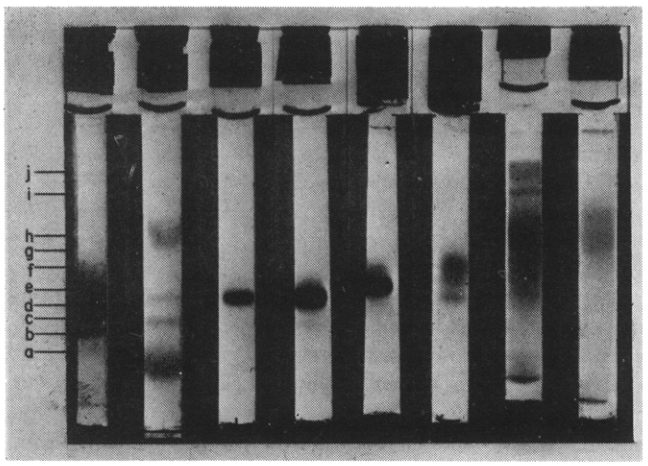

FIGURE 1: Disc electrophoresis of parent tryptic digest of bovine growth hormone and fractions derived therefrom by DEAE-cellulose chromatography. Reading from left samples are parent digest and from Figure 2, fractions eluted with 0.03 M, 0.05 M, 0.07 M, 0.1 M, 0.15 M, 0.3 M, and 0.7 M NH_4HCO_3, respectively.

(0.5 mg/ml), and soybean trypsin inhibitor (5 mg/ml), all in 0.1 N KCl, were prepared immediately before use. All digestions were performed at 25° with continuous mechanical stirring. Proteolysis was initiated by addition of trypsin solution to bovine growth hormone solution to give a ratio of 1 mg of enzyme to 300 mg of bovine growth hormone. Proteolysis was terminated by addition of trypsin inhibitor solution (10 mg of inhibitor to 1 mg of enzyme). All digestions were performed at pH 9.5 in 0.1 N KCl with a recording pH-Stat (Type TTT 1 b Radiometer, Copenhagen). Digestions were carried out in water-jacketed reaction vessels, under nitrogen, with the pH held constant during proteolysis by automatic titration with standard potassium hydroxide. Upon termination of proteolysis, the product was recovered by dialysis at 4° and freeze drying.

The yield (Table I) has been determined from the amount of tryptic digests of bovine growth hormone obtained from bovine growth hormone after proteolysis, dialysis, and freeze drying. The extent of proteolysis estimated from standard base uptake, yield, and biological activity of the preparations employed is listed in Table I.

TABLE I: Preparations of Tryptic Digests of Bovine Growth Hormone.

Lot	Base Uptake (equiv/ 21,000 g)	Yield (%)	Biological Activity (IU units/mg ± SE)
AIP	2.4	94	1.39 ● 0.20
			1.29 ± 0.24
AIS	2.4	93	2.37 ± 0.32
			1.68 ± 0.33
AJN	2.4	86	1.40 ± 0.32
AJX	2.4	85	1.50 ± 0.16
AKF	2.4	91	1.88 ± 0.44
MK 109	2.5	98	1.16 ± 0.26
MK 114	2.5	93	1.79 ± 0.32

Disc electrophoresis revealed the multiple bands (Figure 1) previously noted (Sonenberg *et al.*, 1965; Nadler *et al.*, 1967; Sonenberg *et al.*, 1968). There were five major bands (a,b,c,d,e) and five minor more rapidly migrating anodal bands (f,g,h,i,j) in all lots in approximately equivalent amounts.

Fractionation of Tryptic Digests of Bovine Growth Hormone by Chromatography. DEAE-cellulose was equilibrated with 0.01 M NH_4HCO_3 buffer (pH 8.4) by repeated suspensions and decantations. A tryptic digest of bovine growth hormone (2.36 g) in 0.01 M NH_4HCO_3 was applied to the column (2.5 × 60 cm) and developed first with starting buffer. Stepwise elution was performed with NH_4HCO_3 solutions of the following concentrations and pH: 0.03 M, pH 8.4; 0.05 M, pH 8.4; 0.07 M, pH 8.4; 0.1 M, pH 8.3; 0.15 M, pH 8.2; 0.3 M, pH 8.2; 0.5 M, pH 8.2; 0.7 M, pH 8.1; and 1 M, pH 8.1. A flow rate of 25 ml/hr was maintained. Protein concentration in 10 ml of eluent was measured by ultraviolet absorption at 278 mμ. The pooled fractions were lyophilized directly.

