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Human Pituitary Growth Hormone. Studies of the Tryptophan Residue*

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ABSTRACT: The single tryptophan residue in the human growth hormone molecule has been quantitatively and specifically reacted with 2-nitrophenylsulfenyl chloride and 2-hydroxy-5-nitrobenzyl bromide using 50% acetic acid as reaction media. The derivatives have been characterized by biological, chemical, and biophysical techniques. The nitrophenylsulfenyl derivative (I) retains full growth-promoting activity and its physicochemical properties are similar to those of the native molecule. The monolabeled 2-hydroxy-5-nitrobenzyl derivative (II) possesses much less growth-promoting potency, while the doubly labeled derivative (III) is almost completely devoid of this biological activity. The relative looseness of the molecular structure and the lowered stability of these latter two derivatives (II and III) is shown by the higher rate of tryptic digestion and the irreversibility of their spectrophotometric titration in aqueous media as compared with the native hormone. When tested for lactogenic activity, all these derivatives were inactive. It may therefore be concluded that the tryptophan residue in the human growth hormone molecule is not essential for growth-promoting activity, but, in contrast, may play an important role in the lactogenic activity of the hormone, suggesting that there are two different "active sites" for the two biological activities.

The fact that the phenolic groups of the 2-hydroxy-5-nitrobenzyl moieties in derivatives II and III have an abnormal ionization behavior, and that total alkylation of the tryptophan residue occurs in 50% acetic acid, but not in 0.2 M acetic acid solution suggested that the tryptophan residue is "buried" in the interior of the hormone molecule, and is exposed in 50% acetic acid.

Recent studies on the reduction and alkylation of the disulfide bonds in HGH[1] (Dixon and Li, 1966; Bewley *et al.*, 1968, 1969) have shown that the tetra-*S*-carbamidomethylated hormone retains full growth-promoting and lactogenic activities and that its physicochemical properties are similar to those of the native hormone. The fact that the disulfide bridges are neither essential for the biological activity nor for the three-dimensional structure suggested that the conformation of the HGH molecule must be largely a consequence of noncovalent forces. Since tryptophan residues are known to play an important role in stabilizing the structure of proteins by hydrophobic interactions with other nonpolar residues (Kauzmann, 1959), it is of interest to determine the essentiality of the tryptophan residue for the maintenance of the biological property and structure of HGH.

In this paper we present investigations that have been carried out using two specific tryptophan reagents: NPS-Cl (Scoffone *et al.*, 1968) and HNB-Br (Koshland *et al.*, 1964).

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[1] Abbreviations used are: HGH, human growth hormone; NPS-Cl, 2-nitrophenylsulfenyl chloride; HNB-Br, 2-hydroxy-5-nitrobenzyl bromide; HNB-OH, 2-hydroxy-5-nitrobenzyl alcohol.

Moreover, the environmental sensitivity of the latter reagent has been used to provide information concerning the environment of the tryptophan residue in the molecule.

Materials

Human growth hormone was isolated from fresh human pituitaries by the procedure previously described (Li *et al.*, 1962). The heptadecapeptide, α^{1-17}-ACTH, corresponding to the first seventeen amino acid residues of ACTH was a synthetic product obtained as described by Li *et al.* (1964). The NPS-Cl was obtained from Eastman Organic Chemicals (Rochester) and recrystallized as recommended by Scoffone *et al.* (1968). HNB-Br was obtained from Mann Research Laboratories (New York) and was used without further purification. Trypsin was a commercial preparation rendered essentially free of chymotrypsin with L-1-tosylamido-2-phenylethyl chloromethyl ketone, obtained from Calbiochem (Los Angeles). All other chemicals were reagent grade.

Methods

Amino acid analysis of acid hydrolysates (6 N HCl at 110° for 20–22 hr) were performed in the Beckman–Spinco Model 120B amino acid analyzer, according to the method of Spackman *et al.* (1958). The reactions at a constant pH were performed in a Metrohm Combititrator 3D pH-Stat. All ultraviolet absorption spectra were taken in a Beckman DK-2A ratio recording dual-beam spectrophotometer. Ultrafiltrations were carried out in Amicon ultrafiltration cells with UM-10 membranes.

