Growth Hormone Conformation and Conformational Equilibria†

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ABSTRACT: The circular dichroic spectrum of human, bovine, ovine, and rat pituitary growth hormone has been determined between 202 and 310 nm. Each spectrum was resolved into gaussian components and assignments were made to the various aromatic side-chain chromophores (*i.e.*, tryptophan, tyrosine, and phenylalanine) and to the peptide chromophore. Below 240 nm the circular dichroic spectrum of each growth hormone was similar and indicated about 45–50% α helicity. This agreed well with the predicted α helicity using semiemperical rules for helix formation involving short-range interactions only. Above 240 nm the circular dichroic spectrum of bovine and ovine growth hormone is similar, but is strikingly different from that of both human and rat growth hormone, particularly in the region corresponding to the L_a and L_b tryptophanyl bands. Since a single tryptophan is present in each of these four proteins, the spectral differences indicate a nonidentical localized environment of this particular aromatic group arising either from the sequence differences or from conformational differences. A comparison of the resolved phenylalanyl circular dichroic bands in growth hormone with those of a phenylalanyl-containing peptide indicated that many of these residues are constrained in position in growth hormone, probably as part of the tertiary structure. Reversible equilibrium unfolding studies were performed by measuring the denaturation difference absorption at 290 nm (indicative of tertiary structure) and the ellipticity at 222 nm (indicative of secondary structure) at various concentrations of guanidinum hydrochloride. The data were not consistent with a two-state process, but could be described using a sequential three-state model involving a helical intermediate containing little tertiary structure. An analysis of these denaturation data in terms of the three-state model yielded unfolding free energies in the absence of denaturant of 8–14 kcal/mol for rat, bovine, and ovine growth hormone. These represent the first stability estimates of a polypeptide hormone and, interestingly, the values are comparable to those of noncirculatory proteins (*e.g.*, pancreatic ribonuclease and myoglobin). The demonstrated "conformational flexibility" of growth hormone may be important in membrane interactions leading to the stimulation of particular intracellular processes.

Various studies on the enzymes ribonuclease and staphylococcal nuclease have clearly demonstrated the dependence of the biologically active conformation on the essentially complete amino acid sequence (Lin, 1970; Anfinsen *et al.*, 1971; Puett, 1972a–c), while, in contrast, fragments of several polypeptide hormones retain biological activity. Since a stable conformation is not expected in small peptides in aqueous solution, these results raise interesting questions regarding the relative roles of hormone sequence and conformation on the interaction with target cells leading to a biological response.

GH[1] is of interest in structure–function studies since the intact protein is sufficiently large for a stable tertiary structure to develop, *e.g.*, the molecular weight is about 22,000. Yet there is evidence that some peptides from GH retain varying biological activities (Nutting *et al.*, 1970, 1972; Bornstein *et al.*, 1971; Yamasaki *et al.*, 1970; Chillemi *et al.*, 1972; Sonnenberg *et al.*, 1972). Also, we have recently demonstrated that a large cyanogen bromide fragment of GH containing a significant amount of secondary structure induced the activity of hepatic ornithine decarboxylase, but failed to induce somatomedin (Levine *et al.*, 1973).

The results of the extensive studies on both the sequence of GH from various species (Li *et al.*, 1969, 1972; Santome *et al.*, 1971; Niall, 1971; Fellows *et al.*, 1972) and the conformational aspects of GH (Edelhoch and Lippoldt, 1970; Aloj and Edelhoch, 1972; Bewley and Li, 1972a,b) have provided a strong foundation for more detailed structure–function studies. We have obtained and resolved the CD spectrum of GH from four species, and these results, along with data on the conformational equilibria of GH from three species, are presented herein. Our studies dealing with the effects of protein modification on GH activity will be presented elsewhere.

Experimental Section

Materials. The various hormones used in this study were kindly provided by the Endocrinology Study Section of the National Institute of Arthritis, Metabolic, and Digestive Diseases. The bovine (NIH-GH-B17, 0.92 IU/mg) and ovine (NIH-GH-S10, 0.86 IU/mg) preparations were supplied by the Animal Pituitary Hormone Program. HGH (NIH-GH-ES 1544C, 2.0 IU/mg) and rGH (NIAMD-RAT-GH-B-1 and B-2, 0.9 IU/mg) were donated by the National Pituitary Agency and the Rat Pituitary Hormone Program, respectively. The ovine pituitary lyophilized powder was generously given to us by Dr. David N. Orth and Mr. Wendell E. Nicholson. The peptide, glycyl-L-phenylalanylglycine, was from Cyclo Chemical Co. (lot G-1704) and spectrophotometric grade GdmCl was from Heico, Inc.

Hormone Purification. The ovine, bovine, and rat NIH

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[1] Abbreviations used are: CD, circular dichroic; GdmCl, guanidinium hydrochloride; GH, pituitary growth hormone (the prefixes b, h, o, and r denote bovine, human, ovine, and rat, respectively).

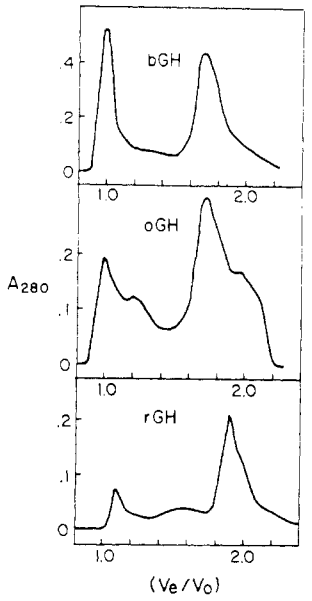

FIGURE 1: Gel filtration of bGH, oGH, and rGH on Sephadex G-100 (2.5 × 94 cm) in 0.126 M ammonium bicarbonate. The abcissa represents the ratio of the elution volume to the void volume.

supplied GH preparations were purified by gel filtration (Burger *et al.*, 1966a) on a calibrated 2.5 × 94 cm Sephadex G-100 column equilibrated and developed with 0.126 M ammonium bicarbonate (pH 8.5) at 25°. Typically, 150 mg of GH was suspended in 4.8 ml of 0.126 M ammonium bicarbonate, stirred for 15–90 min, and centrifuged at 1800*g* to remove any particulate material before application to the column. The effluent was monitored by absorbance at 280 nm and fractions of 3–5 ml were collected. hGH (10 mg) was chromatographed under similar conditions except that a smaller column (1 × 21 cm) was used. Ovine GH was prepared from the lyophilized pituitary powder using the method of Papkoff and Li (1958) and then chromatographed on Sephadex G-100 as described above. The portion eluting in the fractions corresponding to a molecular weight of 44,000 was used.

