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Reaction of Bovine and Ovine Pituitary Growth Hormones with Tetranitromethane†

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ABSTRACT: Bovine and ovine pituitary growth hormones, two structurally similar proteins, were allowed to react with tetranitromethane at 0°. Data obtained from studies of the relative rates of trypsin digestion, elution volumes from exclusion chromatography, circular dichroism spectra, and biological activities indicate close structural similarity of the two modified hormones, both in relationship to their parent compounds, and to each other. Both proteins were shown to have two completely modified, two partially modified, and two unmodified tyrosines at identical sites along the chain. It was also demonstrated that all six tyrosine residues in the bovine hormone are to some extent accessible to the nitrating agent at 25°. The nitrated bovine hormone was also reduced to its amino derivative which, on limited investigation, appears to have similar properties to the native and nitrated proteins.

Comparison of the reported sequences of bovine (Fernández *et al.*, 1972) and ovine (Li *et al.*, 1972) pituitary growth hormones shows a very high degree of homology between these two proteins. In addition, studies of their respective states of aggregation (Dellacha *et al.*, 1968; Bewley and Li, 1972), circular dichroism (Edelhoch and Lippoldt, 1970; Bewley and Li, 1972), and immunological and biological responses (Papkoff and Li, 1958; Moudgal and Li, 1961; Hayashida and Li, 1959) are indicative of a similar molecular architecture. Sedimentation and exclusion chromatographic studies (Dellacha *et al.*, 1968) have shown that BGH[1] exists in slightly basic solutions as a dimer, whereas dissociation to a monomeric form occurs under acidic conditions in low ionic strength media. Osmotic pressure and exclusion chromatography measurements have confirmed this finding for BGH and extended it to SGH (Bewley and Li, 1972). A helix content of 40–50% was found under several conditions (Edelhoch and Lippoldt, 1970; Bewley and Li, 1972). Additional studies performed on BGH have indicated that a molecular transition accompanied by increased unfolding occurs on acidification from pH 5 to 2 (Burger *et al.*, 1966). The effect of urea on the conformation of BGH was studied and several new molecular forms were inferred, based on data from fluorescence, polarization of fluorescence, and ultraviolet difference spectroscopy (Edelhoch and Burger, 1966). In the latter study, optical rotary dispersion indicated that the helical regions remain largely stable, even under conditions leading to considerable disruption of tertiary structure. This may account for the reported stability of hormonal activity under conditions of strong alkali, acid, and heat (Li and Papkoff, 1953; Ellis *et al.*, 1956).

The relationship between the chemical structure of a protein and its biological activity has undergone increased chemical probing in recent years with the advent of milder and more specific reagents for the modification of side-chain residues (Glazer, 1970; Riordan and Sokolovsky, 1971). One of the most successfully employed procedures has been the nitration of tyrosine residues with tetranitromethane (Sokolovsky *et al.*, 1966). Details of this selective chemical reaction on BGH and SGH and the subsequent functional and structural studies of the modified proteins are the subject of this report. Nitrotyrosyl-BGH, prepared at 0°, was also reduced to the corresponding aminotyrosyl derivative and this product was investigated by circular dichroism and bioassay.

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[1] Abbreviations used are: BGH, bovine pituitary growth hormone; SGH, ovine pituitary growth hormone; nitrotyrosyl-BGH, the product formed on reaction of BGH with tetranitromethane; nitrotyrosyl-SGH, the product formed on reaction of SGH with tetranitromethane; aminotyrosyl-BGH, the product formed on reduction of nitrotyrosyl-BGH with sodium hydrosulfite to its amino derivative; $C(NO_2)_4$, tetranitromethane; CD, circular dichroism.

Materials and Methods

The pituitary growth hormones from beef (Li, 1954) and sheep pituitaries (Papkoff and Li, 1958) were isolated according to methods previously described. Each was further purified on Sephadex G-100 (Pharmacia) in 0.05 M Tris buffer, pH 8.5. Glycine and 2-amino-2-(hydroxymethyl)-1,3-propanediol were obtained from Eastman Organic Chemicals, Rochester, N. Y., and used without further purification. Tetranitromethane was purchased from Aldrich Chemical Co., Milwaukee, Wis. The trypsin (TPCK treated, lot no. 900870) was purchased from Calbiochem, Los Angeles, Calif., and α-chymotrypsin (lot No. CDI 61467) was obtained from Worthington Biochemical, Freehold, N. J. All other chemicals were reagent grade. Deionized water was used throughout.

Ultraviolet Spectra. All ultraviolet spectra were read on a Beckman DK-2A recording spectrophotometer using an optical path of 1.00 cm. Absorption in the range 360–340 nm was used to correct for light scattering in solutions as described by Beaven and Holiday (1952). The protein concentration of the unmodified hormone solution was obtained using a value $E^{1\%}_{1\text{ cm }277\text{ nm}} = 7.30$ as determined previously (Bewley and Li, 1972). The protein concentration of nitrated derivatives was obtained by measuring the absorbance at 428 nm, using 4500 as the molar extinction coefficient per nitrotyrosine residue in solutions of pH >9.0. The determination of the degree of nitration for the modified proteins is given below. The concentration of the aminotyrosyl-BGH derivative was measured in the Tris buffer, using $E^{1\%}_{1\text{ cm, }282\text{ nm}} = 8.9$.[2]

Nitration Reaction. The procedure used was as described previously by Sokolovsky *et al.* (1966), with the exception that the reaction was carried out[3] at 0°. The protein (100 mg, 3.95 μmol) was dissolved in 25 ml of Tris buffer (0.05 M, pH 8.5) at 0° and an 0.85 M solution of $C(NO_2)_4$ in 95% ethanol was added to give a fivefold molar excess over tyrosine content. The reaction was allowed to proceed for 50 min and then terminated by desalting in the cold on Sephadex G-25 in the Tris buffer. After dialysis against dilute ammonia (pH 9.0) and lyophilization, the nitroprotein was redissolved in the Tris buffer and passed through a Sephadex G-100 column (BGH, 3.0 × 68 cm; SGH, 2.4 × 78 cm) to separate the dimeric fraction from higher molecular weight aggregates. The buffer was removed by dialysis and the nitrated material obtained on lyophilization.