For the tryptic digestions, a combination of Radiometer pH meter type PHM 26, titrator type TTT 11, titration assembly type TTA 31, and titrigraph recorder type SBR 2 was used. To the protein in aqueous solution (0.6 mg/ml) at 37° ± 0.1 and pH 8.4, trypsin was added to provide an enzyme to substrate ratio of 1:250 (w/w). The pH was automatically maintained by the addition of 0.005 N, CO_2-free NaOH solution, and the alkali uptake was recorded as a function of time.

Spectrophotometric titrations were performed both in water and in 5 M Gu·HCl by the difference spectra technique. An aliquot of the protein solution was maintained at a fixed pH in the reference beam of the spectrophotometer. The pH of the remainder of the protein solution was changed by the addition of HCl or KOH, and difference spectra were taken. In all cases, optical densities at the desired wavelength were corrected for dilution.

The growth-promoting potency of the preparations was estimated by the rat tibia test (Greenspan *et al.*, 1949), and their lactogenic potency by the pigeon crop-sac test (Lyons, 1937; Nicoll, 1967).

Reaction of HGH with NPS-Cl. Two different media were used for the reaction of HGH with NPS-Cl.

A. Acetic acid (50%). In a typical experiment, 1 μmole of HGH was dissolved in 2 ml of 25% acetic acid and 20 μmoles of NPS-Cl dissolved in 1 ml of glacial acetic acid was added with continuous stirring. After 1 hr, the protein was separated from the excess reagent by gel filtration on a column (2.2 × 20 cm) of Sephadex G-25, preequilibrated and eluted with 0.2 M acetic acid. The protein peak was lyophilized or concentrated by ultrafiltration and a final purification was achieved by gel filtration using a Sephadex G-100 column (3 × 60 cm), preequilibrated and eluted with 0.2 M acetic acid solution. The monomeric form of the protein (Bewley *et al.*, 1968) having a relative elution volume, V_e/V_0, between 2.0 and 2.5, was concentrated by ultrafiltration or recovered by lyophilization.

B. Acetic acid (0.2 M). The protein was dissolved in 0.2 M acetic acid and a 20 molar excess of solid reagent was added. The pH was maintained at 4.0 in the pH-Stat. After 2 hr at room temperature, the insoluble excess reagent was removed by centrifugation and the supernatant was submitted to the purification procedure described under part A.

Reaction of the Heptadecapeptide with NPS-Cl. The reaction was performed in 0.2 M acetic acid (pH 4.0) under the conditions described for part B. The product was separated from the excess of reagent by gel filtration on a Sephadex G-10 column (1.2 × 26 cm) preequilibrated with 0.2 M acetic acid.

Determination of the Extent of the NPS Reaction. To estimate the extent of modification, each NPS derivative was dissolved in 80% acetic acid (0.5–1.0 mg/ml) and the amount of NPS chromophore was determined spectrophotometrically (ϵ 4000 at 365 mμ) (Scoffone *et al.*, 1968). The protein concentration was determined on the basis of the dry weight, or using the molar extinction coefficient of HGH at 277 mμ corrected by the value (molar extinction coefficient of Trp-NPS in peptide linkage reported by Scoffone *et al.* (1968) minus the molar extinction coefficient of Trp = $1.51 \times 10^{-4} - 5.5 \times 10^{-3}$) to account for the change in extinction following derivatization of the Trp residue.

Reaction of HGH with HNB-Br. Three different conditions were used for this reaction.

C. HGH (1 μmole) was dissolved in 2 ml of 0.2 M acetic acid and the pH was adjusted to 3.5; 100 μmoles of solid HNB-Br was added with vigorous stirring, and the pH was maintained by the automatic addition of alkali from a pH-Stat. The reaction was allowed to proceed for 1 hr at room temperature with continuous stirring. To remove the excess insoluble reagent and its hydrolytic product (HNB-OH), the reaction mixture was centrifuged and the solution was passed through a Sephadex G-25 column (2.2 × 20 cm) using 0.2 M acetic acid as eluent. The protein peak was collected and dialyzed during 2–3 days against several changes of 0.2 M acetic acid solution and lyophilized or concentrated by ultrafiltration. Further purification was achieved by exclusion chromatography on a Sephadex G-100 column (3 × 60 cm) using 0.2 M acetic acid as eluent. The monomeric form of the derivative was collected and, after concentration, was rechromatographed in the Sephadex G-100 column, and recovered by lyophilization.