Hormone Characterization. The amino acid analyses were performed on a Beckman 120 analyzer using 20-hr protein hydrolysates (6 N HCl, 110°) with norleucine as internal standard. Polyacrylamide gel electrophoresis (pH 9.5) was conducted as described by Davis (1964) except that stacking and sample gels were omitted. Sodium dodecyl sulfate gel electrophoresis in the presence of 2-mercaptoethanol and dithiothreitol was conducted according to method one of Weber *et al.* (1972).

Solution Preparation. Stock solutions containing 1.6–3.9 mg/ml of GH were made in 0.126 M ammonium bicarbonate by stirring for 15–30 min at 25° and then centrifuging for 10 min at 1800*g* (25°). The clear supernatant formed the stock solution. Protein solutions containing various concentrations of GdmCl were normally prepared using dilutions of the GH stock solution with water and 6 M GdmCl to give solutions containing *ca.* 0.20–0.27 mg of GH/ml in 12.6 mM ammonium bicarbonate and the desired GdmCl concentration. The pH of these solutions was in the range of 7.9–8.5. Protein concentrations were determined using an extinction coefficient (1 mg/ml, 278 nm, 1 cm) of 0.70 for bGH (Burger *et al.*, 1966b) and 0.931 for hGH (Bewley and Li, 1972a). The extinction coefficients of bGH and oGH are essentially identical (Bewley and Li, 1972b), and, thus, the value of 0.70 was also used for oGH. The extinction coefficient for rGH was estimated to be 0.763 based on quantitative amino acid analysis of a solution of known absorbance; we assumed 16 mol of aspartic acid plus asparagine per mol of protein of mol wt 21,500 (Groves and Sells, 1968).

Denaturation Difference Spectra. All difference spectra were measured on a Hitachi Perkin-Elmer double-beam spectrophotometer in the 90–110%T mode with a single matched pair of 1-cm cuvets. The sample solution contained GH (0.2–0.4 mg/ml) in 12.6 mM ammonium bicarbonate and a known concentration of GdmCl, and the reference solution contained the same concentration of GH in 12.6 mM ammonium bicarbonate. The absorbance (1 cm) of the 6 M GdmCl stock at 290 nm is less than 2×10^{-3} absorbance unit and thus no correction was necessary for the denaturant. Kinetic studies showed that the GdmCl denaturation of GH is very rapid; however, to ensure that equilibrium was established, a number of the solutions were measured both within an hour after mixing and again after 24 hr had elapsed.

Circular Dichroic Spectra. The CD spectra were obtained with a Cary 60 spectropolarimeter equipped with a CD attachment and calibrated with *d*-10-camphorsulfonic acid. In the near-ultraviolet region (250–320 nm), GH concentrations of 1–3 mg/ml were used in a 1-cm cell and the 40-mdeg full-scale range was used. In the far-ultraviolet region (200–250 nm), the GH concentrations were 0.1–0.4 mg/ml and the cells had path lengths of 0.5 and 1 mm; the full-scale range was either 40 or 100 mdeg. A time constant of 3 (and occasionally 1) sec was used. Reported values represent the average of two to four scans and base lines were recorded regularly. The mean residue ellipticity, $[\theta]$, in (deg cm^2)/dmol, was calculated using a mean residue weight of 113 for bGH and oGH and 115 for rGH and hGH. Curve resolution was achieved as described elsewhere (Puett, 1972a; Zahler *et al.*, 1972). The rotational strengths of the resolved bands were calculated as described previously (Puett, 1972a).

Results

Purification and Characterization of Growth Hormone. Figure 1 shows typical elution profiles for bGH, oGH, and rGH. Based on the elution volumes of proteins of known molecular weight both bGH and oGH elute as dimers. The elution profile of rGH was somewhat variable and was probably influenced by the concentration and time of stirring before application to the column. The major portion of the protein either eluted as a dimer or else eluted as a lower molecular weight fraction. However, both "monomeric" and "dimeric" rGH appeared to have superimposable GdmCl denaturation profiles and identical amino acid compositions. The elution profile of hGH showed a very small amount of material at V_0 and a single symmetrical peak eluting at $V_e/V_t = 0.58$; the yield from this procedure for hGH was 75%.

The amino acid composition of purified bGH, oGH, and rGH was in reasonable agreement with reported values (Fernádez *et al.*, 1972; Li *et al.*, 1972; Ellis *et al.*, 1968).

Polyacrylamide gel electrophoresis on bGH and oGH indicated one major and two minor components as has been reported (Free and Sonnenberg, 1966) and is attributed to deamidation. Both the "monomeric" and "dimeric" rGH yield one major band that migrated with a R_F slightly greater than oGH or bGH. Polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate and a reducing agent gave single bands with rGH, bGH, and oGH as prepared by the method of Papkoff and Li (1958). Using the proteins bovine serum albumin, ovalbumin, myoglobin, lysozyme, and cyto-

TABLE I: Resolved Gaussian Parameters of the Circular Dichroic Spectrum of Human Growth Hormone.

