# Circular Dichroism Studies on Human Pituitary Growth Hormone and Ovine Pituitary Lactogenic Hormone*

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ABSTRACT: The conformations of human pituitary growth hormone and ovine pituitary lactogenic hormone have been compared by means of circular dichroism measurements. Both proteins appear to contain about half of their residues in the form of an $\alpha$ helix. Two dichroism bands in the region of side-chain adsorption have been assigned to the two tryptophan residues in lactogenic hormone, while a single band has been assigned to the tryptophan in growth hormone. In three perturbing solvents, the conformation of lactogenic hormone appeared to be less stable than that of growth hormone. In spite of this difference in stability, all conformational changes in both proteins were found to be reversible upon removal of the perturbants by dialysis.

The generally accepted hypothesis that the native conformation of a protein is primarily a result of noncovalent forces arising from its amino acid sequence (Epstein *et al.*, 1963; Anfinsen, 1964) further predicts that proteins which exhibit high degrees of sequence homology should also show corresponding degrees of conformational homology. The validity of this prediction is strongly supported by the demonstration of great similarity in the three dimensional structures of the $\alpha$ and $\beta$ chains of human hemoglobin in which approximately 50% of the residues are either identical or conservative replacements (Perutz, 1965; Perutz *et al.*, 1965).

The primary structures of two pituitary hormones,[1] HGH and LTH, have been determined (Li *et al.*, 1969a,b), and a comparison of the two sequences (Bewley and Li, 1970a) has shown them to be homologous to the extent of about 45%. In addition, the known overlap in biological activities[2] of these two hormones has suggested (Li, 1968) for some time that structural and conformational homologies may exist.

In this investigation we have concentrated on comparing those aspects of the native secondary and tertiary structures of HGH and LTH which can be inferred from measurements of circular dichroism. The relative stabilities of the two native structures have also been compared by challenging each with three perturbing solvents.

## Experimental Section

*Materials.* Glycine (ammonia free) and guanidine hydrochloride were obtained from Eastman Organic Chemicals, Rochester, N. Y. The guanidine hydrochloride was recrystallized twice from ethanol using Norite decolorizing carbon. Human pituitary growth hormone was prepared as previously described by Li *et al.* (1962). Sheep pituitary lactogenic hormone was prepared by a slight modification of the procedure described by Cole and Li (1955). All other chemicals were of reagent grade and used without further purification.

*Preparation of Samples for Measurement.* Stock solutions (1%) of both HGH and LTH were prepared by dissolving the proteins in very dilute ammonia solutions of pH 8.0–8.5. Aliquots of these stock solutions were carefully transferred by micropipet to a series of volumetric flasks and lyophilized. The concentration of protein in the two stock solutions was determined spectrophotometrically using the relations $\epsilon^{1\%}_{1cm,277nm}$ 9.31 for HGH and $\epsilon^{1\%}_{1cm,277nm}$ 9.09 for LTH. The protein concentrations in each volumetric flask could then be calculated from the concentrations of the stock solutions. The appropriate solvent was then added to the lyophilized protein and carefully made up to the desired volume. Solutions were allowed to stand at room temperature with periodic mixing for at least 1 hr before use. After completion of the circular dichroism spectra, each sample (5–7 ml) was dialyzed against two portions (2.5 l.) of 0.1 M Tris buffer, pH 8.2, total dialysis time being 24 hr. Before taking the circular dichroism spectra of the dialyzed samples, the pH was checked, and its protein concentration was redetermined spectrophotometrically.

*Circular Dichroism Spectra.* 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°. Fused quartz cells were used with path lengths of 1, 5, 10, and 20 mm. Protein concentrations were all between 0.5 and 0.9 mg/ml. No dilutions of the samples were made at any time during the taking of the spectra. Measurements were made from 350 nm to as close to 200 nm as possible, reducing the optical path whenever the dynode voltage reached 700 V. Mean residue molecular ellipticities, $[\theta]_{MRW}$, were calculated using a value of 115 for the mean residue weight in both HGH and LTH. Helical contents were estimated as previously described (Bewley *et al.*, 1969).

## Results

*CD Spectra of the Native Proteins.* Figure 1a shows the circular dichroism spectra of native HGH and LTH (0.1 M

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[1] Abbreviations used are: HGH, human pituitary growth hormone; LTH, ovine pituitary lactogenic hormone; BGH, bovine pituitary growth hormone.