For the other two procedures, the protein was dissolved in 50% acetic acid and a 100 molar (procedure D) or a 10 molar (procedure E) excess of solid reagent was added. In each case, the reaction was allowed to proceed for 1 hr at room temperature and the reaction mixture was treated as described in C.

Determination of the Extent of the HNB Reaction. The number of HNB groups bound per molecule of HGH was determined spectrophotometrically on 0.5–1.0-mg/ml solutions in 1.0 M NH_4OH, using a value of 1.8×10^4 for the molar extinction coefficient at 412 mμ (Horton and Koshland, 1965). The protein concentration was based on dry weight.

In order to know if there still was some HNB-OH absorbed

TABLE I: Reaction of HGH and α^{1-17}-ACTH with NPS-Cl.

Preparation	Acetic Acid	Moles[a] of NPS/ Mole of Trp	Ehrlich Reaction
HGH	50%	1.1–1.2 (5)	–
HGH	0.2 M	0.4–0.5 (3)	+
(α^{1-17}-ACTH)	0.2 M	1.0 (1)	–

[a] Range (number of experiments).

TABLE II: Reaction of HGH with HNB-Br.

Reaction Conditions	Moles[a] of HNB/ Mole of HGH	Ehrlich Reaction
0.2 M AcOH	0.6–1.3 (2)	+
50% AcOH – 100 moles excess of reagent	1.75–2.0 (4)	–
50% AcOH – 10 moles excess of reagent	1.0–1.0 (2)	–

[a] Range (number of experiments).

but not combined to the protein molecule, the molar ratio HNB:HGH was determined before and after the treatment by the following procedures. Aliquots of the derivatives were dissolved in 5 M Gu·HCl at pH 8.4 and dialyzed against 0.01 M NH_4HCO_3 at pH 8.4 or passed through a Sephadex G-25 column preequilibrated and eluted with 5 M Gu·HCl, then dialyzed against 0.01 M NH_4HCO_3 and lyophilized. Aliquots of the products obtained by procedures D or E were dissolved in 50% acetic acid and passed through a Sephadex G-25 column in the same solvent. The protein peak was collected, dialyzed, and recovered by lyophilization.

Control Preparation. Since HGH might be partially denatured during the 50% acetic acid treatment, control experiments were carried out in which HGH was submitted to procedure A described above except that the addition of the reagent was omitted.

All analytical data, recorded below, were obtained with the monomeric form of HGH or its derivatives.

Results and Discussion

As may be seen in Table I, quantitative alkylation of the tryptophan residue in HGH by NPS-Cl was obtained when 50% acetic acid was used as reaction media, but only partial conversion occurred in 0.2 M acetic acid. Since total alkylation of tryptophan by NPS-Cl can be effected in 0.2 M acetic acid (pH 4.0) as is shown by the experiment (see Table I) performed on the heptadecapeptide (α^{1-17}-ACTH), some conformational change in the HGH molecule sufficient to expose the tryptophan residue or to increase its reactivity must occur in 50% acetic acid.

Table II shows the results of the reaction of HGH with HNB-Br. Since identical ratios HNB:HGH were obtained before and after the treatment with 5 M Gu·HCl or 50% acetic acid, and since no free reagent was detected by thin-layer chromatography of chymotryptic digests of these derivatives, the values herein reported represent the actual number of HNB groups covalently attached to the molecule.

When 50% acetic acid was used as reaction medium, two different derivatives were obtained depending upon the molar excess of reagent over the protein. With 10 molar excess (procedure E) a monolabeled derivative (HGH-HNB_1) was obtained, while a 100 molar excess (procedure D) leads to a doubly labeled derivative (HGH-HNB_2). These results (Table II) are in agreement with the findings of Spande *et al.* (1968) and Barman and Koshland (1967). Spande *et al.* report that the major product resulting from the reaction of *N*-acetyl-L tryptophanmethyl ester with 2 equiv of HNB-Br appears to have reacted with two molecules of HNB in positions 1 and 3 of the pyrrole ring. Barman and Koshland show that the labeling process is concentration dependent, only one molecule of HNB per tryptophan residue being incorporated at low concentrations of reagent, followed by a second incorporation at higher reagent concentrations. This can also explain the equivocal results obtained when the reaction was performed in 0.2 M acetic acid; the ratio HNB:HGH of 1.3 together with the presence of unreacted tryptophan (see Table II) as shown by a positive Ehrlich reaction must represent a double incorporation into some molecules and lack of reaction with others. Similar results were reported by Barman and Koshland (1967) for carboxymethylchymotrypsinogen.