Assignment	λ_0 (nm)	Δ (nm)	$[\theta^\circ]$ (deg cm²)/ dmol	$R \times 10^{40}$ (cgs)
π–π^*	206.2	9.5	−14,000	−7.96
n–π^*	223	12.3	−14,900	−10.1
a	229	4	−600	−0.129
a	238	4.2	+1,000	+0.218
Tentative Assignment				$R \times 10^{43}$ (cgs)
0+2(930) Phe	255.3	4.3	−44	−9.14
0+930 Phe	261.7	2.6	−48	−5.88
0–0 Phe	267.7	2.3	−55	−5.83
0+2(800) Tyr	271.7	2.1	−25	−2.38
0+800 Tyr	276.5	3.5	−58	−9.06
0+420 Tyr	281	1.6	−13	−0.913
0–0 Tyr	283	3.5	−50	−7.63
0+1400 ^1L_a Trp	293	5	+29	+6.11
0+400 ^1L_a Trp	298.5	3	+11	+1.36
0–0 ^1L_a Trp	304	4	+4	+0.649

[a] No assignments have been made to these bands.

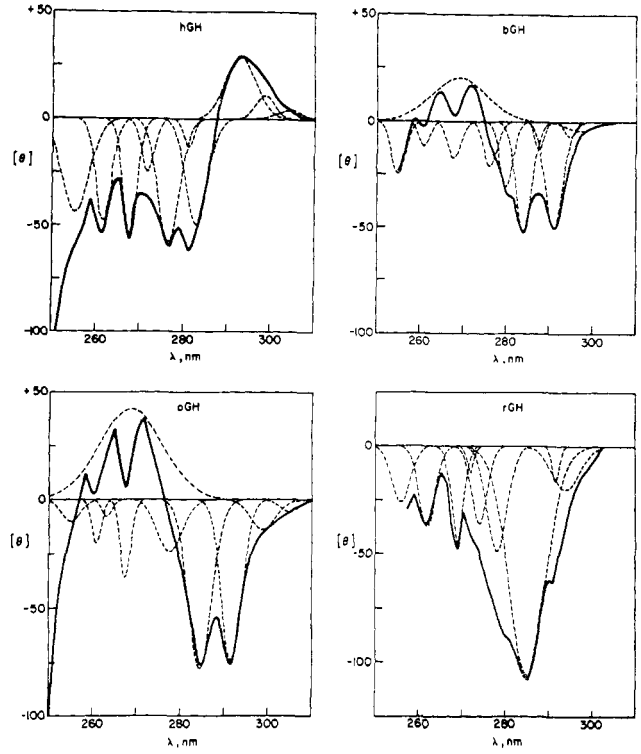

FIGURE 2: The resolved near-ultraviolet CD spectrum of GH from four species in 0.126 M ammonium bicarbonate. The experimental details are given in the Experimental Section. The solid line represents the experimental curve and the dashed lines denote the resolved bands. Protein concentrations were 2.0, 2.7, 1.3, and 1.6 mg per ml for hGH, bGH, oGH, and rGH, respectively.

chrome *c* as standards, the molecular weights of rGH, bGH, and oGH were essentially identical, 22,000. We found that one lot of NIH supplied oGH (lot S10), when incubated at 37° for 2–4 hr at a concentration of 20 mg/ml in 0.126 M ammonium bicarbonate, gave two bands with apparent molecular weights on 12% polyacrylamide gels of 13,500 and 8700 under these denaturing (sodium dodecyl sulfate) and reducing conditions.

In the procedure used to purify the NIH oGH preparation, it is thus possible that some chain cleavage may have occurred in the material used for physicochemical characterization. However, we wish to emphasize that under nondenaturing conditions, this modified oGH exhibited chromatographic (Sephadex G-100) and electrophoretic (polyacrylamide gels) properties identical with those of unmodified oGH. Also, as discussed below, both this material and oGH prepared by the method of Papkoff and Li (1958), which we know is intact, gave essentially the same GdmCl denaturation profiles and reversibility characteristics. The characterization of the proteolytic cleavage will be explored more completely in a subsequent communication (L. A. Holladay and D. Puett, in preparation). The present evidence indicates that the fragments are joined by both noncovalent interactions and a disulfide bridge.

Purified bGH, oGH (purified from NIH-GH-S10), and both "monomeric" and "dimeric" rGH were active in the stimulation of hepatic ornithine decarboxylase[2] in intact rats (Russell and Snyder, 1969). Both oGH purified from NIH-GH-S10 and rGH were assayed[3] for somatomedin induction (Salmon, 1972) in hypophysectomized rats and were found to be highly active.

Ultraviolet Circular Dichroism of Growth Hormone. In order to determine if the resolved gaussian CD bands are useful in understanding species differences and similarities in the four growth hormones examined in this study, the CD spectra were obtained and resolved into component bands as shown in Figures 2 and 3 for the near- and far-ultraviolet spectra, respectively. The parameters of the resolved bands and the associated rotational strengths of each GH are given in Tables I–IV. The assignments of the resolved near ultraviolet bands are tentative at best, and represent extrapolations based on work with model compounds (Horwitz *et al.*, 1969, 1970; Strickland *et al.*, 1969, 1970; Edelhoch *et al.*, 1968). Several cautionary remarks are in order regarding the resolved band parameters. It is known that there are two disulfide bonds in the ovine, bovine, and human growth hormones (Fernández *et al.*, 1972; Li and Dixon, 1971). The CD bands due to disulfide bonds do not show any fine structure (Beychok, 1965, 1966) and, thus, it is not surprising that the curve resolution does not require inclusion of a disulfide CD band. The curve resolution parameters thus represent a minimum set of parameters which can be used to calculate the observed spectrum to an accuracy of a few per cent over the wavelength range 205–310 nm.

We have compared the rotational strengths of the three resolved bands assigned to phenylalanyl bands in GH with the rotational strengths obtained by resolving the near-ultraviolet CD spectrum of Gly-L-Phe-Gly which is shown in Figure 4 for the peptide in aqueous solution and in 80% ethylene glycol. The spectra closely resemble those reported for *N*-acetyl-L-phenylalanylamide (Simmons *et al.*, 1969). The characteristics of the resolved bands are given in Table V and a comparison of three of these bands with the resolved phenylalanyl bands of GH is presented in Table VI. The agreement is satisfactory for the 0 + 930 band resolved for oGH, but poor for the remaining resolved bands. The wave-

[2] It is a pleasure to thank Dr. Jon H. Levine and Mr. Wendell E. Nicholson for performing these assays.