Gel Filtration in 50% Acetic Acid. Gel filtration at 4° was performed on a column (2.5 × 40 cm) of Sephadex G-75 equilibrated with 50% (v/v) acetic acid, to which was added the single component of a tryptic digest of bovine growth hormone in 50% acetic acid, followed by elution with the same solvent. Fractions eluted were read at 280 mμ and an aliquot of each fraction was taken for further identification by ninhydrin reaction after alkaline hydrolysis (Crestfield *et al.*, 1963). Fractions were evaporated under reduced pressure and then freeze dried. Fractions were further purified by repeating the gel filtration in 50% acetic acid. For the study of biological activity, fractions obtained by gel filtration were freeze dried directly from 50% acetic acid.

Gel filtration of a tryptic digest of bovine growth hormone in other solvents, *i.e.*, 1 N, 2 N, 20% acetic acid, etc., was carried out by the same procedure except that the protein was identified by ninhydrin reaction.

Disc Electrophoresis. Analytical electrophoresis on polyacrylamide gel was performed by the disc electrophoretic method of Ornstein (1964) and Davis (1964). Electrophoresis was performed at pH 9.5 in 7.5% polyacrylamide gel. Protein components were stained with 0.5% Amido Schwarz in 7% acetic acid.

Amino Acid Analysis. Proteins or peptides were hydrolyzed in 6 N HCl at 110° under reduced pressure for 24 and 72 hr. Hydrolysates were analzyed on an amino acid analyzer (Spackman *et al.*, 1958). For amino acids partially destroyed during hydrolysis, appropriate corrections were applied by extrapolation to zero time from the values obtained from both 24- and 72-hr hydrolysates.

Determination of the NH_2-Terminal Residues. Dinitrophenylation was performed without alcohol (Levy and Li, 1955) at pH 8.6 at 40° for 3 hr. DNP-protein or -peptide was hydrolyzed by 6 N HCl in a sealed evacuated tube at 105° for 16 hr. Identification and quantitative analysis of DNP[1]-amino acids were performed by two-dimensional

[1] The following abbreviations have been used: FDNB, 2,4-dinitro-1-fluorobenzene; DNP, dinitrophenyl; RCM, reduced and carboxymethylated.

thin-layer chromatography. Two solvent systems were used: solvent 1 (Brenner *et al.*, 1961) toluene–pyridine–ethylene chlorhydrin–0.8 N ammonium hydroxide (100:30:60:60) and solvent 2 (Fittkau *et al.*, 1964) benzene–pyridine–acetic acid (80:20:2). The upper phase of solvent 1 was used for chromatography. DNP spots were eluted with 4 ml of 1% $NaHCO_3$, and their absorptions were read at 360 mμ after removing silica gel by centrifugation. Pure DNP-amino acids were hydrolyzed with the samples followed by thin-layer chromatography with the same procedure as that employed for DNP derivatives. The NH_2-terminal amino acid values have been appropriately corrected for losses during all procedures.

Determination of the COOH-Terminal Residues. To 0.2 μmole of dried samples was added 25 mg of hydrazine sulfate and the mixture was dried in a vacuum desiccator over P_2O_5 for 3 days. Anhydrous hydrazine (0.2 ml) was added and allowed to react in a sealed tube at 60° for 16 hr. The hydrazinolysate was dried in a desiccator over concentrated H_2SO_4.

After treatment with heptanal (Kusama, 1958) the hydrazinolysate was dinitrophenylated in 66% ethanol (Sanger, 1945) and the COOH-terminal DNP-amino acid was fractionally extracted (Kawanishi *et al.*, 1965). Quantitative analysis of DNP-amino acids was performed by thin-layer chromatography as described above for NH_2-terminal amino acids. Appropriate recovery corrections were determined by submitting given amino acids to the hydrazine reaction in the presence of protein.

Reduction and Carboxymethylation. Total reduction and carboxymethylation of the component of a tryptic digest of bovine growth hormone or larger fractions obtained by gel filtration in 50% acetic acid were performed in 8 M urea with 0.2% EDTA in pH 8.6 Tris buffer with β-mercaptoethanol and iodoacetic acid (Crestfield *et al.*, 1963). Fractionation of reduced and carboxymethylated peptides was carried out on a column (2.5 × 40 cm) of Sephadex G-75 in 50% acetic acid covered by aluminum foil. Aliquots of each fraction were taken for identification by the ninhydrin reaction (Crestfield *et al.*, 1963). Peptide fractions were evaporated under reduced pressure and then freeze dried.

