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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.

and the native hormone is reported to contain about 50% of these residues in an α-helical conformation (Bewley and Li, 1967).

Experimental Procedures

Materials. Sodium α-iodoacetate and dithiothreitol were obtained from Calbiochem, Los Angeles, Calif., α-iodoacetamide was from Aldrich Chemical Co., Milwaukee, Wis., and twice-crystallized trypsin was obtained from Worthington Biochemical Corp., Freehold, N. J., lot no. TRL6122. *N*-Acetyl-L-tyrosinamide was from Mann Research Laboratories, New York, N. Y. These reagents were used without further purification. Guanidine hydrochloride (Eastman Organic Chemicals, Rochester, N. Y.) was recrystallized twice from 95% ethanol using Norit decolorizing carbon. Human pituitary growth hormone was prepared as described by Li *et al.* (1962). The reduced-tetra-*S*-carbamidomethylated derivative (RCAM) was prepared as described by Bewley *et al.* (1968). This same procedure was used to prepare the reduced-tetra-*S*-carboxymethylated derivative (RCOM) except for the use of sodium α-iodoacetate rather than iodoacetamide as the alkylating agent. Oxidation of the disulfide bonds of HGH was effected with performic acid as described by Li (1957). Monomeric fractions of these derivatives were obtained by exclusion chromatography on Sephadex G-100 as previously described (Bewley *et al.*, 1968).

Amino Acid Analyses. Acid hydrolysis of the products was performed for 22 hr under vacuum, at 110° in constant-boiling HCl. The hydrolysate was subjected to amino acid analysis according to the procedure of Spackman *et al.* (1958), in the amino acid analyzer (Beckman amino acid analyzer, Model 120-C).

Osmotic Pressure. Measurements of osmotic pressure were made in a Melabs recording membrane osmometer, Model CSM-2. The proteins were dissolved in 0.05 M NH_4HCO_3 (pH 8.4) to a concentration of 3–5 mg/ml and then dialyzed against this same buffer overnight. Three dilutions were made of each sample giving a total of four concentrations of each protein. The osmotic pressure of each dilution was measured to ±0.02 cm of H_2O. Protein concentrations were measured spectrophotometrically. The temperature of the osmometer cell was maintained automatically at 20.00 ± 0.002° by the temperature control unit in the osmometer. The data was plotted in the usual way according to the Van't Hoff limiting law, where M_2 is the number-average

$$\frac{1}{M_2} = \frac{\lim \left(\frac{\pi}{c}\right) c \to 0}{RT}$$

molecular weight, R the gas constant, T the absolute temperature, π the osmotic pressure in centimeters of H_2O, and c the protein concentration in milligrams per milliliter. The value of $\lim (\pi/c)c \to 0$ is equal to the intercept on the ordinate, (π/c), at $c = 0$.

Biological Activity. The growth-promoting activity of these preparations was determined by the rat tibia test (Greenspan *et al.*, 1949), the lactogenic activity by the local crop assay in the pigeon. The response in the pigeon assay was determined both as described by Lyons (1937) and by the quantitative method of Nicoll (1967).

Viscosity. Viscosities were measured in an Ubbelohde viscometer with a flow time for water of 140 sec. The viscometer was suspended in a controlled temperature water bath at 28.00°. The temperature was maintained by a Melabs, Model CTC-1A temperature controller, providing a constant temperature, stable to ± 0.005°. Protein concentrations were determined spectrophotometrically as described below. At least three dilutions of each sample, in 0.01 M NH_4HCO_3 buffer (pH 8.4) were used in extrapolating to zero concentration. Flow times were measured to the nearest 0.1 sec, the average deviation with a single dilution over five to ten readings being ±0.2 sec. Intrinsic viscosities were obtained by the following two relations as described by Tanford (1955)

$$[\nu] = \lim_{c \to 0} [t_i - t_0/t_0 C] \quad (1)$$

$$[\eta] = [\nu] + (1 - \bar{v}\rho_0)/\rho_0 \quad (2)$$

where t_i and t_0 refer to the flow times of solution and solvent, respectively, C is the concentration of protein in grams per milliliter, and $[\nu]$ is the intrinsic kinematic viscosity in milliliters per gram. The value of $[\nu]$ is converted into intrinsic viscosity $[\eta]$ by correcting for the density difference between the solvent and solution. In the absence of direct measurements of these densities this conversion may be performed arithmetically by eq 2, where $\bar{v}$ is the partial specific volume of the protein and ρ_0 is the density of the solvent. In all cases $\bar{v}$ was taken to be 0.731 (Li and Starman, 1964) and ρ_0 was assumed to be that of water at 28°. For purposes of comparison, axial ratios have been computed from contour diagrams (Oncley, 1941) by arbitrarily assigning a value of hydration and using the approximation

$$\nu = \frac{[\eta]}{\bar{v}}$$

where ν is the viscosity increment.