[3] It is a pleasure to thank Dr. William D. Salmon, Jr., for performing these assays.

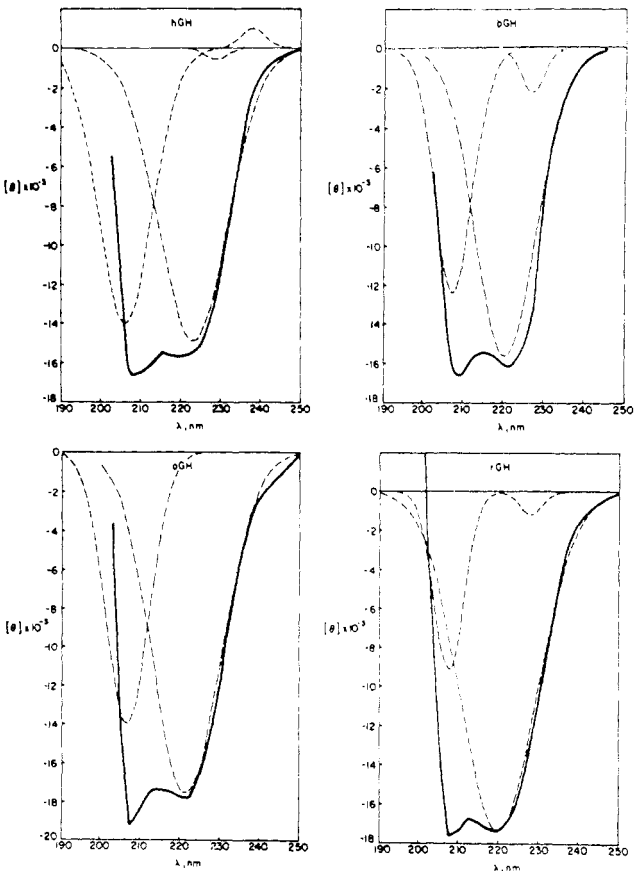

FIGURE 3: The resolved far-ultraviolet CD spectrum of GH from four species in 0.126 M ammonium bicarbonate. The solid line denotes the experimental curve and the dashed lines represent the resolved bands. Protein concentrations were *ca.* 0.4 mg/ml for the various GH's.

TABLE II: Resolved Gaussian Parameters of the Circular Dichroic Spectrum of Bovine Growth Hormone.

Assignment	λ_0 (nm)	Δ (nm)	$[\theta^\circ]$ (deg cm^2)/ dmol	$R \times 10^{40}$ (cgs)
	A. Far Ultraviolet			
$\pi-\pi^*$	207.5	6.7	−12,400	−4.94
n−π^*	220.7	10.7	−15,600	−9.33
a	226.7	4	−2,200	−0.479
Tentative Assignment				$R \times 10^{43}$ (cgs)
	B. Near Ultraviolet			
0+2(930) Phe	255	2.3	−25	−2.78
0+930 Phe	261	1.8	−12	−1.02
0–0 Phe	268	2.2	−17.5	−1.77
a	269	9	+20	+8.26
0+800 Tyr	276.5	2.2	−21	−2.06
a	280	2.1	−31	−2.87
0–0 Tyr and 0+850 ^1L_b Trp	284.2	2.7	−53	−6.21
0+1400 ^1L_a Trp	287.7	1.4	−13	−0.781
0–0 ^1L_b Trp	291.2	2.9	−50	−6.14
0+400 ^1L_a Trp	295	1.7	−7	−0.498
0–0 ^1L_a Trp	297	4.5	−4	−0.748

[a] No assignments have been made to these bands, although the 269- and 280-nm bands may arise from ^1L_a Trp transitions.

TABLE III: Resolved Gaussian Parameters of the Circular Dichroic Spectrum of Ovine Growth Hormone.

Assignment	λ_0 (nm)	Δ (nm)	$[\theta^\circ]$ (deg cm^2)/ dmol	$R \times 10^{40}$ (cgs)
	A. Far Ultraviolet			
$\pi-\pi^*$	206.8	7.8	−14,000	−6.52
n−π^*	222	12.3	−17,500	−12.0
Tentative Assignment				$R \times 10^{43}$ (cgs)
	B. Near Ultraviolet			
0+2(930) Phe	255	3	−10	−1.45
0+930 Phe	260.8	1.5	−20	−1.42
a	263	1.5	−8	−0.563
0–0 Phe	267.5	1.7	−36	−2.82
a	268.5	10	+42	+1.93
0+800 Tyr	277.5	4	−24	−4.27
0–0 Tyr and 0+850 ^1L_b Trp	284.5	3.8	−78	−12.8
0+0 ^1L_b Trp	291.7	3.3	−74	−10.3
0+400 ^1L_a Trp	299	4	−14	−2.31
0–0 ^1L_a Trp	305	3	−5.5	−0.668

[a] No assignments have been made to these bands, although they may arise from ^1L_a Trp transitions.

lengths of the GH phenylalanyl bands do, however, agree very closely with those of Gly-L-Phe-Gly. This, along with the partial agreement obtained for the 0 + 930 band of bGH, oGH, and Gly-L-Phe-Gly, adds support to the interpretation of the CD spectrum in the 250–270-nm region as composed of a broad positive band superimposed on the three negative

TABLE IV: Resolved Gaussian Parameters of the Circular Dichroic Spectrum of Rat Growth Hormone.