[2] Besides its growth-promoting activity, HGH is also a potent lactogenic hormone (Li, 1968).

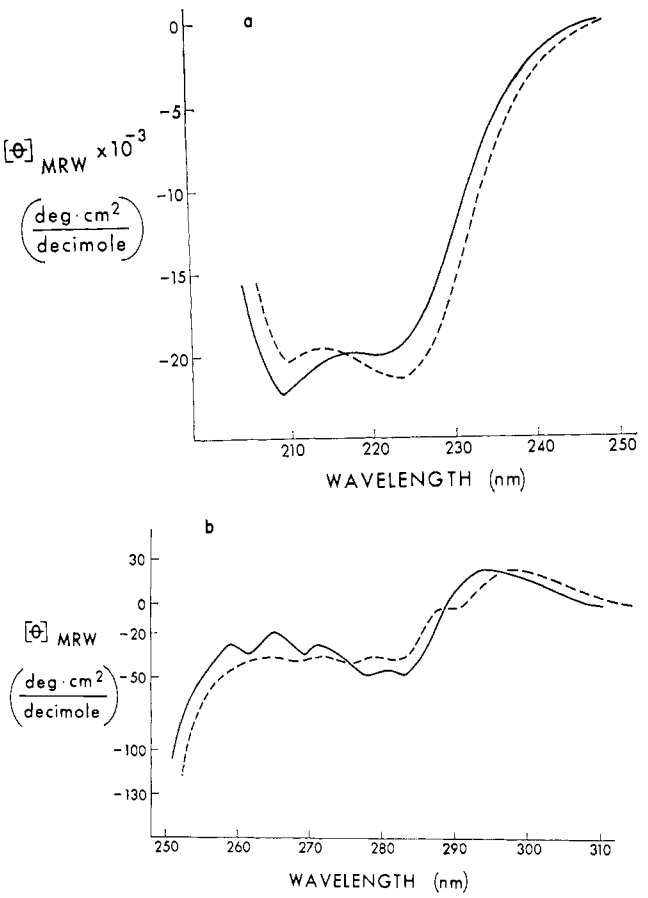


FIGURE 1: Circular dichroism spectra of HGH (——) and LTH (— — —) in 0.1 M Tris buffer, pH 8.2. (a) Amide bond circular dichroism spectra; the curve for HGH is taken from Bewley *et al.* (1969). (b) Side chain circular dichroism spectra; the curve for HGH is taken from Bewley and Li (1970b).