A qualitative estimate of the course of nitration was obtained by measuring the optical density of suitably diluted aliquots at 428 nm in a Zeiss spectrophotometer, Model PM Q II. The amount of nitrotyrosine chromophore per mole of protein was quantitatively determined, after equilibrating the lyophilized product with atmospheric moisture, by measuring the absorbance at 428 nm of a carefully weighed out solution of protein in 0.1 M NaOH. A 15% moisture content was assumed for the protein. In addition, the degree of nitration was obtained from amino acid analysis.

Cyanogen Bromide Cleavage of Nitrotyrosyl-BGH. CNBr (200 mg, 1.9 mmol) was dissolved in 3 ml of 75% formic acid and added to nitrotyrosyl-BGH (100 mg, 3.95 μmol) at 25°. The reaction vessel was allowed to stand in the dark for 24 hr and then diluted and lyophilized. Lyophilization was repeated two times to remove all traces of CNBr. Purification and subsequent studies on this material are described below (see Results).

Reduction of Nitrated BGH to Its Amino Derivative. Nitrotyrosyl-BGH (100 mg, 3.95 μmol) was reduced in 10 ml of the Tris buffer containing sodium hydrosulfite (120 mg, 0.69 mmol) at 25° (Sokolovsky *et al.*, 1967). After 5 min, the yellow color of the nitrophenolate anion was abolished, and the reaction was terminated by the addition of 3-nitrotyrosine. After desalting on Sephadex G-25, the crude material was purified by chromatography on Sephadex G-100. The dimeric product was recovered by dialysis and lyophilization.

Amino Acid Analysis. Amino acid analyses were carried out according to the procedure of Spackman *et al.* (1958), in a Beckman amino acid analyzer, Model 120-C. All samples were hydrolyzed with constant boiling HCl in a sealed, evacuated glass tube for 22 hr at 110°. A ninhydrin color yield of 60.5 relative to Phe = 65.0 and a loss due to hydrolytic destruction of 15% were found for nitrotyrosine under these conditions.

Spectrophotometric Titrations. Spectrophotometric titrations were measured in either a Beckman DK-2A spectrophotometer or a Zeiss Model PM Q II spectrophotometer in stoppered 1.00-cm silica cuvets, using the difference spectra technique previously described (Bewley *et al.*, 1969). A stock solution was prepared at a known protein concentration (~1 mg/ml) in 0.15 M KCl which was 0.05 M in Tris buffer, pH 8.5. Two milliliters of this solution was placed in a stoppered cuvet and kept in the reference cell. The pH of the remaining stock solution was raised in increments of 0.3 pH unit by additions of 10 N KOH, total volume additions never exceeding 3% of the solution volume. The pH of the new solution was measured both before and after scanning from 360 to 260 nm. The measured spectra were corrected for light scattering as described above. Reverse titrations were performed with glacial acetic acid after reaching pH 13. All titrations were carried out under a nitrogen atmosphere and maintained at 25 ± 1° by a water jacket.

Rate of Tryptic Digestion. The digestion was carried out at pH 9.0 in 0.15 M KCl under a nitrogen atmosphere in a pH-stat (Radiometer titrator type TTT11b, pH meter 26C) within a water-jacketed cell at 25 ± 1°. Constant pH was maintained by the automatic addition of 0.002 N CO_2-free KOH. The alkali uptake was recorded as a function of time.

Circular Dichroism Spectra. Spectra were obtained on a Cary Model 60 spectropolarimeter equipped with a Model 6002 circular dichroism attachment. Details of all procedures and methods of calculation have been described previously (Bewley *et al.*, 1969). The concentration of the nitroproteins was determined after the CD spectra were completed by titrating to pH >9.0 with 1 N NaOH and measuring the absorbance at 428 nm.

Ultraviolet Fluorescence Spectra. Measurements were made at room temperature with a Perkin-Elmer Model MPF-2A spectrofluorometer. The excitation and emission monochrometers were set at 286 and 330 nm, respectively. The concentration of nitroprotein solutions in acid (0.1 M Gly-HCl buffer, pH 3.6) was determined on the basis of $E^{1\%}_{1\text{ cm, }277\text{ nm}} = 12.9$ as obtained from weighed samples, after correcting for light scattering, and assuming a 15% moisture content.

[2] This value was calculated by correcting the absorptivity of native BGH for content of aminotyrosine residues in the derivative. An ϵ_m of 2500 was found for aminotyrosine residues at 282 nm and pH 8.5.

[3] A preliminary investigation of the products obtained on nitration of BGH and SGH at 25° was also made and will be discussed at appropriate points in the text.

TABLE I: Amino Acid Composition of Native and Nitrated Beef and Sheep Growth Hormones.

Amino Acid	BGH[a]	NO_2-BGH	SGH[b]	NO_2-SGH
Tryptophan	1	0.9[c]	1	*f*
Lysine	11	11.0	11	10.7
Histidine	3	3.0	3	3.1
Arginine	13	13.0	13	12.3
Aspartic acid	16	16.0	16	16.2
Threonine	12	11.5	12	11.8
Serine	12	12.1	13	11.6
Glutamic acid	24	24.4	24	24.0
Proline	6	4.7	6	6.0
Glycine	10	10.0	9	9.8
Alanine	15	14.4	15	14.1
Half-cystine	4	3.6	4	4.2
Valine	6	6.0	7	7.2
Methionine	4	3.8	4	3.2
Isoleucine	7	7.0	7	6.7
Leucine	26	26.4	27	26.0
Tyrosine	6	2.7[d]	6	3.0[d]
Phenylalanine	13	12.3	13	12.8
Nitrotyrosine		2.8[d] (3.0[e])		2.7[d] (2.8[e])

[a] Taken from the sequence of Fernández *et al.* (1972). [b] Taken from the sequence of Li *et al.* (1972, 1973). [c] Determined from the total enzyme digest (Pronase and chymotrypsin followed by prolidase and leucine aminopeptidase). [d] A 10% correction for Tyr destruction and a 15% correction for nitrotyrosine destruction have been made. [e] Determined spectrophotometrically (see text). [f] Not determined.