Assignment	λ_0 (nm)	Δ (nm)	$[\theta^\circ]$ (deg cm^2)/ dmol	$R \times 10^{40}$ (cgs)
	A. Far Ultraviolet			
$\pi-\pi^*$	207.5	5.5	−9,100	−2.98
n−π^*	220	13.5	−17,300	−13.1
a	228	4.0	−1,200	−0.260
Tentative Assignment				$R \times 10^{43}$ (cgs)
	B. Near Ultraviolet			
0+2(930) Phe	256	3	−26	−3.76
0+930 Phe	262	2.7	−36	−4.58
0+0 Phe	269	2	−46	−4.22
0+2(800) Tyr	272	1.2	−12	−0.653
0+1250 Tyr	274	2.5	−36	−4.05
0+800 Tyr	278	3.25	−49	−7.07
0+0 Tyr and 0+850 ^1L_b Trp	285	6	−106	−27.5
0+0 ^1L_b Trp	291.8	1.2	−16	−0.778
0+400 ^1L_a Trp	294	4.5	−20	−3.78

[a] No assignment has been made to this band.

TABLE V: Resolved Gaussian Band Parameters for Glycyl-L-phenylalanylglycine.

λ_0 (nm)	$[\theta^\circ]$ (deg cm²)/dmol	$R \times 10^{43}$ (cgs)
A. Water, 25°, 10 mM KCl–2 mM Sodium Phosphate (pH 7.5)		
240	−23	−4.14
246.5	+9	+0.541
251	+18	+1.06
254.6	−51	−3.46
257.3	+22	+1.05
260.8	−148	−12.6
263.7	+16	+0.749
267.5	−156	−15.1
λ_0 (nm)	$[\theta^\circ]$ (deg cm²)/dmol	$R \times 10^{43}$ (cgs)
B. 80% Ethylene Glycol, 25° 10 mM KCl–2 mM Sodium Phosphate (pH 7.5)		
238	−355	−106.7
245.5	−35	−3.52
248.5	−30	−2.68
255.3	−68	−5.26
258	+30	+1.00
261.7	−140	−10.6
266	+40	+2.78
268.1	−144	−14.6

phenylalanyl bands as originally proposed by Bewley and Li (1972a).

For rGH, the helix content is estimated to be 45% from $[\theta]$ at 222 nm by the method of Chen and Yang (1971), 47% from $[\theta]$ at 208 nm by the method of Greenfield and Fasman (1969), 51% by the least-squares method of Chen *et al.* (1972) over the 207–243-nm range, and 62 and 48%, respectively, by taking the ratio of the resolved 222-nm n–π^* band and the resolved 208-nm π–π^* band to the resolved bands of the CD spectrum of an entirely α-helical protein as determined by Chen and Yang (1971).

Effect of Guanidine Hydrochloride. Figure 5 gives the relative ellipticity at 222 nm for bGH, oGH, and rGH as a function of GdmCl concentration under equilibrium conditions. (Insufficient material prevented us from performing similar measurements with hGH.) The midpoint of the unfolding profile occurs at a GdmCl concentration of 3.4–3.7 M for all three proteins.

The GdmCl-induced difference spectrum (not shown) is very similar to the acid-induced difference spectrum (Burger *et al.*, 1966b) and is characterized by a 290-nm extremum which is attributed to a masked tryptophan in native GH. Since the magnitude of $\Delta\epsilon_{290}$ arising from GdmCl unfolding was found to be somewhat higher than that expected from a single tryptophan, there may also be some contribution from one or more tyrosines.

Figure 6 gives equilibrium results obtained from difference spectral measurements on bGH, oGH, and rGH at various GdmCl concentrations. For these three GH's the midpoint of the transition occurs at a lower GdmCl concentration than is found when the unfolding is monitored by ellipticity changes at 222 nm. This shows that the denaturation process for GH is not a two-state process, but suggests, rather, that unfolding proceeds in stages with the exposure of the tryptophanyl residue preceding helix unwinding.

Due to the possibility that a portion of the oGH prepared from NIH-GH-S10 had undergone chain cleavage, we repeated the GdmCl denaturation profile measurements of $\Delta A_{290}/A_{278}$ and $[\theta/\theta_0]_{222}$ over the 1.8–4.0 M GdmCl concentration range with oGH purified by the method of Papkoff and Li (1958), which is known to be free of proteolytic cleavage.

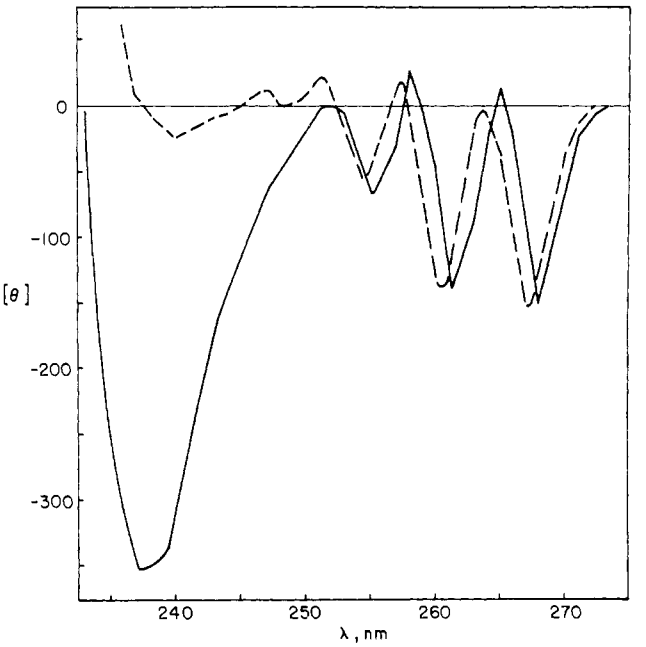

FIGURE 4: The CD spectrum of Gly-L-Phe-Gly in 10 mM KCl–2 mM sodium phosphate buffer (pH 7.5) (- - -) and in 80% ethylene glycol (——). $[\theta]$ is based on the phenylalanine molarity.