Ultraviolet Spectra. All spectra were obtained on a Beckman DK-2A recording spectrophotometer, using matched pairs of far-ultraviolet silica cuvets. The optical path for all spectra was 1.00 cm. Absorption in the range 340–360 mμ, attributed to light scattering, was corrected for, as described by Beaven and Holiday (1952). After correction for any scattering effects, the optical densities at 277 mμ were used to determine the protein concentrations of the various test solutions. The molar extinction coefficient of native HGH was calculated from the optical densities of a series of carefully weighed samples whose total nitrogen contents were known from micro-Dumas nitrogen analyses. The computation was based on a molecular weight of 21,500 and a nitrogen content for HGH (100% protein) of 16.23% (Li and Liu, 1964). A practical working extinction coefficient has also been computed. The native protein concentration is routinely determined, by means of the following relation, $E^{0.1\%}_{1\text{ cm},277\text{ m}\mu} = 0.931$, from a spectrum[2] taken in the pH range 2.0–

[2] We have confirmed the observation of Léonis and Li (1958) that native HGH shows no significant spectral changes in this pH range. However, this is not true of either reduced-alkylated derivative, there being a small acid difference spectrum. For this reason the use of the

8.5. The concentration of solutions of the RCAM and RCOM derivatives was obtained using the same relation, but from spectra taken at pH 8.0–8.4.

Spectrophotometric Titrations. Spectrophotometric titrations were carried out in a Beckman DK-2A spectrophotometer in stoppered 1.00-cm silica cuvets, using the difference spectra technique. Briefly, a 10–20-ml solution of protein at a known concentration (≈1 mg/ml) in either 0.15 M KCl or 6 M guanidine hydrochloride was titrated to pH 8.0 using a Metrohm E-300 pH meter. The combination glass electrode was standardized against a series of six buffers covering the pH range 2.27–12.88 as recommended by Bates (1954); 2 ml of this solution were placed in a stoppered cuvet and kept in the reference beam of the spectrophotometer. The pH of the remainder of the solution was raised approximately 0.2–0.5 pH unit by addition of 10 N KOH from a microburet. A second aliquot, now at a higher pH value was removed and placed in the sample beam of the spectrophotometer. The spectra was scanned from 325 to about 275 mμ. The pH of the sample was measured before and after it was scanned. Reverse titrations were performed by adding 6 N HCl to the same stock solution after reaching pH 13 and taking spectra exactly as described for the forward titration. The temperature of all solutions was 25 ± 1°.

Circular Dichroism. Circular dichroism spectra were obtained on a Cary Model 60 spectropolarimeter equipped with a Model 6002 circular dichroism attachment. The instrument was calibrated with D-10-camphorsulfonic acid (Eastman Organic Chemicals) as recommended by the manufacturer. All spectra were taken at 27° in a 1.00-mm path-length cell. Each preparation was run at three concentrations in the range 0.01–0.1 mg/ml. Dilutions for spectra were made from a stock solution of ≈1 mg/ml whose concentration was determined spectrophotometrically. All solutions were in 0.1 M Tris buffer (pH 8.2). Spectra were taken from 250 mμ to as close to 200 mμ as possible before the dynode voltage reached ≈600 V. Mean residue molecular ellipticities, $[\theta]_\lambda$, were calculated using a value of 115 for the mean residue weight in HGH. The helical content of the samples was estimated from the absolute values of the ellipticities at both 221 and 209 mμ. In these calculations the ellipticity values for 100% α helix and 100% random coil obtained for poly-L-glutamic acid at 222 and 210 mμ (J. Y. Cassim and J. T. Yang, unpublished data) were used as limiting values in the following relations

$$\% \ \alpha \text{ helix from } [\theta]_{221} = \frac{|[\theta]_{221}| + 4{,}800}{45{,}400} \times 100$$

$$\% \ \alpha \text{ helix from } [\theta]_{209} = \frac{|[\theta]_{209}| - 2{,}200}{36{,}200} \times 100$$

where $|[\theta]_\lambda|$ is the absolute value of the ellipticity at wavelength λ.