All solutions were measured at the same concentration, *i.e.*, 0.137 mg/ml, and on the same day.

Peptide Mapping. The nitrated proteins were digested with trypsin or chymotrypsin in 0.1 M NH_4OAc buffer, pH 8.4, for 16 hr at 37°, using an enzyme:substrate ratio of 1:25. After digestion, the peptides were oxidized with performic acid. Chromatography was performed on Whatman No. 3MM paper with *n*-butyl alcohol–acetic acid–water (4:1:5, v/v, upper phase), and this was followed by high voltage electrophoresis in formic acid–acetic acid–water, pH 2.1 (218 ml of 90% HCOOH and 63 ml of CH_3COOH per liter of solution), for 1 hr at 2000 V (Gilson High Voltage Electrophoretor, Model D). The nitrotyrosine peptides were detected as yellow spots after exposure to an ammonia atmosphere and eluted from the paper with 0.1 N NH_4OH.

Biological Studies. The growth promoting potency of the preparations was estimated by the rat tibia test (Greenspan *et al.*, 1949). Immunological properties of nitrotyrosyl-BGH were also investigated by the agar gel double diffusion technique (Ouchterlony, 1949) using rabbit antisera to BGH.

Results

Nitration and Exclusion Chromatography. BGH and SGH showed about the same rate of nitration as evidenced by the absorbance at 428 nm of suitably diluted aliquots. The nitration reaches an apparent plateau in 40–50 min, but small increases in optical density with time were noted. The dimeric fractions obtained from exclusion chromatography on Sephadex G-100 gave V_E/V_O ratios of 1.70–1.75. This compares with a V_E/V_O ratio of 1.60–1.65 for the dimer fractions of BGH and SGH (Bewley and Li, 1972). This retardation may be due to absorption of the protein onto the gel or to a slight shift in equilibrium toward the monomeric form. The final yield of the major product was high (56% for nitrotyrosyl-BGH, 59% for nitrotyrosyl-SGH) as compared to the yields following nitration of human growth hormone (Ma *et al.*, 1971; Maddaiah *et al.*, 1972) and ovine lactogenic hormone (Ma *et al.*, 1970) at 25°. Experiments conducted on BGH at 25° also lead to the isolation of substantially less dimer material, *i.e.*, 25%. The polymeric material formed may, in part, be due to intermolecular cross-linking of tyrosine residues induced by the $C(NO_2)_4$ (Boesel and Carpenter, 1970; Williams and Lowe, 1971). This hypothesis is supported by the amino acid analysis of the aggregated material which consistently failed to give a material balance for the values of nitrotyrosine plus tyrosine.[4]

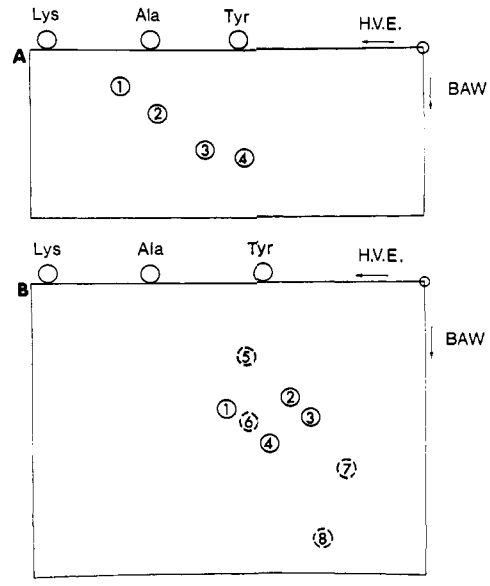

FIGURE 1: Schematic diagram of the pattern of the nitrotyrosine peptides from both beef and sheep pituitary growth hormones: (A) chymotryptic digestion; (B) tryptic digestion. The solid lines represent nitrotyrosine peptides (detected on exposure to ammonia vapor) obtained after nitration at 0°. The dotted lines represent additional peptides obtained when the nitration was carried out at 25°.

The degree of nitration was estimated as described above under Methods. As indicated in Table I, both procedures used show the nitration of approximately three tyrosyl residues.

Identification of the Nitrated Residues in BGH and SGH. The nitrotyrosine-containing peptides obtained after reaction with $C(NO_2)_4$ at 0° and digestion with trypsin or chymotrypsin were located on peptide maps (Figure 1). The corresponding amino acid analyses of the eluted peptides for which assignments could be made are given in Table II. The reported amino acid sequence of SGH is given in Figure 2.[5] No differences were found between BGH and SGH in

[4] Several attempts were made to prepare fully nitrated protein by reaction in 5 M guanidine-HCl. Each effort led to the isolation of polymeric material exclusively, and the amino acid analysis (determined under several conditions) showed approximately three nitrotyrosines and almost no tyrosine residues. Nitration of performic acid oxidized material also gave unsatisfactory results.

[5] For the purposes of clarity in comparing these hormones, the amino acid residues for BGH and SGH are numbered according to the reported sequence of SGH (Li *et al.*, 1972).

TABLE II: Amino Acid Compositions and Assignments for Various Peptide Fragments from Nitrated Beef and Sheep Pituitary Growth Hormones.