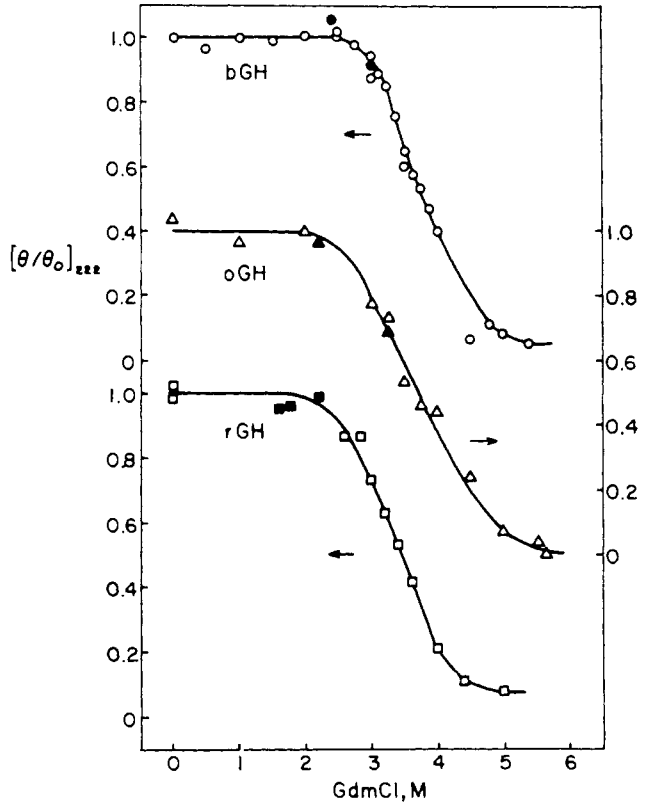

FIGURE 5: The variation of the ellipticity at 222 nm (relative to the value in 12.6 mM ammonium bicarbonate) with GdmCl concentration for bGH, oGH, and rGH under equilibrium conditions. Open symbols denote solutions prepared from GH initially in 126 mM ammonium bicarbonate and closed symbols refer to solutions prepared from GH initially in concentrated GdmCl.

TABLE VI: Comparison of Rotatory Strengths (cgs units) for Resolved Phenylalanyl Circular Dichroic Bands.[a]

Assignment	Gly-L-Phe-Gly in H_2O, $R \times 10^{43}$	Gly-L-Phe-Gly in 80% Ethylene Glycol, $R \times 10^{43}$	bGH, $R \times 10^{43}$ × (190/13)	oGH, $R \times 10^{43}$ × (190/13)	rGH, $R \times 10^{43}$ × (190/12)	hGH, $R \times 10^{43}$ × (190/11)
0–0	−15.1 (267.5)	−14.6 (268.1)	−26 (268)	−41 (267.5)	−67 (269)	−101 (267.7)
0+930	−12.6 (260.8)	−10.6 (261.7)	−15 (261)	−21 (260.8)	−72 (262)	−102 (261.7)
0+2(930)	−3.46 (254.6)	−5.26 (255.3)	−41 (255)	−21 (255)	−59 (256)	−158 (255.3)

[a] The value in parentheses after R denotes the wavelength in nm of the CD band. The rotational strengths of the phenylalanyl bands in the GH's have been corrected to yield R based on the phenylalanine molarity.

The transition profiles were superimposable within the limits of experimental error.

Free Energy of Unfolding. Using the same approach as for a twofold transition, one can convert the unfolding profiles in Figures 5 and 6 to the apparent (*i.e.*, experimentally determined) unfolding free energy, ΔG_{app}, at each GdmCl concentration. As discussed below, the data can then be analyzed using a more realistic model for unfolding. The apparent equilibrium constant, K_{app}, follows from

$$K_{app} = (Y_N - Y)/(Y - Y_D) \quad (1)$$

where Y, Y_N, and Y_D refer to the optical properties of GH at any GdmCl concentration, native GH, and denatured GH, respectively, with due base-line allowances being made for native and denatured GH. In order to estimate the apparent free energy of unfolding in the absence of denaturant, ΔG°_{app}, one can use transfer free energies for side chains and peptide units from water to the particular denaturant concentration, and fit the various ΔG_{app} points to eq 2 by summing over the number of peptide units and the various side chains using $\Delta\alpha_{app}$ and ΔG°_{app} as adjustable parameters (Tanford, 1964, 1970; Puett, 1972a).

$$\Delta G_{app} = \Delta G^\circ_{app} + \Delta\alpha_{app}\Sigma_i n_i \Delta G_i^t \quad (2)$$

We have shown the moles of each type of group per mole of GH, n_i, explicitly in eq 2; earlier it was considered implicit in the summation (Puett, 1972a). The parameters $\Delta\alpha_{app}$ and ΔG_i^t refer to the apparent average difference in the degree of exposure of the side chains and peptide groups in the denatured and the native conformation and the transfer free energy of these groups, respectively.

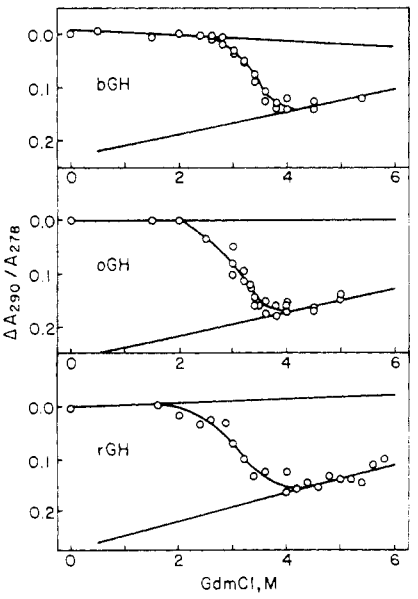

FIGURE 6: The effect of GdmCl on the magnitude of the denaturation difference spectrum at 290 nm (relative to the absorbance of GH in 12.6 mM ammonium bicarbonate at 278 nm) for bGH, oGH, and rGH under equilibrium conditions.