Tryptic Digestion. Tryptic digestions were carried out in the pH-Stat (Radiometer Titrator type TTT 11b, pH meter type 26c), at pH 8.4–8.5 within a water-jacketed cell at 37.0 ± 0.1°, and under a nitrogen atmosphere. Control of the pH was maintained automatically by the addition of standard 0.005 N, CO_2-free KOH. The alkali uptake was recorded as a function of time, indicating both the extent and rate of reaction. In all digestions the enzyme:substrate ratio was 1:500 (w/w). Each solution contained 5–6 mg of hormone dissolved in 4.5 ml of 0.15 M KCl. Since these were short-term digestions, no calcium ion was added to supress autodigestion of the enzyme.

extinction coefficient for the derivatives is probably valid only in the narrower pH range shown above. A full account of the ultraviolet spectral properties of these proteins will be the subject of a future publication.

TABLE I: Amino Acid Analyses of HGH and Derivatives.

	No. of Residues/21,500 Mol Wt[a]			
Amino Acid	Native	RCAM	RCOM	Theor[b]
Lys	9.1	8.7	9.2	9
His	2.8	2.9	3.0	3
Arg	10.5	10.0	10.6	10
SCM-Cys[c]		4.0	3.8	
Asp	21.5	21.9	21.1	20
Thr	9.8	10.4	9.8	10
Ser	17.0	18.1	16.1	18
Glu	27.6	29.7	27.3	26
Pro	9.3	9.4	8.4	8
Gly	9.5	9.5	9.1	8
Ala	8.0	8.3	7.8	7
Half-Cys	3.8	0.0	0.0	4
Val	6.6	6.6	7.0	7
Met	2.6	2.9	2.6	3
Ile	6.2	6.0	6.5	8
Leu	24.1	23.4	24.7	25
Tyr	7.0	7.2	7.3	8
Phe	12.5	12.1	12.9	13

[a] Average values for two preparations. [b] Taken from the sequence (Li *et al.*, 1966). [c] *S*-Carboxymethylcysteine.

Results and Discussion

Amino Acid Analysis. The chemical compositions of these various preparations as determined by amino acid analysis, shown in Table I, are typical results obtained from 22-hr hydrolysates, compared with 22-hr hydrolysates of the native protein. Comparing the compositions of the derivatives with that of the native HGH, the only important differences are in the contents of cystine and *S*-carboxymethylcysteine. In particular, the close correlation in the contents of those amino acids which might have been expected to undergo side reactions with either of the two alkylating agents (*e.g.*, lysine, methionine, histidine, and tyrosine) indicates that such unfavorable reactions cannot have progressed to any significant extent under the conditions of these preparations. The complete absence of unreduced cystine and the essentially stoichiometric recoveries of *S*-carboxymethylcysteine clearly show that the reduction and alkylation reactions have been complete.

Osmotic Pressure. In order to confirm that these materials actually represent the monomer form of each protein, molec-

TABLE II: Number-Average Molecular Weights of "Monomer" Fractions of HGH and Derivatives as Determined by Osmotic Pressure.

Protein	M_n (obsd)[a]	M_n (calcd)
Native	23,800 ± 500	21,500
RCAM	22,800 ± 500	21,700
RCOM	19,800 ± 500	21,700

[a] Determined in 0.05 M NH_4HCO_3 buffer (pH 8.4). Each value is a single determination. The uncertainty in these values has been estimated from the scatter of points about the Van't Hoff plot line.

TABLE IV: Lactogenic Activity of HGH and Derivatives as Measured by the Pigeon Local Crop Assay.

Protein	Dry Mucosal Wt (mg)[a] for Dose (μg)	
	2	8
Native	13.5 ± 3.1 (6)	18.2 ± 4.7 (5)
RCAM	12.6 ± 1.8 (5)	20.1 ± 5.8 (5)
RCOM	11.9 ± 2.8 (6)	15.1 ± 3.9 (4)

[a] Expressed as mean plus and minus standard error of the mean followed by the number of test animals in parentheses. Uninjected controls give mucosal weights of 8–9 mg.

ular weights of the lyophilized "monomer" fractions were determined by measurements of osmotic pressure. In all three cases the Van't Hoff limiting law plots gave straight lines which when extrapolated to zero concentration gave number-average molecular weights as shown in Table II. The values of 23,800 for the native, 22,800 for the reduced-carbamidomethylated, and 19,200 for the reduced-carboxymethylated materials are close enough to the theoretical monomer weights (21,500 for the native and 21,700 for the derivatives) to assure that these fractions indeed represent monomers of the proteins.