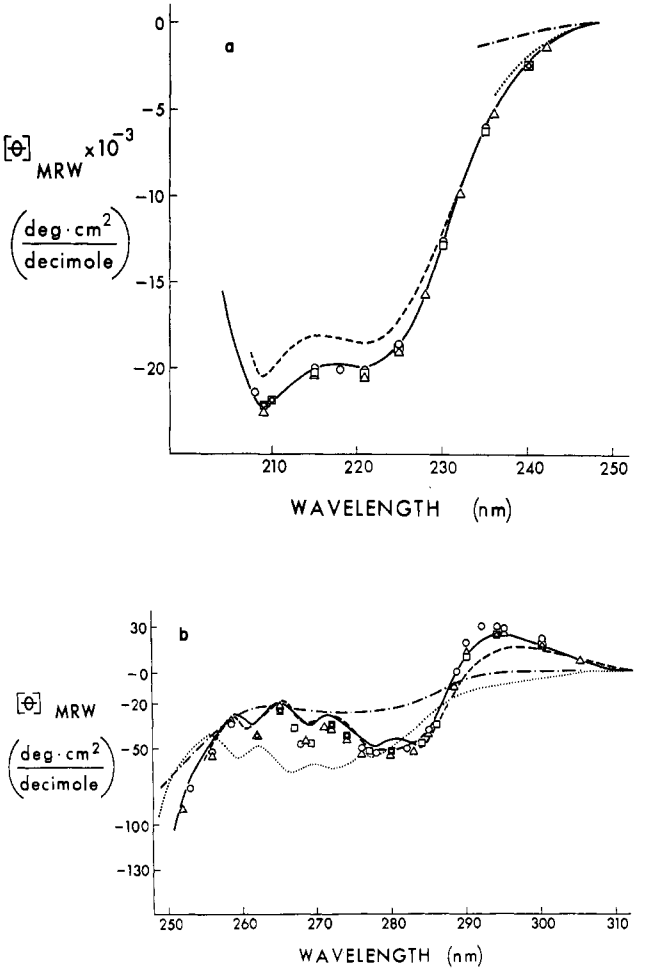


FIGURE 2: Circular dichroism spectra of HGH in: 0.1 M Tris buffer, pH 8.2 (——); 0.1 M glycine hydrochloride buffer, pH 3.6 (——); 50% acetic acid (· · ·); and 5 M guanidine hydrochloride–0.1 M Tris buffer, pH 8.2 (–·–). Ellipticities at selected wavelengths following removal of the perturbing solvents by dialysis against 0.1 M Tris buffer, pH 8.2 are indicated by: glycine hydrochloride, pH 3.6 (△); 50% acetic acid (○); and 5 M guanidine hydrochloride (□). (a) Amide bond circular dichroism spectra. (b) Side-chain circular dichroism spectra.

Tris buffer, pH 8.2) in the region of amide bond absorption. The spectrum of HGH has been described previously (Bewley *et al.*, 1969). The LTH spectrum shows a strong negative band at 223 nm and a second slightly weaker band around 209 nm. Due to excessive absorption below 205 nm, it was not possible to extend the measurements further into the ultraviolet.

Spectra of the same two samples in the region of side-chain absorption are presented in Figure 1b. The LTH spectrum contains a single asymmetric positive band with an apparent maximum around 297–298 nm and a badly resolved system of negative bands showing at least three negative maxima at 281–282 nm, 275–276 nm, and 268 nm. The spectrum for HGH has been described (Bewley and Li, 1970b).

*Effect of Perturbing Solvents on HGH.* The effects of three perturbing solvents on the spectra of HGH are shown in Figure 2a. For the samples in 5 M guanidine hydrochloride and 50% acetic acid, strong absorption by the solvents made accurate measurements below 235 nm impossible. It can be seen, however, that 5 M guanidine hydrochloride produces a marked decrease in the negative dichroism below 250 nm. The 50% acetic acid also produced a decrease in negative dichroism, but the effect of this solvent is quite small compared to that of the guanidine hydrochloride. In glycine buffers of pH 3.6, only a small decrease in the negative dichroism was observed, this being apparent only at wavelengths below 235 nm. Despite this small change in intensity at pH 3.6, both bands have retained their positions with regard to peak wavelength, and the relative intensities are the same as in the native protein. It may also be seen from Figure 2a that when these perturbing solvents are removed by dialysis against 0.1 M Tris buffer of pH 8.2, there is essentially complete return of all three circular dichroism spectra to that of the native protein.

Figure 2b presents the circular dichroism spectra of these same samples in the region of side-chain absorption. Treatment with 5 M guanidine hydrochloride results in the loss of almost all the dichroism above 260 nm associated with the native protein, with only a broad, weakly negative band between 295 and 260 nm remaining. Glycine buffer of pH 3.6 results in a decrease in intensity of the positive band found in the native protein and a shift in its apparent maximum from 294 to 296 nm. In this solvent there are also four negative bands at 284 nm, 278–280 nm, 268–269 nm, and 260–261 nm, with intensities quite comparable to the four similar bands found in the native protein. HGH in 50% acetic acid shows no positive dichroism at all, there being a somewhat increased negative dichroism, however, and, again, this system of bands is resolved into four negative maxima at 277 nm, 272 nm, 266–267 nm, and 259–260 nm. Removal of the solvents by