Amino Acid Composition. In order to ascertain that only the tryptophan residue was modified by HNB-Br or NPS-Cl,

TABLE III: Amino Acid Composition of HGH Treated with 50% Acetic Acid, HGH-NPS, and HGH-HNB_2.

Amino Acid[a]	HGH-AcOH	HGH-NPS	HGH-HNB_2	Theoretical[b]
Lysine	8.9	8.5	8.6	9
Histidine	2.8	3.2	2.9	3
Arginine	10.6	9.5	9.9	10
Aspartic acid	20.2	19.9	21.6	20
Threonine	9.9	10.0	10.4	10
Serine	16.7	17.3	17.5	18
Glutamic acid	27.7	27.3	29.0	26
Proline	8.1	8.4	8.4	8
Glycine	8.4	8.7	8.4	8
Alanine	6.8	7.5	7.8	7
Half-cystine	2.9[c]	3.1[c]	2.3[c]	4
Valine	6.5	6.6	6.8	7
Methionine	2.3	2.9	2.6	3
Isoleucine	6.8	6.8	7.0	8
Leucine	24.6	24.6	25.5	25
Tyrosine	7.1	7.0	6.4	8
Phenylalanine	12.8	13.1	13.9	13

[a] No tryptophan peaks were observed in the chromatograms; it is probable that both tryptophan and its derivatives are destroyed during acid hydrolysis. [b] Taken from the sequence, see Li *et al.* (1966). [c] These values were obtained from samples which were not oxidized with performic acid.

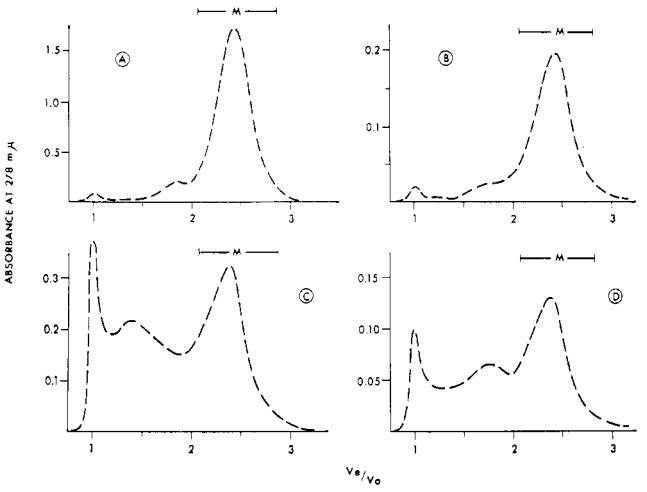

FIGURE 1: Elution patterns of gel filtration on Sephadex G-100 (column 3 × 60 cm) in 0.2 M acetic acid (A) untreated HGH, (B) 50% acetic acid treated HGH (control experiment), (C) HGH-NPS derivative, and (D) HGH-HNB_2 derivative. Fraction M corresponds to the monomeric form. V_e/V_0 = relative elution volume.

the derivatives were submitted to amino acid analysis after acid hydrolysis. Results, summarized in Table III, indicate that the amino acid content of HGH-NPS and HGH-HNB_2 is in good agreement with the data for the untreated hormone.

Gel Filtration on Sephadex G-100. Figure 1 presents the elution patterns which result from fractionation by gel filtration on Sephadex G-100 of (A) HGH untreated, (B) HGH treated with 50% acetic acid (control preparation), (C) HGH-NPS derivative obtained by procedure A, and (D) HGH-HNB_2 derivative obtained by procedure D. As in the case of urea-treated and the reduced carbamidomethylated HGH (Dixon and Li, 1966), reactions of the hormone with NPS-Cl and with HNB-Br introduce some heterogeneity due to aggregation. The monomeric fractions (M) of the control preparation and the two derivatives have elution volumes identical with that of the untreated hormone, indicating that no gross irreversible changes in the size or shape of the HGH molecule occur as a consequence of the 50% acetic acid treatment, or by the alkylation of the tryptophan residue.