Biological Activity. The growth-promoting activity of the reduced-alkylated derivatives compared with the native hormone is shown in Table III. It is evident that the reduced-carbamidomethylated derivative retains nearly full potency while the carboxymethylated preparation is essentially devoid of this activity. In contrast to this behavior, both carbamidomethylated and carboxymethylated HGH appear to retain considerable lactogenic activity when measured in the pigeon local crop assay; these assay data, shown in Table IV, must be considered as preliminary due to the small number of test animals, but they do indicate the retention of lactogenic activity by both derivatives.

TABLE III: Growth-Promoting Activity of HGH and Derivatives as Measured by the Rat Tibia Test.[a]

Prepn	Tibia Width (μ) for Total Dose (μg)			
	0	20	60	80
Saline	168 ± 2 (12)			
Native		211 ± 4 (12)	269 ± 5 (11)	
RCAM		197 ± 6 (12)	257 ± 4 (12)	
RCOM			194 ± 14 (5)	199 ± 22 (4)

[a] Expressed as the mean plus and minus standard error of the mean followed by the number of test animals in parentheses.

Viscosity. In the following treatment of viscosity measurements, no attempt will be made to interpret the data in terms of ascribing a physically significant axial ratio or hydrodynamic volume to the protein in question. Axial ratios have been calculated by applying the usual assumptions but only for the purpose of comparison, they are not meant to be taken as absolute parameter assignments.

Figure 1 shows the plots of the reduced kinematic viscosity *vs.* concentration used to obtain the intrinsic kinematic viscosity, from which the intrinsic viscosity and viscosity increment were calculated. These data are presented in Table V along with axial ratios of equivalent hydrodynamic ellipsoids of revolution (prolate) at various assumed degrees of hydration. As can be seen from the data in this table, there is only a small difference between the native protein and both reduced alkylated products, and essentially no difference between the two derivatives. When these data are interpreted as axial ratios at any assumed degree of hydration, they show a small increase in asymmetry. This small change may be misleading, however, since some part, or even all of it may be due to a small increase in the extent of hydration of the reduced-alkylated derivatives.

The performic acid oxidized protein shows a considerably greater difference in shape from any of the three proteins mentioned above. When the viscosity data for this preparation

TABLE V: Viscosity of HGH and Derivatives.[a]

Protein	$[\eta]$ (ml/g)	ν	Axial Ratio of Prolate Ellipsoid of Revolution at Various Deg of Hydration (% w/w)		
			0	30	100
Native	4.3	5.5	5.0	3.3	1.0
RCAM	5.0	6.4	5.5	3.8	1.6
RCOM	5.0	6.4	5.5	3.8	1.6
Performic acid oxidized	10.4	13.9	10	8	6

[a] Determined in 0.01 M NH_4HCO_3 buffer (pH 8.4).

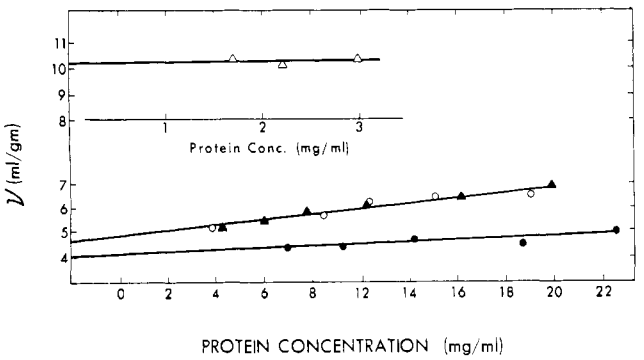

FIGURE 1: Viscosity of native (●), RCAM (○), RCOM (▲), and performic acid oxidized (△) HGH, determined in 0.01 M NH_4HCO_3 buffer (pH 8.4).

are converted into an axial ratio, it would indicate a definite increase in asymmetry since rather unrealistic degrees of hydration (180–200%) would have to be used to explain the increase in viscosity by hydration alone.