	Tryptic Peptides after Modification at 0° with $C(NO_2)_4$[a,b]						Chymotryptic Peptides after Modification at 0° with $C(NO_2)_4$[a]								Tryptic Peptides of BGH after Modification at 25° with $C(NO_2)_4$[a,g]			CNBr Fragments of BGH after Modification at 0°with $C(NO_2)_4$[j]		
	BGH			SGH			BGH				SGH							OCNBr-2[k]	CNBr-3	OCNBr-4[k]
Amino Acid[c]	2	3	4	2	3	4	1	2	3	4[e]	1	2	3	4[e]	5	7	8			
Lysine							1.8	1.2			1.8	1.1			1.3	0.7		5.4	1.8	2.8
Histidine							1.0	0.9			0.8	0.8						2.0		1.0
Arginine	1.0		0.9	1.3		0.8	1.0		0.9	1.0	0.9		0.9	1.6	0.9	0.9		6.0	1.8	2.9
Aspartic acid			0.5	0.7			1.1				1.1			1.0	3.2	1.2	0.8	9.4	3.6	3.5
Threonine	1.0	1.5	1.4	1.5	1.6	1.8	1.8	1.8	1.0		2.0	2.1	1.0	0.9				8.1	2.5	1.8
Serine														1.0		2.1		8.8		1.8
Glutamic acid	2.0	1.2	1.1	3.1	1.2	1.2	1.1	1.0	0.9	2.2	1.1	1.1	1.1	2.5	1.9	1.0		17.7	4.2	1.9
Proline	0.6			2.7						1.0				1.0				4.6	0.7	
Glycine	1.0			1.1						1.1				2.0		2.0	1.1	6.2	2.0	1.2
Alanine																		9.0	1.3	1.1
Half-cystine																1.0[i]		1.4		1.1
Valine				0.7										0.9				4.5	0.4[l]	0.7
Methionine															1.0[h]			0.9[m]	0.8[m]	0.8[m]
Isoleucine	1.0			1.0						1.0				1.0				5.3	1.0	
Leucine			1.0	0.7		1.0	1.0				1.0					2.0	2.4	16.7	2.0[l]	5.3
Tyrosine[d]														0.4		0.3		1.2	0.9	0.7
Phenylalanine															0.9	1.0		7.5	0.9	1.2
Nitrotyrosine[d]	1.1	0.8	1.0	1.1	1.0	1.0	0.9	0.9	1.1	0.8	1.0	0.9	1.0	0.8	0.9	0.8	0.8	1.7		0.8
Assignment[n] positions	35–42	172–175	172–177	35–42 + 126–133 (35% impurity)	172–175	172–177	166–175	170–175	33–36	37–43	166–175	170–175	33–36	37–43[f] (50%) + 104–110[f] (50%)	140–150	158–167 + Ser, Glu, Gly impurities	158–162	6–124	125–149	150–174

[a] The positions of these peptides in peptide maps are illustrated in Figure 1. [b] Tryptic peptide 1 was impure and was obtained in very small quantities from both nitroproteins. However, the amino acid composition was consistent with the tetrapeptide 109–112. [c] Tryptophan was not determined. [d] A 10% correction for tyrosine destruction and a 15% correction for nitrotyrosine destruction have been made. [e] According to the reported sequences of BGH and SGH, these peptides differ in the relative positions for Pro and Glu (residues 38 and 39). This sequence variation might be reflected in small differences in chromatographic and/or electrophoretic behavior. This may explain our ability to isolate the pure peptide only in the BGH experiment. [f] The relative distribution of tyrosine and nitrotyrosine in these two peptides could not be determined from this analysis. [g] Tryptic peptide 6 was impure and no assignment was possible. [h] Identified as methionine sulfone. [i] Identified as cysteic acid. [j] CNBr fragments 1 (residues 1–5) and 5 (residues 180–191) contain no tyrosine and were not investigated. [k] The purified fragments were obtained after performic acid oxidation. [l] Seavey *et al.* (1971) have reported microheterogenicity in position 125 of BGH, with both valine and leucine present. [m] Determined as homoserine and homoserine lactone. [n] See Figure 2.

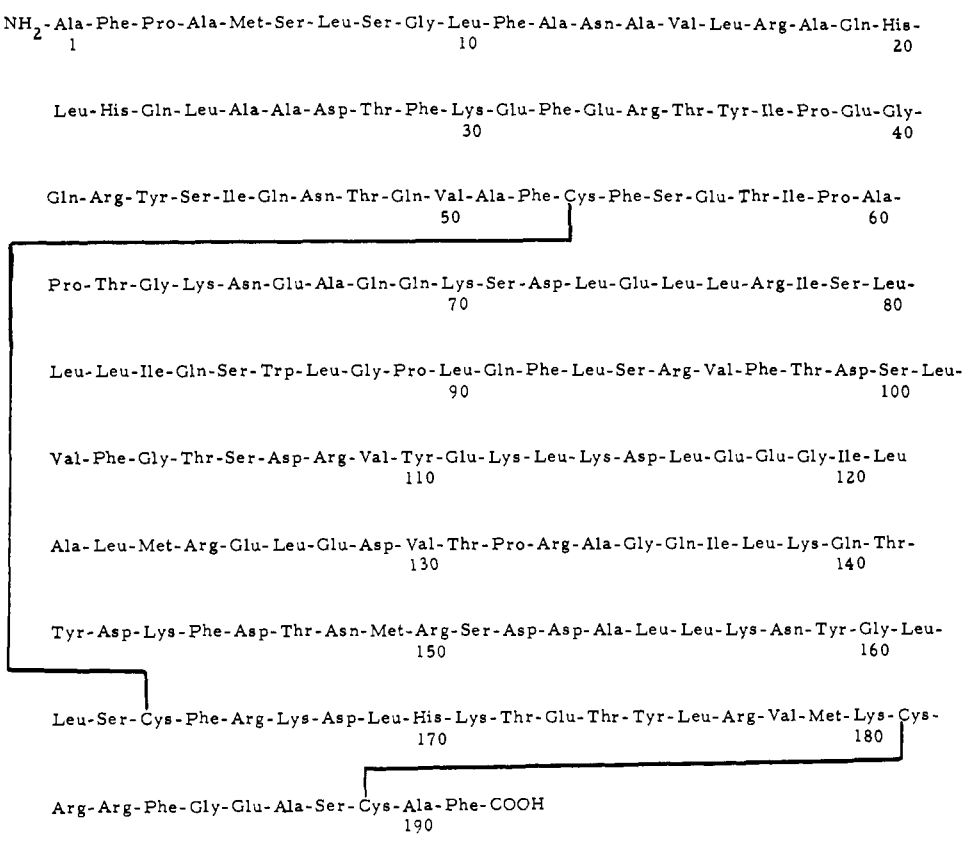

FIGURE 2: The amino acid sequence of sheep pituitary growth hormone (Li *et al.*, 1972).