Determination of Molecular Weight. Apparent weight-average molecular weights were determined by sedimentation equilibrium (Yphantis, 1964) at 4 to 10° in a Spinco Model E ultracentrifuge equipped with ultraviolet optics and an automatic scanner. Three determinations were made simultaneously in an Yphantis six-hole centerpiece; three holes contained the solution, and the other three, the respective solvents. The optical density of the solvent is automatically subtracted from that of the solution, and a graph of protein concentration is produced by the scanner in absolute density units measured at 280 mμ. Molecular weights were calculated with the equation

$$\text{mol wt} = \frac{2RT\text{d ln (OD)}}{2(1 - \bar{v}\rho)\text{d}x^2}$$

where OD is the optical density at 280 mμ read directly from the tracings, $\bar{v}$ is the partial specific volume, ρ is the density of the solution, and x is the distance from the center of rotation to the point in question. In most cases the plot of log OD *vs.* x^2 was linear for approximately 90% of the length of the liquid column indicating homogeneity of the sample. As another test for homogeneity, the determinations were carried out at at least two speeds. In all cases the calculated molecular weights were identical within experimental error. The reproducibility of the low-speed method which is here used is within 5% for any one sample.

The high-speed or meniscus depletion method of Yphantis was not employed because of the necessary limitation of rotor speeds.

Biological Responses. The growth response of the component of a tryptic digest of bovine growth hormone as well as the fractions obtained by gel filtration in 50% acetic acid was determined in hypophysectomized rats (Marx *et al.*, 1942) and the weight gain for 10 days was recorded. The tibial width response was also determined (Geschwind and Li, 1955).

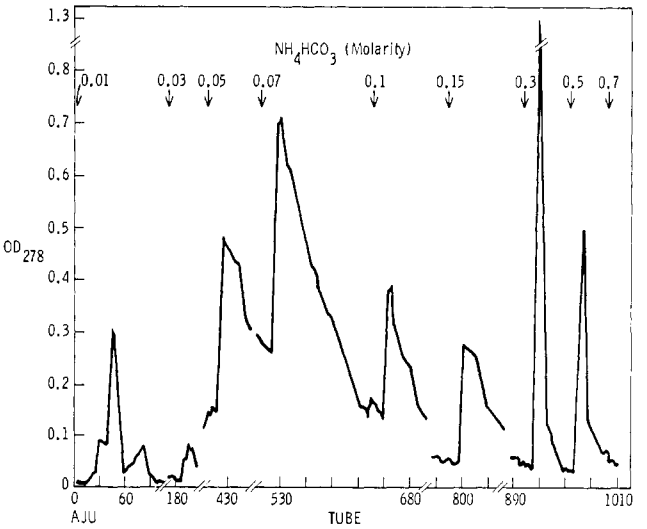

FIGURE 2: Chromatography of tryptic digest of bovine growth hormone on DEAE-cellulose with discontinuous increases in the concentration of eluting solutions of NH_4HCO_3.

Results

Fractionation of a Tryptic Digest of Bovine Growth Hormone. The chromatogram of a tryptic digest of bovine growth hormone on a DEAE-cellulose column is shown in Figure 2. Elution of each component was performed by stepwise increase in the NH_4HCO_3 concentration. Approximately 72% of the protein applied to the column was recovered in the collected fractions as measured by absorbance at 278 mμ.

Disc electrophoretic patterns of the chromatographic fractions of a tryptic digest of bovine growth hormone are shown in Figure 1. The parent tryptic digest of bovine growth hormone and the fractions eluted with 0.01 M and 0.03 M NH_4HCO_3 contained five components (a, b, c, d, and e) as designated in previous reports (Sonenberg *et al.*, 1965; Nadler *et al.*, 1967; Sonenberg *et al.*, 1968) as well as minor components. The component of a tryptic digest of bovine growth hormone with which subsequent studies were done appeared in fractions eluted with 0.05 M NH_4HCO_3. It was of intermediate electrophoretic mobility, previously designated component d. It was obtained as a single component

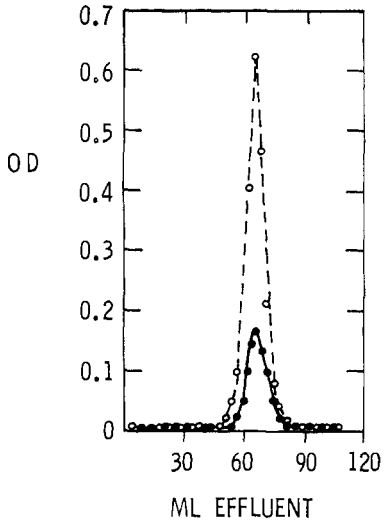

FIGURE 3: Gel filtration of 7 mg of a component of tryptic digest of bovine growth hormone in 0.1 M carbonate buffer (pH 9.5) on Sephadex G-75: column size—2.5 × 42 cm; (-●-) OD_{280}; (-○-) OD_{570} (ninhydrin color).