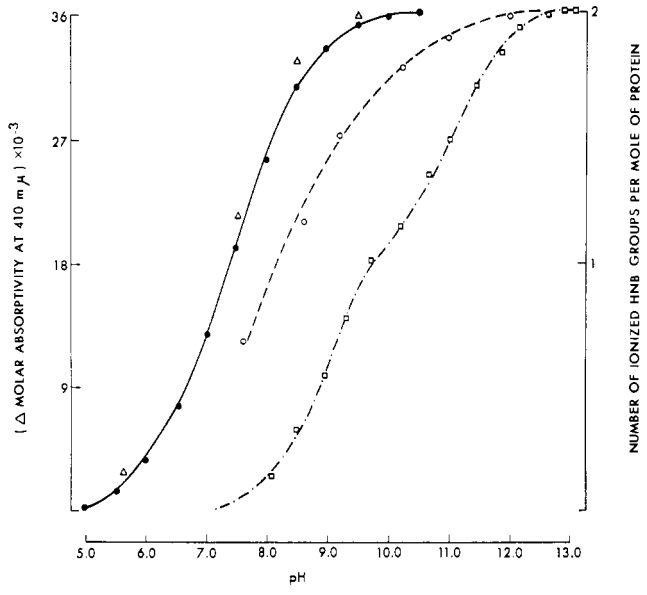

FIGURE 2: Spectrophotometric titrations. Ionization curves for the HNB chromophore in the HGH-HNB_2 derivative in (a) aqueous media (□–·–·–·–□), reverse titration (○– – –○); and (b) 5 M Gu·HCl (●——●), reverse titration is represented by triangles (△). The number of ionized HNB groups per mole of protein was calculated using the value of 18.000 M^{-1} cm^{-1} for the molar absorptivity at 410 mμ, reported by Horton and Koshland (1965).

TABLE IV: Spectrophotometric Titrations of the Phenolic Group in the HNB Moiety of HGH Derivatives.

Compound	Solvent	App pK_1	Reversibility of Titration
HNB-OH	Aqueous	6.85	+
α^{1-17}-ACTH-HNB	Aqueous	7.45	
HGH-HNB_2[a]	Aqueous	8.9	−
HGH-HNB_2	5 M Gu·HCl	7.40	+
HGH-HNB_1	Aqueous	8.9	−

[a] pK_2 = 11.0–11.2.

Spectrophotometric Titration. HNB DERIVATIVES. The possibility that the tryptophan residue is buried in an internal hydrophobic region of the HGH molecule as suggested by its sluggish reactivity in 0.2 M acetic acid may be probed by studying the environmental state of the alkylated residue. For this purpose, spectrophotometric titrations of the phenolic chromophore present in the HNB moiety were performed. Figure 2 shows the data obtained for HGH-HNB_2 in aqueous media and in 5 M Gu·HCl solution, respectively. A comparison between the ionization characteristics of these derivatives and reference compounds is presented in Table IV. In both HNB derivatives, the ionization in aqueous solution is highly abnormal. The HGH-HNB_2 derivative shows an ionization curve with apparent pK's of 8.9 and 11.0–11.2; this behavior is completely normalized in 5 M Gu·HCl solution, where the

TABLE V: Spectrophotometric Titrations of the Tyrosine Residues in Native and NPS Derivative of HGH.

Protein	Solvent	App pK	Total No. of Ionizable Tyr[a]
HGH-NPS	Aqueous	10.90	
HGH-NPS	5 M Gu·HCl	10.25	7.0–7.1
HGH (untreated)	Aqueous	10.95[b]	6.1
HGH (untreated)	6 M Gu·HCl	10.25	7.5

[a] Calculated as explained in the legend of Figure 3. [b] Taken from Bewley *et al.* (1969).