the patterns obtained for the nitrotyrosine-containing peptides in the peptide maps, and analysis of the peptides showed nearly identical results for the two nitroproteins. Peptides corresponding to the nitration of tyrosine residues 36 and 175 are found from both the tryptic and chymotryptic digestions. Tryptic peptide 1 was impure and obtained in very low yield; a conclusive assignment was not possible but the predominance of Val, Lys, Glu, and NO_2Tyr make it likely that Tyr-110 in the tetrapeptide Val-Tyr-Glu-Lys of the molecule has been nitrated to a limited extent. Peptide 4 from the chymotryptic digest of BGH was pure and indicates the nitration of Tyr-43. The corresponding SGH peptide was not obtained in pure form and the amino acid analysis shows the presence of both Tyr and NO_2Tyr; a tentative assignment indicates the presence of peptides containing Tyr-110 and Tyr-43 but the relative degrees of nitration could not be determined from this analysis.

Peptide maps of BGH and SGH which had been nitrated at 25° and digested with trypsin were identical with each other and these revealed the presence of several new nitrated peptides as compared to the 0° reaction (Figure 1). Amino acid analyses of the eluted peptides from the nitrotyrosyl-BGH peptide map (25° reaction) show convincing evidence for the nitration of all tyrosine residues under these conditions (Table II), although the total degree of nitration was incomplete, *i.e.*, 4.9 residues/mol of protein.

In order to examine more closely the degree of nitration of each tyrosine residue, cyanogen bromide cleavage of nitrated bovine growth hormone (from the 0° reaction) was carried out. The fragments were purified in good yield as described for SGH (Li *et al.*, 1973) (Table II). Fragments 1 (residues 1–5) and 5 (residues 180–192) contain no tyrosine residues and therefore were not considered further. Fragment 3 (residues 125–149) contains 0.85 residue of Tyr-142 and no nitrotyrosine, confirming the results from peptide mapping. Fragment 4 (residues 150–179) contains Tyr-159 and Tyr-175. The recovered peptide contains 0.7 residue of Tyr and 0.8 residue of NO_2Tyr. Since it has been established that Tyr-159 is nonreactive under the conditions of the modification, Tyr-175 must be essentially fully nitrated. Fragment 2 (residues 6–124) contains tyrosines 36, 43, and 110. The amino acid analysis reveals the presence of 1.7 residue of NO_2Tyr and 1.2 residue of Tyr per mole of protein. The fragment was subjected to tryptic digestion and exclusion chromatography on G-50 Sephadex (Superfine). The tyrosine- or nitrotyrosine-containing fractions were found mainly in two peaks. The first fraction (higher molecular weight peptides) contained primarily the peptide comprising residues 43–64 (80% pure) and was about 50% nitrated (*i.e.*, Tyr 36%, NO_2Tyr 40%) in Tyr-43. Small amounts of several other non-tyrosine-containing peptides were also identified. The second tyrosine-containing peptide fraction showed the presence of several peptides. Amino acid analysis gave a Tyr : NO_2Tyr ratio of 1 : 2.4. Peptide mapping of this fraction showed the presence of two NO_2Tyr-containing peptides. One component on elution and analysis was identified as the tetrapeptide comprising residues 109–112 containing Tyr-110 in fully nitrated form. The yield, however, was very low. A Pauli positive peptide adjacent to this tetrapeptide on the map was shown on analysis to contain the same peptide in much larger amounts and in the nonnitrated form. The second nitrated peptide on analysis was shown to be the octapeptide comprising residues 35–42 containing Tyr-36 in its fully nitrated form. Therefore, it is concluded that, at 0°, tyrosine residues 36 and 175 are completely nitrated, Tyr-43 is about 50% nitrated, Tyr-110 is nitrated only to a limited extent (20–35%), and Tyr-142 and Tyr-159 are fully resistant to the chemical modification.

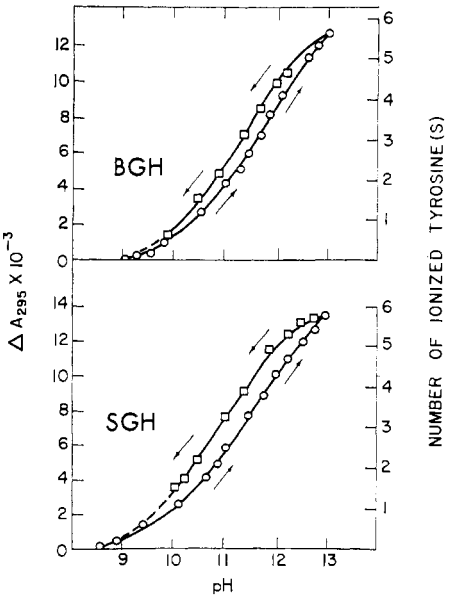

FIGURE 3: Spectrophotometric titration of beef and sheep growth hormones. The procedure for the forward and reverse titrations is described in the text.

Ionization of Tyrosyl Residues in BGH and SGH. Figure 3 shows the spectrophotometric titration curves of the tyrosyl residues in the native hormones. The pK_{app} values of 11.45 for BGH and 11.35 for SGH as determined from the midpoints of the ionization curve are considerably higher than the reported value of 10.2 found for free tyrosine (Nasaki and Tanford, 1967). Using a value of 2310 at 295 nm, for the change in molar extinction coefficient of tyrosine upon ionization (Bewley *et al.*, 1969), 5.5 tyrosines in BGH and 5.8 tyrosines in SGH were found to be ionized at pH 13. This compares with a previous report of complete ionization at pH 13 using an extinction coefficient of 2340 (Edelhoch *et al.*, 1966). The back titrations show the irreversibility of the denaturation caused by alkali. The midpoint of the back titration curve is closer to the "normalized" condition indicative of tyrosine residues more fully exposed to the solvent.

The spectrophotometric titration curves of the nitrated proteins could not be determined due to the insolubility of the products in much of the pH range of interest (*i.e.*, pH 5–9). However, it was ascertained that the nitrophenol chromophore was fully ionized at pH 9.0 in both derivatives.