Spectrophotometric Titration. Spectrophotometric titrations of the native protein and reduced-alkylated derivatives were performed to determine whether the ionization behavior of the tyrosine residues had been affected by the reduction and alkylation reactions. Figure 2 shows a family of difference spectra obtained from a titration of the native protein in 0.15 M KCl. The difference peak is at 295 mμ with a definite isosbestic point at 280 mμ; both facts indicating that these spectra arise only from ionization of the tyrosine residues and therefore that the changes in absorptivity at 295 mμ are directly proportional to the degree of tyrosyl ionization. Similar families of spectra are generated on titration of both reduced-alkylated derivatives. In order to estimate the number of tyrosines ionized, it is necessary to know the ΔE_{295}/mole for peptide-linked tyrosine. To obtain these values under the same experimental conditions we have used for the titrations, spectra have been taken of solutions of *N*-acetyl-tyrosinamide at pH 8.0 and 13.0 in both 0.15 M KCl and 6 M guanidine hydrochloride. The extinction coefficient changes obtained in this way are: 2310 /mole in 0.15 M KCl and 2390/mole in 6 M guanidine hydrochloride. These values compare favorably with those already reported in the literature (Beaven and Holiday, 1952; Nozaki and Tanford, 1967), for peptide-linked tyrosyl groups in these solvents.

Figure 3 shows the data from the titration of native, RCAM, and RCOM-HGH in 0.15 M KCl, plotted as ionization curves. Only about six of the eight tyrosines in native HGH can be titrated up to pH 13. The apparent p*K* (pK_a) of these six residues, estimated from the midpoint of the ionization curve, is 10.9–11.0. Since a pK_a of 10.1–10.3 would have been expected for normal tyrosine ionization behavior (Nozaki and Tanford, 1967), we may conclude that the ionization of these six residues is abnormal to a significant extent.

Figure 3 also shows the ionization curve for the RCAM and RCOM derivatives. The points for these two proteins fall so closely together within experimental error they seem to follow the same ionization curve. These derivatives show an increased ionization over that of the native, there being closer to seven residues titrated up to pH 13 or 13.2. Again,

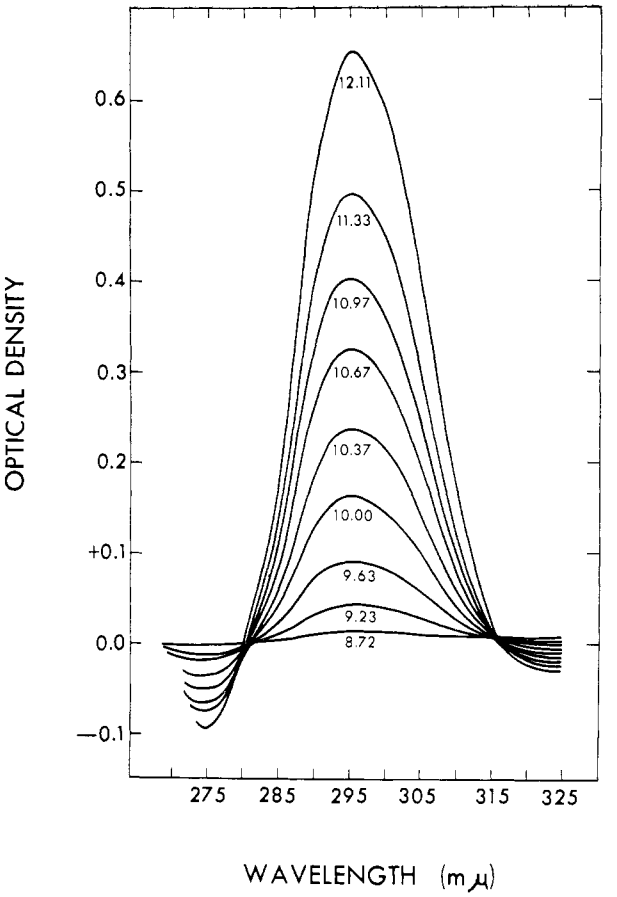

FIGURE 2: Tracing of difference spectra for native HGH in 0.15 M KCl. Reference solution pH 8.25, sample solution pH values are indicated under each curve. Protein concentration is 1.15 mg/ml.

the midpoint of the curve would indicate a pK_a of 10.8–10.9 for the titratable tyrosines. It should be noted that all three titrations were perfectly reversible from pH 13.4, indicating that although these ionizations are somewhat abnormal with respect to p*K*, they are not accompanied by any noticeable irreversible conformational change throughout this pH range. It is rather difficult to say for certain that the differences in the number of ionizing groups between the two derivatives and the native protein actually represent one residue of tyrosine which has been rendered titratable by reduction and alkylation of the protein but it is tempting to consider. The sequence of HGH shows that Tyr_{159} is adjacent to Cys_{158}. Since the reduction and alkylation of this Cys in the absence of denaturant can be accomplished without any gross conformational changes in the molecule (see below), we may assume that it lies on or very near the surface rather than buried deeply within the molecule. If the phenolic group of Tyr_{159} were directed into the interior of the protein, it would not ionize readily even though its next neighbor lay on the surface. It might be this tyrosine residue which becomes ionizable following reduction and alkylation of the disulfide bond between Cys_{158} and Cys_{68}, due to a small localized conformational change.