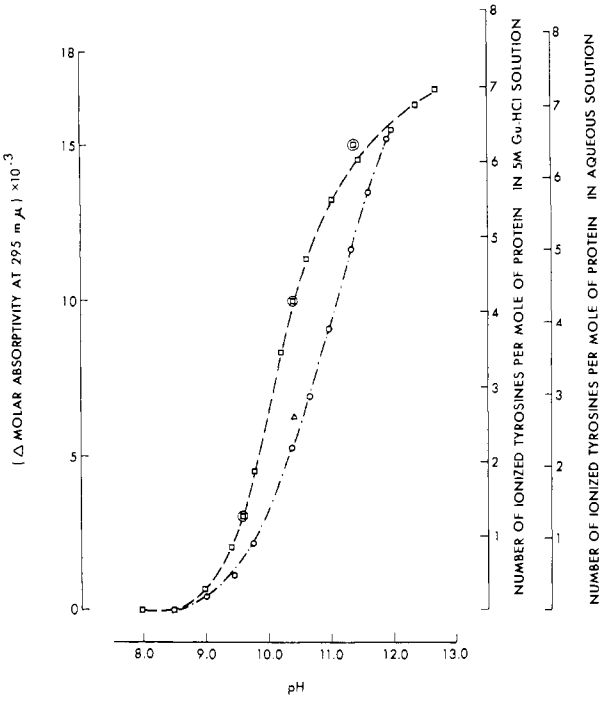

FIGURE 3: Spectrophotometric titration. Ionization curve for the tyrosines in the HGH-NPS derivative, in aqueous solution (○-·-·-·-○), reverse points (Δ); and in 5 M Gu·HCl solution (□-–-–-□), reverse points (◎). The number of ionized tyrosines per mol of protein was calculated using for $\Delta\epsilon_{295}$ for each tyrosine the value of 2.310 and 2.395 for aqueous solution and 5 M Gu·HCl solution, respectively, as reported by Bewley *et al.* (1969).

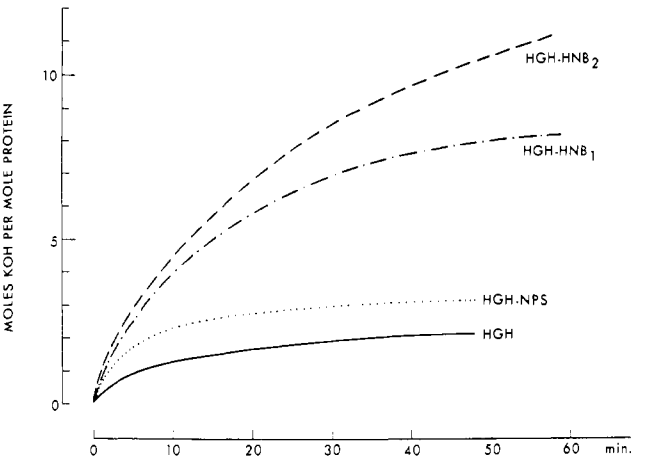

FIGURE 4: Rate of tryptic hydrolysis of HGH (control experiment), HGH-NPS, HGH-HNB$_1$, and HGH-HNB$_2$ derivatives. Enzyme: hormone ratio is 1:250 (w/w). Temperature 37° ± 0.1, pH 8.4. Protein concentration 0.6 mg/ml.

apparent pK is 7.40 identical with that (apparent pK = 7.45) of the Trp-HNB in the model peptide, α^{1-17}-ACTH. This highly altered ionization behavior might be explained by assuming that the HNB groups attached to the tryptophan residue are partially or totally buried in the interior of the molecule, and therefore not normally ionized. In 5 M Gu·HCl, however, those phenolic groups are liberated by denaturation of the protein, allowing them to ionize normally. This strongly supports the possibility that the tryptophan residue is located in the interior of the HGH molecule.

It must be pointed out, however, that the results herein reported were obtained on the HNB derivative. The extrapolation to the untreated molecule is based on the assumption that the location of the tryptophan residue within the three-dimensional architecture of the molecule does not irreversibly change as a consequence of the alkylation reaction. The studies performed with the HGH-NPS derivative support this assumption. In contrast with the native hormone (Bewley *et al.*, 1969) and the HGH-NPS derivative (see below), the HNB derivatives are irreversibly denatured in alkaline solution as shown by their titration curves (Figure 2).