Rates of Tryptic Digestion. The relative rates of alkali uptake of BGH, SGH, their nitro derivatives, and their performic acid oxidized derivatives are shown in Figure 4. The nitro derivatives (prepared at 0°) are digested at rates comparable to those of the starting materials and much slower than either products obtained from nitration at 25° or performic acid oxidized materials.

Fluorescence. The relative fluorescence intensities for the modified and native materials in two solvents are shown in Figure 5. The native hormones have identical patterns at pH 8.5 and very similar spectra at pH 3.6. For both, acidification shifts the maximum intensity from 328 to 334 nm and increases the intensity threefold. Nitration leads to a considerable decrease in quantum efficiency. The two nitrated derivatives have similar spectra with maxima at 325 nm for the nitrated beef hormone and 330 for the nitrated sheep material, the latter showing 10% more fluorescence at its maximum position. Acidification has almost no effect on the

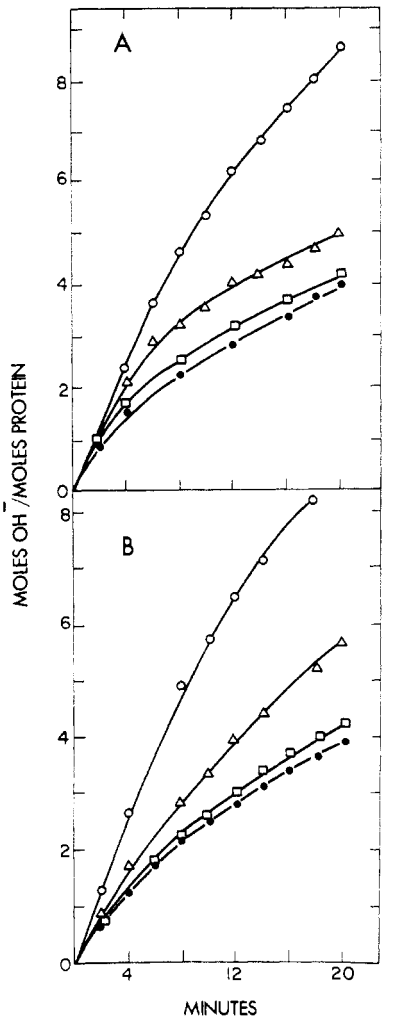

FIGURE 4: (A) Kinetics of the tryptic digestion of SGH (●), nitrotyrosyl-SGH obtained from a 0° reaction (□), nitrotyrosyl-SGH obtained from a 25° reaction (△), and performic acid oxidized SGH (○). The enzyme:hormone ratio is 1:118 at pH 9.0 and 25 ± 1°. (B) Kinetics of the tryptic digestion of BGH (●), nitrotyrosyl-BGH obtained from a 0° reaction (□), nitrotyrosyl-BGH obtained from a 25° reaction (△), and performic acid oxidized BGH (○). The enzyme:hormone ratio is 1:100 at pH 9.0 and 25 ± 1°.

fluorescence of nitrotyrosyl-BGH while nitrotyrosyl-SGH increases its fluorescence[6] intensity ~40%.

Circular Dichroism. The CD spectra of nitrotyrosyl-BGH, nitrotyrosyl-SGH, and aminotyrosyl-BGH are shown in Figure 6. The spectrum of unmodified BGH (Bewley and Li, 1972) has been added for comparison. In the region of amide bond absorption all these spectra show a negative peak near 220–222 nm. The spectra of unmodified BGH and the aminotyrosyl-BGH derivative are essentially identical. Similarly, nitrotyrosyl-BGH and -SGH exhibit nearly

[6] The increase in fluorescence at 330 nm was also studied in the presence of a large molar excess of dithiothreitol (1000-fold over cystine content). Following addition of the reducing agent, SGH and its nitrated derivative increased fluorescence at substantially greater rates than BGH and its derivative. For example, SGH showed a 50% increase in intensity in 13 min as compared to 33 min for BGH. It was not possible to follow the reactions to their end points due to precipitation of the reduced protein. This increase in fluorescence probably reflects a greater denaturation around the microenvironment of the tryptophan in SGH, which results in a reduction in the internal quenching. The loss of rigidity around tryptophan may be one measure of a higher rate of unfolding and/or a higher rate of disulfide bond reduction of the ovine proteins in comparison to the bovine.

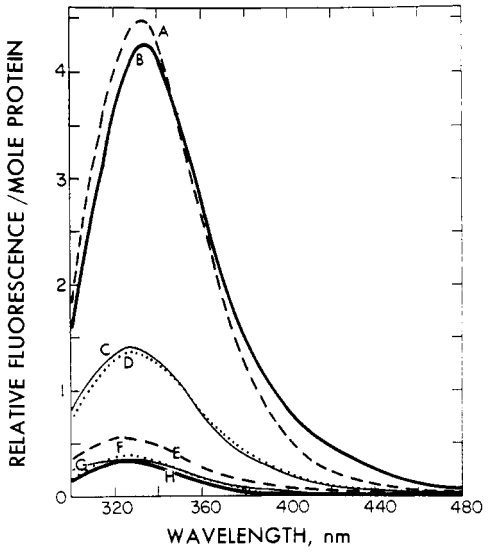

FIGURE 5: Relative fluorescence intensity of BGH, SGH, and their nitro derivatives at pH 3.6 (0.1 M Gly-HCl) and pH 8.5 (0.05 M Tris): (A) SGH (pH 3.6); (B) BGH (pH 3.6); (C) SGH (pH 8.5); (D) BGH (pH 8.5); (E) nitrotyrosyl-SGH (pH 3.6); (F) nitrotyrosyl-SGH (pH 8.5); (G) nitrotyrosyl-BGH (pH 3.6); (H) nitrotyrosyl-BGH (pH 8.5).

equivalent spectra, although these latter two derivatives show slightly more negative ellipticities than the unmodified protein. From the ellipticity values (Table III) at 220–222 nm, α-helix contents of 50% for the unmodified and aminotyrosyl-BGH and 55% for the two nitrotyrosyl derivatives have been calculated. In the region of side-chain absorption, the aminotryrosyl-BGH exhibits a spectrum that is very similar to that of unmodified BGH, except for an overall shift toward more positive values of ellipticity. Again, in this region of the spectrum, the two nitrotyrosyl derivatives give identical spectra, with a broad, weak negative band(s) between 380 and 315 nm, a complex system of overlapping bands between 310 and 265 nm, and a strong positive band with an apparent maximum at 250 nm.