The values used for ΔG_i^t were determined from solubility data (Robinson and Jencks, 1965; Nozaki and Tanford, 1970) as previously described (Puett, 1972a). The amino acid composition of oGH was taken from the primary structure given by Li *et al.* (1972). The amino acid composition of bGH was presumed to be identical with that of oGH, except for a glycyl for valyl substitution as described by Fernández *et al.* (1972) and Seavey *et al.* (1971). For rat GH, the amino acid composition was determined and the data were calculated on the basis of residues per 187 residues; assuming one tryptophanyl residue per mole (Ellis *et al.*, 1968), a molecular weight of 21,500 results. This value agrees with that of 20,500 ± 500 determined by Groves and Sells (1968). The assumed integral values we obtained from the amino acid composition of rGH are in reasonable agreement with those of Ellis *et al.* (1968) computed on the basis of residues per 24,000 grams of protein. For the purpose of calculating values of $\Sigma_i n_i \Delta G_i^t$, it was assumed that one-half of the glutamic acid residues is present as glutamine residues and that 5 of the 16 aspartic acid residues are present as asparagine residues. However, these assumptions have little effect on the final values for ΔG_{app}.

Values for ΔG°_{app} and $\Delta\alpha_{app}$ were obtained by plotting ΔG_{app} as a function of $\Sigma_i n_i \Delta G_i^t$ as shown for bGH in Figure 7. (For brevity the data are shown for bGH only.) As expected from the unfolding data, the plots using ellipticity at 222 nm and ΔA at 290 nm give different values for both ΔG°_{app} and $\Delta\alpha_{app}$. Table VIIA summarizes the values obtained for bGH, oGH, and rGH. The ellipticity data yields ΔG°_{app} values about 3.5 kcal lower than that obtained from difference spectral data.

It is difficult to rationalize a glycyl for valyl substitution resulting in free-energy changes of this magnitude and we know, of course, that the transition is not two state. Moreover, there is other evidence that bGH is able to exist in several conformational states under varying solution conditions (Burger *et al.*, 1966a,b; Edelhoch and Burger, 1966; Edelhoch *et al.*, 1966).

More realistic models to account for the unfolding of GH could involve any of the following

$$N \rightleftharpoons X \rightleftharpoons D \quad (3a)$$

$$N \rightleftharpoons D \rightleftharpoons F \quad (3b)$$

$$N \rightleftharpoons X \rightleftharpoons D \rightleftharpoons F \quad (3c)$$

as well as other, more complicated, schemes. In the above, X represents a stable intermediate on the reversible pathway of

TABLE VII: Comparison of the Conformational Free Energy (kcal/mole) of Growth Hormone from Three Species.

	$[\theta/\theta_0]_{222}$		$(\Delta A_{290}/A_{278})$	
	ΔG°_{app}	$\Delta\alpha_{app}$	ΔG°_{app}	$\Delta\alpha_{app}$
	A. Based on a Two-State Model, N $\rightleftharpoons$ Y[a]			
Bovine	10.1 (0.7)[b]	0.11 (0.008)	12.7 (0.6)	0.15 (0.007)
Ovine	5.9 (0.4)	0.07 (0.004)	9.1 (0.6)	0.12 (0.008)
Rat	7.4 (0.2)	0.09 (0.002)	7.0 (0.2)	0.09 (0.003)

	N → X		X → D		N → D	
	ΔG°_{app}	$\Delta\alpha_{app}$	ΔG°_{app}	$\Delta\alpha_{app}$	ΔG°_{app}	$\Delta\alpha_{app}$
	B. Based on a Three-State Model, N $\rightleftharpoons$ X $\rightleftharpoons$ D					
Bovine	11.9 (0.9)	0.13 (0.011)	2.1 (1.1)	0.03 (0.013)	14.0 (0.7)	0.16 (0.017)
Ovine	10.1 (0.8)	0.12 (0.009)	−0.4 (0.8)	0.00 (0.009)	9.7 (1.1)	0.12 (0.014)
Rat	6.3 (0.2)	0.07 (0.002)	1.7 (0.2)	0.03 (0.004)	8.0 (0.2)	0.10 (0.003)

[a] Here Y refers to a state which probably represents a composite of states X and D (*cf.* text). [b] The values in parentheses are the standard errors for ΔG°_{app} and $\Delta\alpha_{app}$.

native (N) to denatured (D) protein, and F represents an incorrectly folded or partially folded form of GH.

Kinetic data are required to establish the existence of forms X and F and, indeed, scheme 3c appears to be applicable to both the acid unfolding of myoglobin at pH values not near the transition region (Shen and Hermans, 1972) and the GdmCl unfolding of cytochrome *c* (Ikai *et al.*, 1973; Henkens and Turner, 1973). We have attempted to follow the kinetics of unfolding with GH, but the majority of the conformational changes occur too rapidly to monitor using manual methods, and recourse will have to be taken to stopped-flow measurements. We did find, however, that using manual methods (*ca.* 20 sec after mixing were required before measurements could be made) the refolding kinetics of bGH from 4 to 2 M GdmCl were linear when the logarithm of ΔA_{290} (corrected for the equilibrium value) was plotted as a function of time.

It is possible to describe our equilibrium data using either scheme 3a or 3b. For example, with scheme 3a one can assume that state X represents a partially unfolded conformation with the tryptophanyl residue exposed to solvent, but with the secondary structure still mainly intact. According to this sequential model, states X and D would possess identical far-ultraviolet CD spectra. This highly simplified model can then be used to combine the unfolding data in terms of both ellipticity at 222 nm and differential absorbance at 290 nm in the following manner. Ellipticity measurements at 222 nm would result in the fraction of protein in state D, where f_D is given by

$$f_D = (\theta - \theta_D)/(\theta_N - \theta_D) \quad (4)$$

with $\theta = [\theta/\theta_0]_{222}$. The fraction of protein in the native state is determined as $1 - (f_X + f_D)$, where the sum of f_X and f_D is given by

$$f_X + f_D = (A - A_{X,D})/(A_N - A_{X,D}) \quad (5)$$

with $A = \Delta A_{290}/A_{278}$. Thus, by combining the values for f_D and $(f_X + f_D)$, values for the equilibrium constants for the transitions between states N, X, and D may be calculated.