NPS DERIVATIVE. In order to establish the ionization behavior of the tyrosine residues in the HGH-NPS derivative, and to obtain information about the conformation of this protein molecule, spectrophotometric titrations in 5 M Gu·HCl and aqueous media were performed. In Table V, results obtained are compared with those of the untreated hormone reported by Bewley *et al.* (1969). The respective ionization curves are shown in Figure 3.

The same apparent pK's obtained for the native hormone in both solvents were obtained for the NPS derivative. Thus, no appreciable differences in the tyrosine ionization can be detected, between the native protein and the NPS derivative. The derivative molecule appears to maintain much of the native conformation as assessed by the equivalent tyrosine ionizations supporting the assumption that if some structural change occurs during the alkylation reaction in 50% acetic acid, it must be reversible.

As the pH increases above 11, the ionization in aqueous media approximated that in 5 M Gu·HCl, showing a normalization of these somewhat abnormal tyrosine residues to a state similar to that obtained when the protein is dissolved in a denaturant like Gu·HCl. However, the titration shows that this alkaline denaturation of the NPS is not irreversible.

Tryptic Digestion. Figure 4 shows the rate of tryptic digestion of the control HGH preparation and the three derivatives. While the HGH-NPS derivative is digested somewhat more rapidly than native HGH, the two HNB derivatives are much more rapidly digested. These rate differences may be interpreted (Markus, 1965) as indicating a lower degree of rigidity of the structure in the HGH-HNB$_2$ and HGH-HNB$_1$ derivatives, whereas the reaction of HGH with NPS causes only slight alterations in the conformation of the hormone.

Bioassay. The evaluation of the growth promoting potency of 50% acetic acid treated HGH, HGH-NPS, HGH-HNB$_1$, and HGH-HNB$_2$ as measured by the rat tibia test are presented in Table VI. The statistical evaluation of these results shows that full growth-promoting activity is retained in the 50% acetic acid treated control preparation and in the HGH-NPS derivative. Substantially less potency is present in the HGH-HNB$_1$ derivative (0.30 as potent as the native hormone, with fiducial limits of 0.15–0.46), while the doubly labeled HGH-HNB$_2$ is almost completely devoid of growth-promoting activity.

The results of the pigeon crop-sac assay of these preparations are presented in Table VII. While the 50% acetic acid treated hormone is as active as the native in this bioassay, the NPS and HNB derivatives of HGH are almost devoid of lactogenic activity.

TABLE VI: Rat Tibia Test of NPS and HNB Derivatives of HGH.

Preparation	Mean Responses (μ) ± Std Dev, for Total Doses (μg) of:		Statistical Evaluation	
	40	100	Potency	Fiducial Limits for $P = 0.05$
HGH (untreated)	253 ± 6 (10)[a]	271 ± 15 (10)		
HGH (50% AcOH treated)	259 ± 5 (6)	271 ± 20 (6)		
HGH-NPS	250 ± 7 (5)	282 ± 24 (6)	0.98	0.49–1.83
HGH-HNB_1	196 ± 20 (5)	248 ± 4 (5)	0.30	0.15–0.46
HGH-HNB_2		206 ± 26 (6)		

[a] Number of test animals in parentheses.

Conclusion

Chemical modifications at specific sites in the molecule, for studies of structure–biological activity relationship, can have great significance for native proteins only if the conformation is not changed by the modification and/or the procedure used. This is clearly not the case with the HGH-HNB_1 and HGH-HNB_2 derivatives. As was shown by the rate of tryptic digestion, important alterations seem to occur in their structure as a consequence of the reaction with HNB-Br. In contrast, all the studies performed on the HGH-NPS derivative show that its conformation is very close to that of the native hormone.

In the rat tibia bioassay, the preparation to be tested must reach the receptor site through the circulatory system, where it may come in contact with proteolytic enzymes. Under these conditions, the two HNB derivatives may never reach the target, due to their faster enzymatic degradation. This may be an additional explanation for their lower growth-promoting activity.

The fact that HGH-NPS is fully active in growth promotion (Table V) but its lactogenic potency is almost abolished (Table VI) indicates that the tryptophan residue plays different roles in these activities or that the lactogenic activity is highly sensitive to minor changes in the molecular conformation which cannot be detected by the biophysical techniques herein employed. These results may be taken to mean that two different active sites exist in the HGH molecule: one for the growth-promoting activity and the other for the lactogenic activity.