Figure 4 shows the ionization data for all three proteins in 6 M guanidine hydrochloride. We have not been able to obtain reliable values for the ionization above pH 12.5 in this solvent.

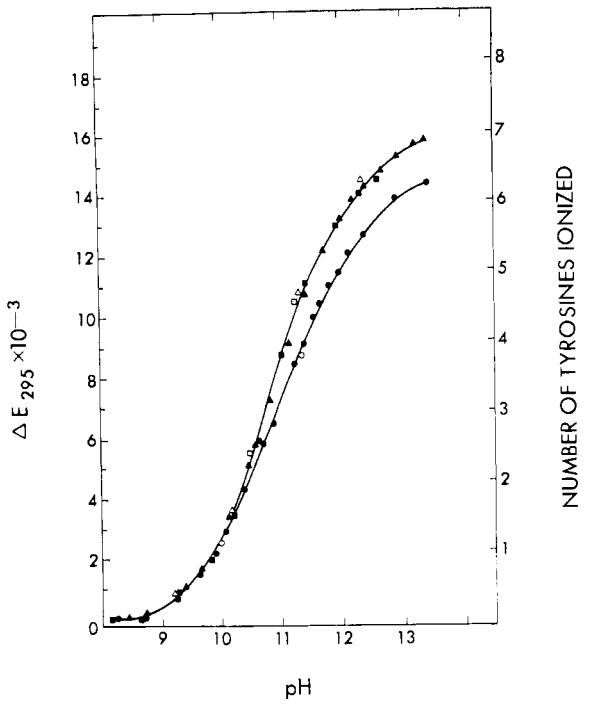

FIGURE 3: Tyrosyl ionization curves from spectrophotometric titrations of native (●), RCAM (▲), and RCOM (■) HGH in 0.15 M KCl. Points from reverse titration are indicated by open figures: native (○), RCAM (△), and RCOM (□). The number of ionized tyrosines is calculated from $\Delta E_{295}/2310$.

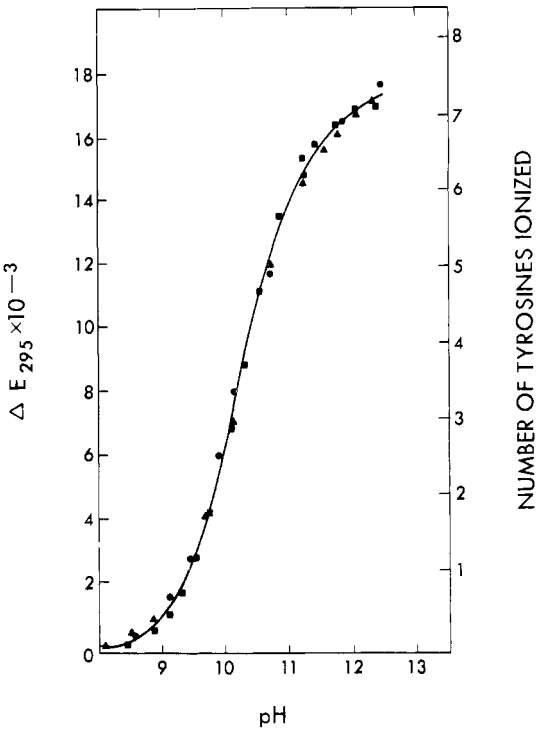

FIGURE 4: Tyrosyl ionization curves from spectrophotometric titrations of native (●), RCAM (▲), and RCOM (■) HGH in 6 M guanidine hydrochloride. The number of ionized tyrosines is calculated from $\Delta E_{295}/2390$.

The points for all three proteins lie fairly close together and we assume that the ionization curve is equivalent for all three within experimental error. The pK_a for these 7–8 tyrosines is 10.25 which is quite close to what has been reported for normalized tyrosines in this solvent (Nozaki and Tanford, 1967).