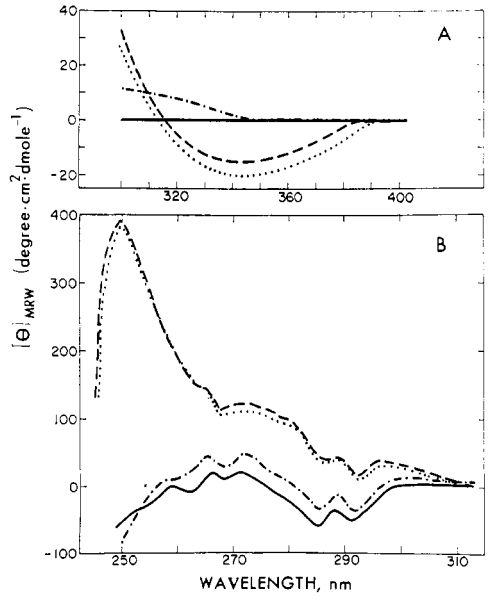

FIGURE 6: Circular dichroism spectra in the long-wavelength region (A) and the region of side-chain absorption (B) of: nitrotyrosyl-BGH (···), nitrotyrosyl-SGH (- - -), and aminotyrosyl-BGH (·-·-). The spectra of BGH (—) have been reproduced for comparison; it has no dichroism above 310 nm and is identical with the base line in this region. The solvent in all cases was 0.1 M Tris buffer (pH 8.2).

TABLE III: Mean Residue Molecular Ellipticities and α-Helix Contents of BGH, Nitrotyrosyl-BGH, Nitrotyrosyl-SGH, and Aminotyrosyl-BGH.[a]

Preparation	$-[\theta]_{221\ nm}$	% Excess Right-Hand α Helix
BGH	18,700	50
Nitrotyrosyl-BGH	20,300	55
Nitrotyrosyl-SGH	19,700	55
Aminotyrosyl-BGH	18,850	50

[a] All spectra were taken at pH 8.2, 0.1 M Tris-Cl buffer. The CD spectra of BGH are essentially identical with SGH under these conditions (Bewley and Li, 1972). [b] Calculated to the nearest 5%.

Biological Studies. Table IV shows the results of the tibia assays. The growth-promoting activity is retained in both nitrohormones, as well as in the aminotyrosyl-BGH. In the agar gel double diffusion experiment, the antisera to BGH produced identical precipitin lines with the native and modified hormones, indicating that the antigenic sites remain largely intact.

Discussion

The finding that two out of six tyrosine residues (Tyr-36 and Tyr-175) of bovine and ovine growth hormones are completely nitrated under mild conditions with $C(NO_2)_4$, while two others (Tyr-43 and Tyr-110) are only partially modified and two (Tyr-142 and Tyr-159) are completely unreactive, suggests that there are three different types of tyrosine residues, *i.e.*, on the exterior of the protein and thereby exposed to solvents and reagents, partially exposed and thereby only slowly modified, or buried within the interior

TABLE IV: Growth Promoting Potency of Native, Nitrotyrosyl, and Aminotyrosyl Beef and Sheep Pituitary Growth Hormones as Measured by the Rat Tibia Test.

Preparation	Dose (μg)	Response[a] (μ)
BGH	20	216 ± 5 (5)
	60	262 ± 8 (5)
Nitrotyrosyl-BGH	20	227 ± 3 (5)
	60	272 ± 6 (5)
SGH	20	224 ± 6 (5)
	60	249 ± 2 (6)
Nitrotyrosyl-SGH	20	222 ± 4 (5)
	60	267 ± 9 (5)
Aminotyrosyl-BGH	20	232 ± 4 (5)
Saline	0	168 ± 2 (12)

[a] Expressed as the mean ± the standard error of the mean, followed by the number of test animals in parentheses.

hydrophobic region and thereby inaccessible to reagent. The spectrophotometric titrations support this conclusion, BGH having a pK_{app} = 11.45 while SGH shows a pK_{app} = 11.35. These abnormally high values compared to the ionization of "free" tyrosine with a pK_{app} = 10.1–10.3 indicate either that a portion of the tyrosine residues is incompletely exposed or that their ionization is affected by neighboring groups or charges. Another point to be considered is that both native hormones exist in a solution as dimers (Dellacha *et al.*, 1968; Bewley and Li, 1972) under the conditions of the nitration (pH 8.5 0.05, M Tris buffer). It is likely that these dimers are stabilized primarily by attractive forces between hydrophobic residues. Some of the tyrosine residues may be partially or fully protected from the nitration reaction by being buried in this region.

Exclusion chromatography of both nitrated proteins (Sephadex G-100, 0.05 M Tris, pH 8.5) gave elution patterns which were slightly retarded in comparison with the native materials. This may be a result of a tendency toward absorption of the nitrotyrosine residue by the resin; alternatively, nitration might result in a product whose state of aggregation in solution has a small component of monomer in dynamic equilibrium with the dimeric form. Reduction of nitrotyrosyl-BGH to its amino derivative does not result in any change in gel filtration pattern.