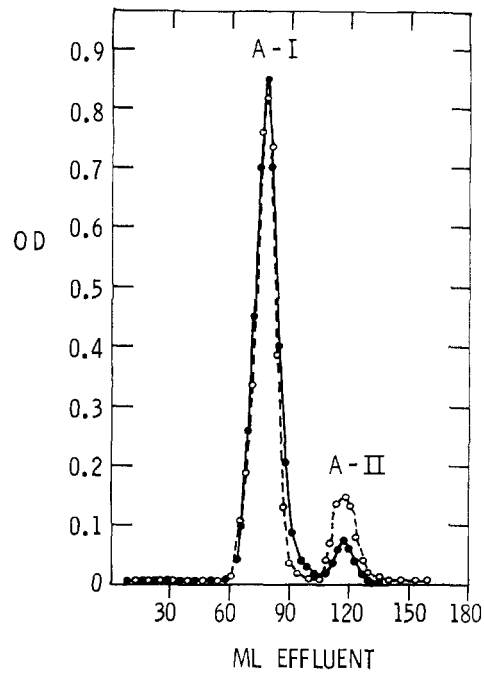

FIGURE 5: Gel filtration of 30 mg of a component of tryptic digest of bovine growth hormone in 50% acetic acid on Sephadex G-75: column size—2.5 × 40 cm: (-●-) OD_{280}; (-○-) OD_{570} (ninhydrin color).

by preparative polyacrylamide electrophoresis (Sonenberg *et al.*, 1968). The fractions eluted with 0.07 M and 0.1 M NH_4HCO_3 contained one major component (d or e) and two or three minor components (c, f, and g). The fraction eluted with 0.15 M NH_4HCO_3 contained components f and g mainly and minor components (d or e, and h).

Gel Filtration. The component (d) obtained from a tryptic digest of bovine growth hormone by elution with 0.05 M NH_4HCO_3 revealed a single peak Sephadex G-75 gel filtration in 0.1 M carbonate buffer at pH 9.5 (Figure 3). In 6 M urea, however, this component was separated into two peaks on Sephadex G-75 (Figure 4). Figure 5 shows the typical elution pattern of this component from the Sephadex G-75 column eluted by 50% acetic acid. Two fractions, A-1 and A-11, were obtained with increasing elution volume, respectively. From 1 g of bovine growth hormone the yield was 920 mg of unfractionated tryptic digest of bovine growth hormone. The latter yielded 119 mg of the single component of a tryptic digest of bovine growth hormone. The latter, in turn gave rise to 84 mg of A-1 and 28 mg of A-11. In 1 N or 2 N acetic acid there was no separation by gel filtration of A-1 from A-11. When equimolar amounts of previously isolated A-1 and A-11 were dissolved together and subjected to gel filtration in 1 N acetic acid, a single peak was noted. Gel filtration of bovine growth hormone on Sephadex G-75 yielded a single peak in 50% acetic acid. Gel filtration of totally reduced and carboxymethylated A-1 produced two fractions (Figure 6).

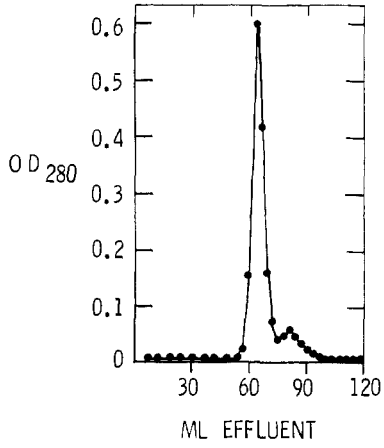

FIGURE 4: Gel filtration of 15 mg of a component of tryptic digest of bovine growth hormone in 0.1 M carbonate buffer (pH 9.5)–6 M urea on Sephadex G-75: column size—2.5 × 43 cm.

Disc Electrophoresis. Analytic disc electrophoresis revealed essentially homogeneous components A-1 and A-11. Component A-1 had slightly greater anodal mobility than A-11.