In summary it may be concluded that for the native protein in 0.15 M KCl one or two tyrosine residues cannot be titrated up to pH 13, and those that do ionize, do so abnormally but reversibly. The two reduced-alkylated derivatives seem to behave identically with perhaps one more ionizable tyrosine than in the native protein but with the same abnormal and reversible properties. It seems quite clear that reduction and alkylation of the disulfide bonds does not cause any gross conformational changes in the protein resulting in a general normalization of the tyrosine ionization.

Circular Dichroism. The ultraviolet circular dichroism spectra of the native protein and the two reduced-alkylated derivatives are shown in Figure 5. All three proteins show two of the three dichroism bands observed for α-helical polypeptides (Holzwarth, 1964; Holzwarth and Doty, 1965). Because of excessive absorption at wavelengths below ≈202 mμ we have not yet been able to demonstrate the positive α-helix band at 191 mμ. Nevertheless, the values of the mean residue molecular ellipticities for the two negative bands, which in these proteins occur at 209 and 221 mμ, indicate that all three contain a significant and essentially equivalent percentage of their amino acid residues in an α-helical conformation. We have estimated the helix contents from the ellipticities of both bands using values for 100% α helix and 100% random coil obtained from measurements of poly-L-glutamic acid (J. Y. Cassim, and J. T. Yang, unpublished data). These helix contents, shown in Table VI rounded off to the nearest 5%, are identical for both bands in all three proteins. This value of 55% α helix is in excellent agreement with an α-helix content of 50–60% obtained for native HGH from measurements of optical rotatory dispersion (Bewley and Li, 1967). Except for the small differences[3] around the 221-mμ band, the three spectra are remarkably similar. We conclude that the secondary structure of HGH is only very little if at all perturbed by reduction and alkylation of the disulfide bonds.

Tryptic Digestion. Figure 6 shows the alkali uptake in moles of OH^- per mole of protein as a function of time for tryptic digestions of native, RCAM, RCOM, and performic acid oxidized HGH. From the content of lysine and arginine residues in HGH (Li *et al.*, 1966), it can be concluded that about 20 moles of OH^- should be consumed upon complete digestion. It is quite evident from Figure 6 that these proteins are considerably different in their rates of proteolysis by trypsin. The RCOM and performic acid oxidized hormone are digested at the same rate, the reaction being approximately 40% complete in 30 min. The RCAM protein is digested much more slowly being about 25% complete in 30 min. The native hormone is even slower to digest, being only about 15% complete in the 30-min period.

[3] As mentioned above, these small differences cannot be meaningfully interpreted as any change in α-helix content. Whether they are due to contributions from a weakly dichroic band somewhere between 220 and 240 mμ or to experimental error is not presently known but is under investigation.

TABLE VI: Results of Circular Dichroism Measurements on HGH and Derivatives.

Protein	$[\theta]_\lambda$[a] 221 mμ	$[\theta]_\lambda$[a] 209 mμ	α-Helix Content from $[\theta]_\lambda$[b] % α Helix$_{221\,m\mu}$	α-Helix Content from $[\theta]_\lambda$[b] % α Helix$_{209\,m\mu}$
Native	19,700 ± 100	21,800 ± 300	55	55
RCAM	20,500 ± 300	22,300 ± 400	55	55
RCOM	20,100 ± 100	22,300 ± 500	55	55

[a] Expressed as the mean of three determinations plus and minus average deviation. Units are (deg cm²)/dmole. [b] Rounded off to the nearest 5%.

Markus (1965) has suggested that the susceptibility of a protein to proteolysis is a function of the looseness of the protein's conformation and its ability to assume many closely equivalent structural states. Thus, a given amino acid sequence would show different rates of proteolytic digestion depending on its particular state of rigidity.

Since the four proteins used in these digestions have essentially the same primary structure (the only alterations being in residues not attacked by trypsin), any differences in the rates of enzymatic hydrolysis are most probably indicative of an increased susceptibility of digestible bonds. Such increased susceptibility may be brought about by denaturation of the protein or as Markus suggests simply by removing some of the physical restraints normally imposed which might tend to hold the protein in a more rigid conformation.

It would appear that the RCAM-HGH while somewhat more susceptible to proteolysis than the native is significantly less susceptible than either the RCOM or performic acid oxidized derivatives. The viscosity data presented above indicate that performic acid oxidation irreversibly denatures HGH, producing an expanded, disorganized molecule. The rapid enzymatic digestion of this oxidized protein is consistent with this picture. On the other hand, the data relating to the secondary and tertiary structure of the RCOM derivative show that its structure is equivalent to that of the RCAM derivative and not very different from that of the native. The rapid proteolysis of this RCOM protein must indicate a difference in the stability of the secondary and tertiary structures of this molecule relative to the RCAM protein, perhaps due to a very small conformational difference which escapes detection by the other criteria. Thus, the marked increase in proteolysis of the RCOM over the RCAM protein may be due to an increased susceptibility of only one or two potential sites of hydrolysis, the cleavage of which promotes a gross conformational change resulting in rapid hydrolysis of the entire molecule. The proximity of the negative charges on the two carboxymethyl groups may be the perturbing element in this derivative.