Table VIIB gives the ΔG°_{app} values we obtained for the various transitions in terms of this three-state model. The small differences found in ΔG°_{app} for the N → X transition of oGH and bGH are seen to be well within the range of experimental error, but appear to be significantly higher than for rGH.

Discussion

Circular Dichroic Properties of Growth Hormone. The GH from the four species studied herein each possesses a single tryptophanyl residue. Thus, most of the optical activity of these proteins above 285 nm should arise from the localized environment of this group. It is noteworthy that three distinct types of CD patterns are seen between 285 and 300 nm. Whereas, the magnitude of the spectrum differs for oGH and bGH, the overall shape is remarkably similar. The difference in magnitude may reflect in part the different concentrations used or the possible mainchain cleavage of oGH. Strickland *et al.* (1969) have described four types of tryptophanyl CD spectra which are distinguished by the relative prominence and sign of the L_a and L_b transitions. These are dependent on both the type of model compound used and the solvent composition.

At this point, the safest interpretation of the spectra shown

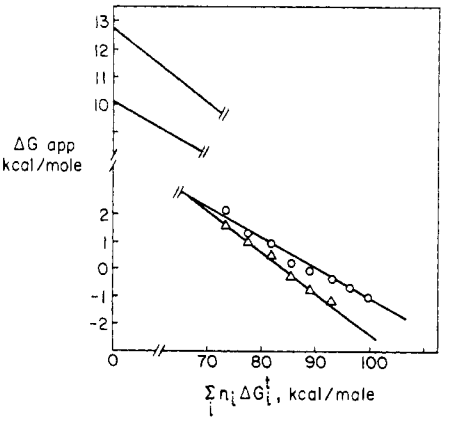

FIGURE 7: The apparent free energy of bGH (as obtained from the data in Figures 5 and 6) is plotted as a function of $\Sigma_i n_i \Delta G_i^t$ (see eq 1 for details). Circles denote CD date at 222 nm and triangles refer to difference absorbance data at 290 nm. The solid lines are theoretical and are based on the parameters given in Table VIIA in conjunction with eq 1.

in Figure 2 is that the single tryptophan, and probably the other aromatic residues as well, are in a different environment in rGH, hGH, and o,bGH. This may be due to differences in the amino acid sequence or to small conformation differences. Interestingly, human chorionic somatomamotropin, which has an amino acid sequence similar to that of hGH (Bewley *et al.*, 1972), has a CD spectrum (Bewley and Li, 1971) like that we found for rGH. Our finding that the resolved phenylalanyl CD bands are considerably higher than the corresponding values in the low molecular weight phenylalanyl-containing peptide indicates that many of the phenylalanyl residues in GH are constrained in position, *e.g.*, as part of a stable tertiary structure.

The far-ultraviolet spectrum of each of the GH's is very similar and indicates about 45–50% α helix. The spectra for hGH, bGH, and oGH are in good agreement with published data (Bewley and Li, 1972a,b).

Since there is a possibility that the α-helical regions of GH are stable even when the tertiary structure is destroyed, we have attempted to predict the α-helical segments based on the sequence of GH (that of oGH, Bewley and Li, 1972b, was primarily used) and the semiempirical rules of Kotelchuck and Scheraga (1969) which are based on short-range interactions. The result of this analysis indicates that oGH should be slightly greater than 50% α helical in good agreement with the experimental value. In particular, this model predicts α-helical regions involving residues 14–26, 29–34, 49–60, 73–97 with a possible bend involving residues Gly-88 and Pro-89 (*cf.* Crawford *et al.*, 1973), 108–113, 116–130, 133–140, and 176–191. If the carboxyl-terminal disulfide induces unfavorable constraints on α-helix formation, then the latter α-helical region is probably not realized and the expected α helicity would decrease to about 44%. Thus, most of the predicted α helicity is within about the first two-thirds (from the amino terminus) of the protein. This is in agreement with our finding that the A–B cyanogen bromide fragments of GH, which comprise approximately two-thirds of the amino-terminal portion of GH, have essentially the same degree of secondary structure as the intact molecule (Levine *et al.*, 1973).

Growth Hormone Conformational Equilibria. The results of our equilibrium studies on the reversible unfolding of GH by GdmCl conclusively demonstrates that denaturation is not a two-state process. Our treatment of the data in terms of a three-state process is only a working hypothesis and the identification of the number and type of stable intermediates or incorrectly folded conformations must await detailed kinetic studies involving rapid-reaction absorbance and optical activity measurements. The three-state model does indicate that the free energy of unfolding of oGH and bGH is identical within experimental error, a result that is in accord with the considerable homology in the amino acid sequence for these two proteins and the known similarity of their physical and spectral properties. Interestingly, the amino acid composition of rGH differs from that of the other GH's and the estimated stability also differs. Similar results have been noted for myoglobin (Puett, 1973a; Puett *et al.*, 1973) and cytochrome *c* (Puett, 1973b) from several species.

The values obtained for ΔG°_{app} for the X $\rightarrow$ D transition are very close to zero, indicating that once the protein has lost much of its tertiary structure, helix unfolding proceeds easily. This result is not unexpected since short helices, which are not stabilized by long-range interactions arising from a stable tertiary structure, are often unstable in aqueous solution (Hermans and Puett, 1971; Puett, 1972c).

In the context of the three-state model, the unfolding free energy of GH is comparable to that of other proteins of only slightly lower molecular weight, *e.g.*, lysozyme, ribonuclease, and myoglobin (Tanford, 1970; Puett, 1972a, 1973a). This represents the first estimate of the thermodynamic stability of a polypeptide hormone. It will be of interest to determine if the "conformational flexibility" of GH is important in membrane interactions and such studies are now in progress in this laboratory.

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