Molecular Weight. By sedimentation equilibrium, the molecular weight of the larger fraction (A-1) separated by Sephadex G-75 in 50% acetic acid was approximately 16,000 and that of the smaller component (A-11) was 5000. The ultracentrifugal pattern of each fraction suggested homogeneity. The molecular weight of the larger fraction of reduced and carboxymethylated A-1 (RCM-A-1_1) was approximately 11,000 and that of the smaller peak (RCM-A-1_2) 5000. Both ultracentrifugal patterns indicated monodispersity.

Amino Acid Composition. Amino acid analysis of the single component of a tryptic digest of bovine growth hormone is presented and compared with that of bovine growth hormone in Table II. Comparison of the data for these materials reveals almost identical compositions within experimental error, no differences greater than one residue being noted between the component of a tryptic digest of bovine

TABLE II: Amino Acid Composition.

Amino Acid	BGH[a]	TBGH-d[b]	A-1[c]	A-11[d]	RCM-A-1$_1$[e]	RCM-A-1$_2$[f]
Lys	10.5	10.8	8.8	2.0	4.3	4.4
His	3.0	3.4	3.2	0	2.4	1.1
Arg	12.2	12.3	10.5	2.8	6.9	4.4
Asp	15.1	15.4	10.8	3.8	6.3	4.3
Thr	11.3	10.9	7.1	2.6	5.6	2.2
Ser	11.7	12.5	10.0	1.9	8.9	2.9
Glu	21.6	21.7	18.1	4.9	17.6	2.2
Pro	6.1	5.9	5.9	0.9	6.9	0
Gly	9.8	10.8	6.5	2.9	5.3	2.1
Ala	13.5	12.6	12.1	1.0	10.6	2.8
Cys (1/2)	4.6	4.5	3.9	0	0	0
Val	6.4	6.8	3.0	3.0	3.1	1.0
Met	4.4	4.2	2.3	1.0	1.1	0.9
Ile	6.5	6.4	5.5	0.9	5.3	0
Leu	24.3	23.9	19.5	5.1	14.9	5.8
Tyr	6.0	5.7	4.6	1.0	3.9	2.4
Phe	11.6	11.4	9.5	1.8	7.5	2.8
CM-Cys	0	0	0	0	1.4	2.8

[a] Molecular weight 21,000 bovine growth hormone. [b] Molecular weight 21,000 component of tryptic digest of bovine growth hormone. [c] Molecular weight 16,000. [d] Molecular weight 5000. [e] Molecular weight 11,000. [f] Molecular weight 5000.

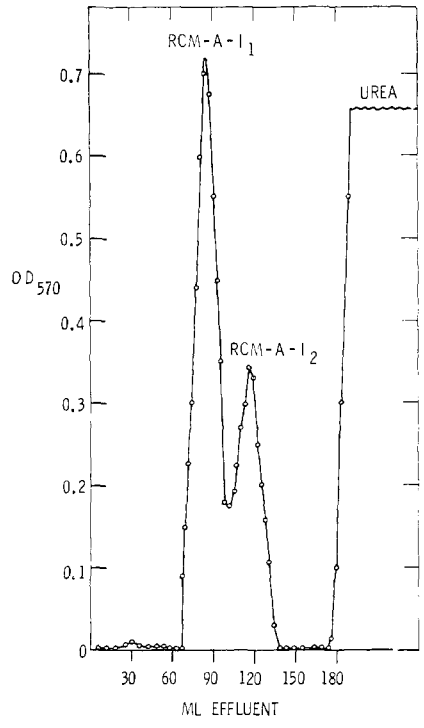

FIGURE 6: Gel filtration on Sephadex G-75 in 50% acetic acid of fraction A-1 after reduction and carboxymethylation in urea. Column size—2.5 × 40 cm. Color developed with ninhydrin.

growth hormone and bovine growth hormone. In Table II, amino acid analyses of A-1 and A-11 are also presented and compared with that of bovine growth hormone and a component of a tryptic digest of bovine growth hormone. Two disulfide bonds were found in A-1 and none in A-11. Only one tyrosine residue was present in A-11. The sum of amino acid residues of A-1 and A-11 corresponds to that of bovine growth hormone and a component of a tryptic digest of bovine growth hormone within experimental error. The amino acid compositions of the reduced and carboxymethylated A-1, RCM-A-1$_1$, and RCM-A-1$_2$ are shown in Table II. The sum of the amino acids in RCM-A-1$_1$ and RCM-A-1$_2$ agreed well with the composition of A-1 (Table II).