The rate of digestion of the native hormones, their nitrated derivatives, and their oxidized derivatives was investigated as an indication of conformational change. It has been proposed that the flexibility of a protein is reflected in the efficiency with which it is digested by proteolytic enzymes (Markus, 1965). Since nitration should have no effect on the lysine and arginine residues, the relative rates of tryptic digestion should be a measure of the availability of peptide bonds for digestion. The equal rate of digestion of each hormone, as compared to the nitrated derivative (prepared at 0°), is evidence that little or no loss of rigidity has resulted from the nitration. On the other hand, products derived from nitrations at 25° are digested considerably more rapidly, while performic acid oxidized proteins demonstrate the largest disruption of conformational rigidity. It has been reported (Bewley and Li, 1972) that BGH and SGH exhibit nearly identical CD spectra in the regions of amide bond and side-chain absorption. This was interpreted as indicating that the overall conformations of these molecules are essentially identical, with equivalent contents of secondary structure(s), as well as similarly asymmetric microenvironments for the various side-chain chromophores. From its CD spectrum, aminotyrosyl-BGH appears to be very similar in conformation to the unmodified proteins. In BGH, the two negative maxima at 291 and 285 nm have been assigned to a ^1L_b indole transition and a composite of indole and tyrosyl transitions, respectively. Most of the fine structure seen between 275 and 255 nm has been assigned to phenylalanine residues (Bewley and Li, 1972). These same chromophore assignments may be given to the bands in aminotyrosyl-BGH, although the nature of the overall shift toward somewhat more positive ellipticity values is uncertain. In parallel with the behavior of the unmodified hormones, nitrotyrosyl-BGH and nitrotyrosyl-SGH exhibit essentially identical spectra. The pattern produced in the spectra of the unmodified protein by the indole transition at 291 nm and the composite band at 285 nm, as well as part of the fine structure near 260–270 nm, can still be discerned in the spectra of the nitrotyrosyl derivatives, although in these derivatives the corresponding bands appear superimposed on a strong, positive envelope. This envelope and the intense positive band at 250 nm are probably associated with a transition of the nitrotyrosyl chromophores.[7] Clearly, the nitration of these hormones yields products which are as similar in conformation to each other as were the unmodified forms. This is consistent with the chemical evidence presented above indicating that nitration occurs on the same tyrosine residues in both proteins.

The immunochemical cross-reactivity of BGH and its nitrated derivative using the agar gel double diffusion technique with rabbit antisera to bovine growth hormone showed equivalent precipitin lines indicating that the nitration has not produced any noticeable immunochemical changes. The biological response, as measured by the rat tibia assay, showed a high degree of activity for both nitroproteins as well as for the amino bovine growth hormone derivative.

Our results indicate that the mild nitration of both beef and sheep pituitary growth hormones gives products which are apparently similar in physical, chemical, and biological properties, both to each other and to the native hormone. In addition, these parameters seem largely unaffected by reduction of nitrated bovine growth hormone to its amino derivative. Three types of tyrosine residue, *i.e.*, very reactive, slowly reactive, and unreactive, have been identified.

Acknowledgments

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Immunochemistry of a Synthetic Peptidoglycan–Precursor Pentapeptide†

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ABSTRACT: A protected pentapeptide with the sequence found in several precursors to a major class of bacterial peptidoglycans, Ala-γ-DGlu-Lys-DAla-DAla, has been synthesized by classical methods. After selective deprotection of the α-amino group, the pentapeptide was covalently linked to the random polypeptide $(Glu^{60}Ala^{40})_n$. The fully deprotected conjugate evoked an antibody response in each of two rabbits immunized. The specificity of the antibodies obtained from a single bleeding was studied by radioimmunoassay, using related polypeptides and haptens as inhibitors. The immunodominant region comprised the carboxyl terminus of the pentapeptide, *X*-Lys-DAla-DAla. The tripeptide with the *tert*-butoxy-protecting group in position *X* was almost as effective an inhibitor as the precursor pentapeptide with Ala-DisoGlu in position *X*. The antibody was absorbed specifically by a natural peptidoglycan from group C *Streptococcus*.

A major class of bacterial peptidoglycans is synthesized from several glycopeptide precursors containing the pentapeptide, Ala-γ-DGlu-Lys-DAla-DAla. This peptide is attached by the α-amino group to the glycan, which consists of alternating residues of *N*-acetylglucosamine and *N*-acetylmuramic acid (for recent reviews, see Osborn, 1969; Schleifer and Kandler, 1972). Several modifications of this peptide can occur prior to the final transpeptidation step, including amidation of D-glutamic acid and peptidation of the ε-amino group of lysine to form the so-called "peptide bridge." In the transpeptidation, a bond is formed between the penultimate D-alanine of one chain (with the release of the terminal D-alanine) and the ε-amino group of lysine, or the α-amino group of the peptide bridge of a second chain. This process can be repeated with the formation of polymers of the tetrapeptide, (Ala-γ-DGlu-Lys-DAla), plus the modifications which had previously occurred. In *Staphylococcus aureus* at least, this polymerization may not be complete. Tipper and Strominger (1965) have shown the peptidoglycan to consist of 7% of the material, *N*-acetylglucosaminyl-*N*-acetylmuramyl(Ala-γ-DGln-Lys-DAla-DAla)$(Gly)_5$.

Schleifer and Krause (1971) have studied the immune response of rabbits to group A variant *Streptococcus*. Inhibition of the precipitin reaction of this antiserum with solubilized peptidoglycan from *Staphylococcus epidermidis* was far greater with the pentapeptide, Ala-γ-DGlu-Lys-DAla-DAla, than with the tetrapeptide, Ala-γ-DGlu-Lys-DAla. By inhibition studies with smaller haptens, they could show that the dominant antibody specificity was directed against the carboxyl terminal, D-alanyl-D-alanine. This implied that the unpolymerized peptide portion of the peptidoglycan is immunodominant to the polymerized peptide portion.

We wish to study the immune response of rabbits to this peptidoglycan–precursor pentapeptide in the form of a branched copolymer and compare the antibody specificity with the results obtained by Schleifer and Krause from group A-variant *Streptococcus*. Group A streptococci have been shown to have materials with endotoxic activity (Stetson, 1956; Rotta *et al.*, 1965), as well as materials with determinants whose antibodies cross-react with several mammalian cell types (Kaplan and Meyeserian, 1962; Zabriskie and Freimer, 1966; Rotta and Bednar, 1970; Kingston and Glynn, 1971; Wagner and Weppe, 1972). Presumably, these materials will be absent from the synthetic immunogen. The present report includes the synthesis and immunochemistry of the peptidoglycan–precursor pentapeptide (Ala-γ-DGlu-Lys-DAla-DAla).

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