TABLE VII: Pigeon Crop Sac Assay of NPS and HNB Derivatives of HGH.

Preparation	Total Dose (μg)	Response	
		Visual[a]	Mean Wt[b] ± Standard Error
HGH (untreated)	2	2.3	17.2 ± 2.9
	16	3.4	30.0 ± 8.9
HGH (50% AcOH treated)	2	2.2	17.5 ± 6.3
	16	3.5	28.0 ± 8.0
HGH-NPS	5	0	9.7 ± 2.8
	40	1.6	12.4 ± 3.4
HGH-HNB_1	2	0	9.3 ± 2.2
	16	1.1	13.5 ± 4.6

[a] Subjectively evaluated using the scale: 0, no stimulation; 1, minimal stimulation; 2, moderate stimulation; 3, marked stimulation; 4, maximum stimulation. Values shown are the means of ten observations. [b] Dry weight of crop-sac mucosal epithelium in milligram.

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Human Pituitary Growth Hormone. Physicochemical Investigations of the Native and Reduced-Alkylated Protein*

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ABSTRACT: Results of investigations into the biological and physicochemical properties of native, reduced-tetra-*S*-carbamidomethylated, and reduced-tetra-*S*-carboxymethylated human pituitary growth hormone are presented. While both derivatives appear to retain lactogenic activity, only the carbamidomethylated product retains growth-promoting potency. Measurements of chemical composition, molecular weight, viscosity, spectrophotometric titration of tyrosyl groups, and circular dichroism have failed to uncover any significant differences between the two derivatives. In addition, differences between either derivative and the native hormone appear to be very small. Accordingly, we conclude that the disulfide bonds in this molecule are not necessary for the manifestation of biological activity nor are they required for the formation of the secondary and tertiary structure. However, investigation of the relative rates of proteolysis by trypsin would indicate that the presence of these bonds does serve to stabilize the molecular architecture against perturbing forces. The carbamidomethylated derivative is digested about 1.5 times as fast as the native, while the carboxymethylated product, under identical conditions, is digested at 2.5–3 times the rate of the native hormone.

The general acceptance of the hypothesis that the native conformation of a protein is mostly a result of noncovalent intramolecular forces arising from its amino acid sequence (Epstein *et al.*, 1963; Anfinsen, 1964) raises an interesting question as to the specific role of disulfide bonds in either achieving and/or stabilizing these conformations by strategic placement of a few nonpeptidic covalent links. In addition to their functioning as structural restraints, there is also the question of whether some disulfide bonds may be intrinsically involved in the active site(s) of the proteins which contain them. The general approach to these problems is the application of a selective chemical modification followed by biological and physicochemical evaluation of the derivative.

Experimentally, disulfide modification by the use of harsh and relatively nonspecific oxidative techniques or reductive cleavage in the presence of strong denaturing agents makes it very difficult if not impossible to determine whether or not subsequent changes in either conformation and/or biological activity are solely a consequence of disulfide cleavage. Ideally, one must be able to separate those effects resulting purely from the modification employed from those effects resulting purely from the conditions under which the modification was carried out. This ideal would seem to be approachable only in those cases where the conditions of modification are so passive that they do not in themselves contribute to the effects of modification.

It has been demonstrated that the two disulfide bonds in HGH[1] may be quantitatively reduced at pH 8.1 with dithiothreitol in the complete absence of denaturant (Bewley *et al.*, 1968). The reduced product, following alkylation of the thiol groups with iodoacetamide was found to retain essentially full biological activity as previously reported by Dixon and Li (1966). Subsequently, it was found (Bewley, 1968) that when iodoacetic acid was used as the alkylating agent instead of iodoacetamide, the product displayed no growth-promoting activity. In view of the very mild reaction conditions employed these two reduced-alkylated derivatives, along with the native hormone, provide an interesting system for studying the relationship between structure and activity and the influence of the disulfide bonds on both. Such a study is even more attractive with these molecules since the amino acid sequence of HGH is known (Li *et al.*, 1966)

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[1] Abbreviations used are: HGH, native human growth hormone; RCAM, reduced tetra-*S*-carbamidomethylated HGH; RCOM, reduced tetra-*S*-carboxymethylated HGH.