Amino- and Carboxyl-Terminal Amino Acids. Thin-layer chromatography of the DNP derivatives revealed in bovine growth hormone two NH_2-terminal amino acids, phenylalanine and alanine. In addition to phenylalanine and alanine analysis of the DNP component of a tryptic digest of bovine growth hormone revealed valine and serine as additional NH_2-terminal amino acids.

Quantitative NH_2-terminal amino acid determination (Table III) of bovine growth hormone revealed 0.51 mole of phenylalanine and 0.51 mole of alanine per mole of bovine growth hormone of molecular weight 21,000 (Sonenberg *et al.*, 1968). In contrast, the number of moles of NH_2-terminal amino acids in the component of a tryptic digest of bovine growth hormone was 0.51 for phenylalanine, 0.48 for alanine, 0.90 for valine, and 0.90 for serine. The number of moles of NH_2-terminal amino acids in A-1 was 0.48 for phenylalanine, 0.44 for alanine, and 0.83 for serine per mole of A-1 of molecular weight 16,000. Valine was found as the NH_2-terminal amino acid of A-11 with 0.92 mole/mole of molecular weight of 5000.

Whereas undigested bovine growth hormone had 1 carboxyl-terminal amino acid/21,000 g, the component of a tryptic digest of bovine growth hormone had 1 phenylalanine and 2 arginines as the carboxyl-terminal amino acids. A-1 had 1 mole of arginine and 1 mole of phenylalanine per molecular weight of 16,000. In contrast, 1 mole of arginine was found in A-11 as a carboxyl-terminal amino acid (Table III). The larger reduced and carboxymethylated fraction

TABLE III: NH_2- and COOH-Terminal Amino Acids.[a]

	BGH	TBGH-d	A-1	A-11	RCM-A-1$_1$	RCM-A-1$_2$
			NH_2-Terminal Amino Acids			
Phe	0.51	0.51	0.48	0	0.49	0
Ala	0.51	0.48	0.44	0	0.45	0
Val	0	0.90	0	0.92	0	0
Ser	0	0.90	0.84	0	0	0.95
			COOH-Terminal Amino Acids			
Phe	0.85	0.93	0.76	0	0	0.84
Arg	0	1.94	0.92	1.1	0.95	0

[a] See Table II for identification of fractions.

TABLE IV: Biological Response of Growth Hormone Derivatives.[a]

Expt	Fraction	Lot	Number of Rats	Daily Dose (μg/rat)	Weight Gain (g ± SE)
		Weight Gain			
1	Solvent		9	0	0 ± 0.7
	BGH	Int std	8	25	12.0 ± 0.8
			8	100	18.1 ± 1.2
	TBGH-d	ALK	8	18	9.5 ± 0.9
			8	70	16.6 ± 0.8
	TBGH-d (50% acetic acid)	NY-11-124-0	8	18	3.6 ± 0.9
			8	70	6.2 ± 0.5
	A-1	NY-11-124-1	8	170	5.8 ± 1.0
	A-11	NY-11-124-4	8	170	12.5 ± 1.4
2	Solvent		8	0	1.5 ± 0.7
	BGH	Int std	8	25	9.5 ± 0.6
			8	100	18.1 ± 0.8
	TBGH-d	ALO	8	16	11.0 ± 0.9
			8	25	18.9 ± 1.4
	TBGH-d (50% acetic acid)	NY-11-238-0	8	25	1.0 ± 0.8
			8	100	8.0 ± 0.9
	A-1	NY-11-238-2	5	250	3.6 ± 0.7
	A-11	NY-11-238-5	8	165	8.5 ± 0.7
		Tibial Width			Epiphyseal Width, μ (±SE)
1	Solvent		5	0	135 ± 13
	BGH	NIH-GH-B7	8	6.25	160 ± 11
			11	18.75	191 ± 15
			9	56.25	222 ± 20
	A-11	NY-11-124-4	8	25	162 ± 6
			7	75	177 ± 13
2	Solvent		6		165 ± 3
	BGH	Int std	6	6	218 ± 9
			6	15	277 ± 19
			6	37.5	309 ± 13
	A-1	AMF-2	6	120	174 ± 5
			6	300	208 ± 8
	A-11	AMF-5A	6	30	233 ± 15
			6	75	259 ± 9
			6	187	286 ± 10

[a] See Table I for identification of fractions.

of A-1 contained equimolar amounts of alanine and phenylalanine as its NH_2-terminal amino acids in amounts similar to bovine growth hormone (Table III). The smaller component of reduced and carboxymethylated A-1 (RCM-A-1_2) had as its NH_2-terminal amino acid 1 mole of serine (Table III).