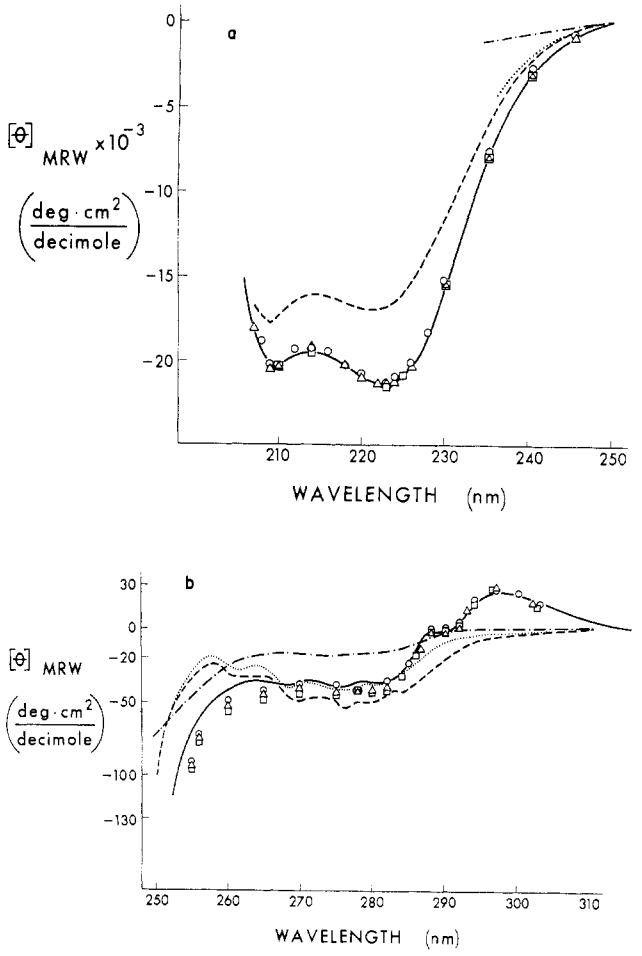


FIGURE 3: Circular dichroism spectra of LTH in: 0.1 M Tris buffer, pH 8.2 (——); 0.1 M glycine hydrochloride, pH 3.6 (— — —); 50% acetic acid (· · ·); and 5 M guanidine hydrochloride–0.1 M Tris buffer, pH 8.2 (– · –). Ellipticities at selected wavelengths following removal of the perturbing solvents by dialysis against 0.1 M Tris buffer, pH 8.2, are indicated by: glycine hydrochloride, pH 3.6 (△); 50% acetic acid (○); and 5 M guanidine hydrochloride (□). (a) Amide bond circular dichroism spectra. (b)Side-chain circular dichroism spectra.

dialysis results in essentially complete return of all three spectra to that of the native protein, the only difference being a small increase in the intensities of the two negative peaks at 269 and 262 nm, and a loss of resolution of the two peaks at 283 and 278 nm. Despite these small changes from the native protein, all three dialyzed samples appear to be equivalent.

*Effects of Perturbing Solvents on LTH.* Figure 3a shows the effect of these three solvents on the amide bond circular dichroism spectra of LTH. As in the case of HGH, 5 M guanidine hydrochloride results in a marked loss of negative dichroism over the narrow spectral range in which accurate measurements can be made. Over the same limited spectral range, 50% acetic acid produces a much smaller loss and is only slightly more effective in this respect than the glycine buffer of pH 3.6. However, this latter solvent produces considerably greater change in the LTH spectrum than it did in the case of HGH. The negative peak occurring at 223 nm in the native protein has been shifted to 221 nm along with a 20–25% decrease in intensity. Repeated measurements have shown this small blue shift to be quite reproducible. A similar shift is not observed for the peak at 209 nm although its intensity is decreased by about 15%, resulting in a reversal in the relative intensity of the two peaks. As in the case of HGH, if these solvents are removed by dialysis against 0.1 M Tris buffer of pH 8.2, the circular dichroism spectra of all three samples return to essentially that of the native protein.

In Figure 3b the effect of these solvents on the side-chain dichroism is shown. Only a broad, weakly negative band between 295 and 260 nm remains in 5 M guanidine hydrochloride. Both the glycine buffer of pH 3.6 and 50% acetic acid result in the complete loss of the positive band at 298 nm and increased resolution of the two negative bands around 268–269 nm and 261–262 nm. The 50% acetic acid has no significant effect on the spectrum between 284 and 268 nm, but the glycine buffer produces a small increase in the negative bands in this region. Following dialysis, all three samples show side-chain circular dichroism spectra which are at most only very slightly altered from that of the native protein. The complete return of the positive band at 298 nm in all three cases is especially noteworthy.

TABLE I: α-Helix Contents[a] of HGH and LTH.

| Protein | pH[b] | λ (nm) | % Excess Right-Hand α Helix Calculated From Ellipticity at λ |
|---|---|---|---|
| HGH | 8.2 | 209 | 55 ± 5 |
| | | 221 | 55 ± 5 |
| | 3.6 | 209 | 50 ± 5 |
| | | 221 | 50 ± 5 |
| LTH | 8.2 | 209 | 55 ± 5 |
| | | 223 | 55 ± 5 |
| | 3.6 | 209 | 45 ± 5 |
| | | 221 | 50 ± 5 |

[a] Calculated as described in Bewley *et al.* (1969). [b] 0.1 M Tris buffer, pH 8.2; 0.1 M glycine hydrochloride buffer, pH 3.6.