Conclusion

It is clear that the disulfide bonds in HGH are not intrinsically necessary for biological activity, nor are these bonds

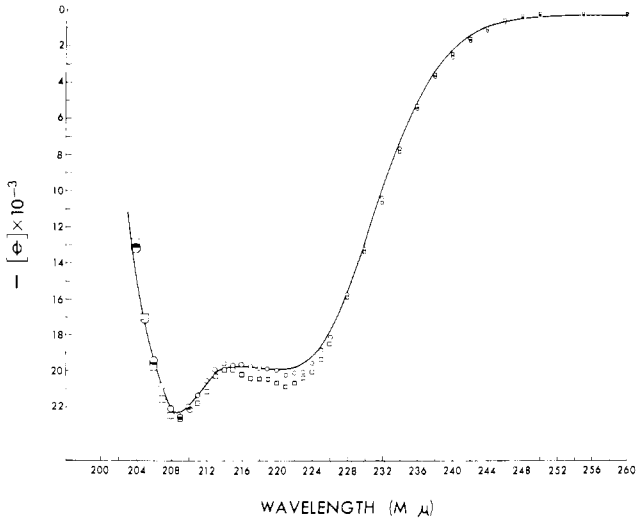

FIGURE 5: Circular dichroism spectra of native (———), RCAM (□), and RCOM (○) HGH in 0.1 M Tris buffer (pH 8.2). Each point is the mean of three determinations. The size of each point indicates the average deviation from the mean. The signal to noise ratios are: 16:1 at 230 mμ, 13:1 at 220 mμ, 6:1 at 210 mμ, and 1.6:1 at 205 mμ.

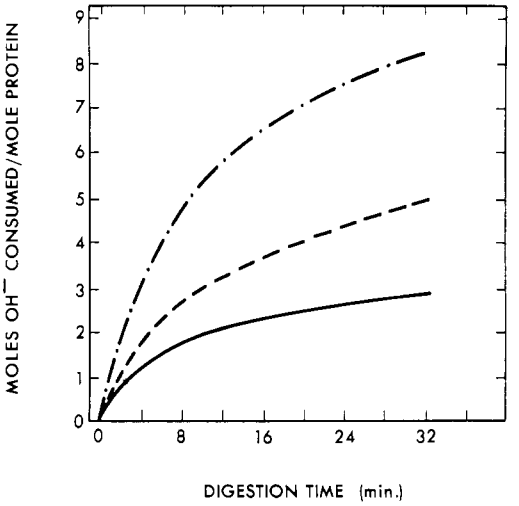

FIGURE 6: Rates of tryptic hydrolysis of native (———), RCAM (– – – –), and RCOM and performic acid oxidized (–·–·) HGH. Conditions of hydrolysis are as described in the text under Experimental Procedures. The RCOM and performic acid oxidized proteins were digested at the same rate and are shown as a single curve.

required to forcibly link portions of the peptide chain together. These bonds do serve to provide physical restraints which induce added rigidity to the conformation determined by the primary structure. Such added rigidity may be of considerable importance to the molecule in terms of being able to survive within the organism in an active form from the time it is synthesized in the pituitary until it performs its intended function at the receptor site.

One must be very careful in interpreting investigations of this type. For instance, if iodoacetic acid had been the only alkylating agent employed, it would have been very easy to conclude that one or both of the disulfides are necessary for the retention of the growth-promoting activity. The rates of enzymatic digestion, however, would indicate that there have been pronounced changes in the stability of the structure in the RCOM derivative. It is very likely that the loss of growth-promoting activity is due to destruction of the derivative, probably by enzymatic degradation, during its journey from the site of injection to the receptor site, rather than being due to an unfavorable perturbation of the active site of the hormone. In addition, the maintenance of considerable lactogenic activity may reflect the fact that in this assay the test substance is injected directly into the tissue which contains the lactogenic receptor. Since in this case the protein does not go through the blood stream, but acts locally at the site of injection, there is considerable less opportunity for degradation to occur before the protein has performed its function.

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