Biological Response. Since, in repeated experiments, parallel increases in weight gain or tibial width were not established, the biological responses have not been quantitated as per cent of standard. As shown in Table IV, the component of a tryptic digest of bovine growth hormone dissolved in 50% acetic acid lost much of its ability to increase the weight and tibial width of the hypophysectomized rat. Whereas, A-11 retained significant capacity to increase weight and tibial width, this capability was decreased in the A-1 fraction.

Discussion

From a total tryptic digest of bovine growth hormone with an average of two bonds hydrolyzed we have isolated a fraction which appears as one component by disc electrophoresis and sedimentation equilibrium, the latter being a less sensitive criterion of homogeneity. As previously reported (Sonenberg *et al.*, 1968) the component had a molecular weight similar to that of bovine growth hormone, *i.e.*, 21,000, as determined by sedimentation equilibrium. The amino acid composition of this component was identical with that of

bovine growth hormone within experimental error (Mills and Wilhelmi, 1965; Wolfenstein *et al.*, 1966; Free and Sonenberg, 1966). The DNP derivative of this component showed the presence of DNP-valine and DNP-serine in addition to phenylalanine and alanine as NH_2-terminal amino acids. Two new COOH-terminal residues, both arginine, were detected in addition to the one residue of phenylalanine of undigested bovine growth hormone. Assuming that there is only one component, the above result suggests that arginyl–valine and arginyl–serine peptide bonds in a loop of the polypeptide chain occurring between the –S–S– bonds of bovine growth hormone are cleaved by trypsin. It would appear that these arginyl–valine and arginyl–serine bonds are most susceptible to tryptic proteolysis under the conditions of these experiments. There are theoretically 23 peptide bonds, *i.e.*, 11 lysyl and 12 arginyl bonds that can be split by trypsin. If we assume the molecular weight of the component of a tryptic digest of bovine growth hormone to be 21,000, approximately 0.5 mole of DNP-phenylalanine, 0.5 mole of DNP-alanine, 0.9 mole of DNP-valine, and 0.9 mole of DNP-serine were obtained in the acid hydrolysate of 1 mole of the DNP derivative of the component of a tryptic digest of bovine growth hormone (Table III).

Gel filtration of this component of a tryptic digest of bovine growth hormone on Sephadex G-75 revealed a single component at pH 9.5 (Figure 3). The results presented above demonstrate that this stable complex was separated into two fractions, A-1 and A-11. Because the larger fraction (A-1) was eluted slightly faster than egg white lysozyme (mol wt 14,000) and the smaller one (A-11) at the same position as porcine insulin (mol wt 6000) from the column of Sephadex G-75 in 50% acetic acid, the estimated molecular weight of A-1 and A-11 was 15,000–16,000 and 5,000–6,000, respectively. By sedimentation equilibrium the separated fractions, A-1 and A-11, have molecular weights of 16,000 and 5,000, respectively. The molecular weight of this component and undigested bovine growth hormone were previously found to be 20,000–23,000 by sedimentation equilibrium and gel filtration. Like the disc electrophoretic patterns the sedimentation equilibrium patterns of both fractions revealed a high degree of homogeneity.

We previously demonstrated that the molecular weight of this component of a tryptic digest of bovine growth hormone decreased from 22,000 to 16,000 at pH 9.5 in the presence of 5 M urea and suggested the possibility of the existence of two fractions in this component (Sonenberg *et al.*, 1968). In the present study we observed (Figure 4) gel filtration patterns of this component in the presence of urea with two peaks. Dissociation of the fraction A-1 from A-11 also is promoted by increased concentrations of acetic acid (Figure 5). This dissociation is reversible as indicated by the reconstitution of A-1 and A-11 to give a single peak on gel filtration.

Amino-terminal amino acid analysis of bovine growth hormone revealed equimolar amounts of phenylalanine (0.5 mole) and alanine (0.5 mole) per mole of protein of molecular weight 21,000. Two additiional NH_2-terminal amino acids, serine and valine, were found in the component of a tryptic digest of bovine growth hormone. In this study, the isolated fraction A-1 contained 0.5 mole of phenylalanine, 0.5 mole of alanine, and 1 mole of serine as NH_2-terminal amino acids per mole of A-1 of 16,000 molecular weight. Only 1 mole of valine was found as NH_2-terminal amino acid of A-11. The carboxyl-terminal amino acids of the component of a tryptic digest of bovine growth hormone were found to be 1 mole of phenylalanine and 2 moles of arginine. The carboxyl-terminal amino acids (Table III) of A-1 were 1 mole of phenylalanine and 1 mole of arginine while A-11 had 1 mole of arginine. This is consistent with the total carboxyl-terminal amino acids of this component. Amino acid analysis of each fraction demonstrated that the two disulfide bonds of bovine browth hormone or a tryptic digest of bovine growth hormone are in the larger fraction, A-1, and no cystine residue is in the small fraction A-11. The amino acid compositions of each fraction account for not only that of this component but also that of bovine growth hormone (Table II).