## Discussion

The circular dichroism spectra described above indicate that while, as expected, these two proteins possess conformations which have certain features in common, there are, also as expected, some distinct differences. At the present time we are not able to make completely unequivocal interpretations of the nature of all these similarities and differences. Nevertheless, we wish to present a discussion of our present thinking in this regard.

Comparisons of the solution properties of LTH and the growth hormone isolated from bovine pituitaries (BGH) have already shown some areas of significant similarity. Early studies (Jirgensons, 1960) of the optical rotatory dispersion properties of LTH and BGH indicated that both proteins contained an unusually high proportion of their residues in an α-helical conformation. Very recently, a more definitive comparison of BGH and LTH has appeared (Aloj and Edelhoch, 1970) in which the authors conclude that the conformations of these two molecules are indeed very similar. From circular dichroism studies they estimated the α-helix content of BGH to be 52% and that of LTH to be 60%. Since no circular dichroism spectra were shown it is somewhat difficult to compare their results on LTH with those reported herein. Previous circular dichroism measurements on HGH (Bewley

*et al.*, 1969) have placed its content of $\alpha$ helix at 55 ± 5%, emphasizing the rather striking similarity in these molecules' unusually high content of this type of secondary structure.

The three perturbants in this investigation were chosen for their known abilities to effect conformational changes either in the two molecules under study or in closely related ones. Thus, 50% acetic acid has been shown (Brovetto-Cruz and Li, 1969) to induce a conformational change in HGH which renders its otherwise unreactive tryptophan residue susceptible to chemical modifications. Dellacha *et al.* (1968) have shown that BGH may be prepared as a relatively stable monomer in 0.1 M glycine hydrochloride buffer of pH 3.6. This buffer was then chosen so that we might extend our comparisons in the future to include this molecule. Finally, 5 M guanidine hydrochloride of pH 8.2 was used to bring about a more pronounced denaturation of the two molecules.

*Amide Bond Spectra.* The positions of the two negative bands in the amide bond circular dichroism spectrum of HGH (209 and 221 nm) are typical of $\alpha$-helical polypeptides (Holzwarth, 1964; Holzwarth and Doty, 1965). Helix contents[3] estimated from the intensity of either band indicate a value of 55 ± 5% excess right-hand $\alpha$ helix (Bewley *et al.*, 1969). While the same $\alpha$-helix content (55 ± 5%) may be calculated from the two negative bands in the LTH spectrum, there are, nevertheless, significant differences in these two spectra. The lower energy band, usually assigned to an $n \rightarrow \pi^*$ transition of the amide bond when in an $\alpha$-helical conformation, ordinarily occurs around 221–222 nm (Holzwarth and Doty, 1965; Schellman and Oriel, 1962). In the LTH the apparent negative maxima of this band is found at 223 nm compared to 221 nm in HGH. Evidence that this small difference is real is provided by the spectra of LTH taken in the glycine buffer (Figure 3a) in which this band appears at 221 nm and then shifts back to 223 nm on removal of the perturbing solvent by dialysis against 0.1 M Tris buffer at pH 8.2. This same perturbant has no significant effect on the wavelength of the 209-nm maximum in LTH, nor any effect on the wavelength of either maxima in HGH. Moreover, in the native proteins, the relative intensities of these two bands are reversed. Studies with model compounds (Greenfield and Fasman, 1969; Chapman and Wallach, 1968) indicate that polypeptides with conformations consisting of approximately 50% $\alpha$ helix and 50% random coil usually exhibit a spectrum similar to that of HGH with the 209-nm band somewhat stronger than the 221-nm band. The 221-nm band becomes the stronger of the two only at significantly higher $\alpha$-helix contents (*e.g.*, 80–100%). Such an interpretation must be ruled out, however, in the case of LTH, since the ellipticities of both bands are too low to be consistant with such high helical contents (Greenfield and Fasman, 1969; Chapman and Wallach, 1968).

Two alternative interpretations of the LTH spectra are now under investigation in our laboratory. Visual comparison with computer-generated curves [see Greenfield and Fasman (1969), Figure 5] for hypothetical mixtures of conformational forms suggests that a polypeptide with 45–50% $\alpha$ helix, 40% random coil, and 10–15% $\beta$ structure might result in an amide bond circular dichroism spectrum very much like that of LTH. However, this would require that the negative maximum of the $\beta$-structure circular dichroism in LTH occur around 225 nm rather than 218 nm as found in poly-L-lysine (Greenfield and Fasman, 1969). This is still a possibility in view of the known sensitivity of the $\beta$-structure circular dichroism to solvent and side-chain effects (Fasman and Potter, 1967; Kay, 1970). The $\alpha$-helix content indicated in this alternative is very close to that shown in Table I for LTH in the glycine buffer. We might then interpret the reversible transition displayed by LTH between pH values 8.2 and 3.6 as the destruction and re-formation of some 10–15% $\beta$ structure, with little or no effect on the $\alpha$-helix content.