Reduction and carboxymethylation of A-1 yielded two fractions, RCM-A-1_1 and RCM-A-1_2 (Figure 6) on a column of Sephadex G-75 in 50% acetic acid. The larger peptide of reduced and carboxymethylated A-1 (RCM-A-1_1) has a molecular weight of approximately 11,000 and contains 0.5 mole of phenylalanine and 0.5 mole of alanine as NH_2-terminal amino acids and 1 mole of arginine as COOH-terminal amino acid. On the other hand, 1 mole each of serine and phenylalanine was found as NH_2- and COOH-terminal amino acids, respectively, in RCM-A-1_2 of molecular weight 5000.

The finding of two different NH_2-terminal amino acids, phenylalanine and alanine, in bovine growth hormone suggests two molar species in bovine growth hormone preparations which differ only in their NH_2-terminal residue (Fellows and Rogol, 1969; Wallis, 1969). Only phenylalanine occurs as the NH_2-terminal residue of human growth hormone (Li *et al.*, 1966). Consequently the two different NH_2-terminal amino acids phenylalanine and alanine in RCM-A-1_1 can be considered to be the original amino-terminal residues of bovine growth hormone.

The carboxyl-terminal amino acid sequence of bovine growth hormone has been reported. RCM-A-1_2 is most likely the peptide at the COOH-terminal end of bovine growth hormone since it has the same amino acid composition as the COOH-terminal peptide originating at serine 138 (J. M. Dellacha, personal communication).

Although A-11 and other growth hormone derivatives had similar biological responses, *e.g.*, weight gain and tibial width increases, the responses were not parallel to a growth hormone standard. Whether this lack of parallelism is due to differences in metabolism of the derivatives and the standard or some other mechanisms is not apparent at this time. It is interesting that the smaller peptide A-11 obtained from the component of a tryptic digest of bovine growth hormone by gel filtration in 50% acetic acid had significant biological activity. Since much of the growth-promoting activity of this component was lost when the latter was dissolved in 50% acetic acid and then freeze dried, it may be that methods other than gel filtration of this component in 50% acetic acid may yield A-11 with greater biological activity. This is under study. These studies suggest that the "active site" of bovine growth hormone may be present in a limited part of the whole protein molecule.

All results described herein support our previously reported postulation (Sonenberg *et al.*, 1968) that the component of a tryptic digest of bovine growth hormone was

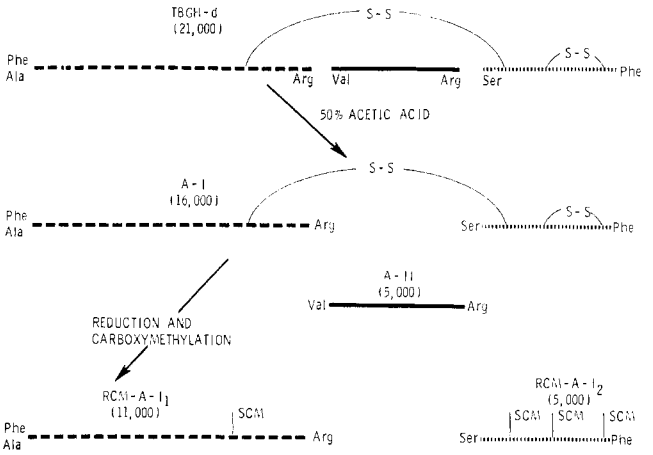

FIGURE 7: Proposed changes in a component of tryptic digest of bovine growth hormone.

formed by the cleavage of arginyl–valine and arginyl–serine peptide bonds in a loop of the polypeptide chain occurring within the disulfide loops of bovine growth hormone (Figure 7). There are no arginyl–valine or arginyl–serine bonds in the small disulfide loop of bovine growth hormone (Dellacha *et al.*, 1968). Hence, the 5000 molecular weight biologically active peptide occurs in the large disulfide loop.

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