Alternatively, the LTH spectrum, and in particular the transition between pH values 8.2 and 3.6, might be due to the side-to-side aggregation of $\alpha$-helical segments as described by Cassim and Yang (1967) for poly-L-glutamic acid. Such aggregates show both hyperchromic and bathochromic shifts in the $n \rightarrow \pi^*$ amide transition, with only a relatively small hyperchromic shift in the 209–210-nm $\pi \rightarrow \pi^*$ band. The magnitudes of these shifts are quite consistant with the spectra shown in Figure 3a for LTH. It has been ascertained by exclusion chromatography that the LTH is in a monomeric form before and after the circular dichroism spectra were taken in both solvents. Therefore, any aggregation would have to involve the formation of intramolecular aggregates of $\alpha$-helical segments at pH 8.2, and their reversible dissociation at pH 3.6. No comparable conformational change has been found for HGH.

Both sequences have been analyzed for potential $\alpha$ helix by a method described in detail elsewhere for HGH (Bewley, 1968). In brief, this procedure designates as potentially helical those sections of the peptide chain which, when folded into a $\alpha$ helix, produce a cylindrical column, one side of which is predominantly hydrophobic and the other predominantly hydrophilic. This analysis predicts 57 ± 5% $\alpha$ helix for HGH and 45 ± 5% for LTH. The agreement between these predicted helical contents and the experimentally determined values is most encouraging.

Figures 2a and 3a indicate that there is a pronounced decrease in the helical contents of both HGH and LTH when dissolved in 5 M guanidine hydrochloride of pH 8.2. However, in both molecules this large conformational change is completely reversed upon removal of the denaturant by dialysis. Despite the relatively limited spectral range available for study, Figures 2a and 3a further indicate that 50% acetic acid results in a much less pronounced loss of secondary structure than that found in 5 M guanidine hydrochloride. In both proteins this conformational change is also freely reversible upon removal of the denaturant.

*Side-Chain Spectra.* The circular dichroism spectra of HGH and LTH in the region of side-chain absorption again indicate that although there is considerable similarity in the conformations of these two molecules, they are not completely identical. The two peaks shown by HGH at 261–262 nm and 269 nm, and assigned in a previous publication (Bewley and Li, 1970b) to phenylalanine residues, are almost entirely absent from the spectrum of native LTH (Figure 1b). At the present time we cannot make definite chromophore assignments to the negative dichroism exhibited by both proteins between 270 and 288 nm. Tyrosine and tryptophan residues and the disulfide bonds[4] may all contribute to the spectrum in this region (Beychok, 1966; Strickland *et al.*, 1969).

[3] We have arbitrarily assigned a minimum uncertainty of ±5% in the estimation of all helix contents. This uncertainty is primarily due to a lack of precise information about the most suitable reference states for 0 and 100% $\alpha$ helix. From the spread of reference values currently in use, we estimate the maximum uncertainty in HGH and LTH to be ±10%.

[4] HGH contains 13 phenylalanine, 8 tyrosine, and 1 tryptophan residue per mole. In addition, there are two disulfide bonds in each molecule. In LTH there are 6 phenylalanine, 7 tyrosine, and 2 tryptophan residues, with 3 disulfide bonds per mole.

We can however be somewhat more certain about the region above 290 nm. Since fully reduced HGH shows the same positive band as the native protein in this region (Bewley and Li, 1970b) the disulfide bonds may be ruled out as a major contributor. The other potentially optically active chromophores in these proteins (*e.g.*, amide bond, phenylalanine, and tyrosine) do not contribute significantly to the dichroism above 290 nm (Strickland *et al.*, 1969; Horwitz *et al.*, 1970) and may also be ruled out. Tryptophan however is known to exhibit several circular dichroism bands (both positive and negative) between 290 and 305 nm, the amplitudes and positions being very sensitive to the immediate environment of the chromophoric group (Strickland *et al.*, 1969). Therefore, in the region above 290 nm, the major part of the dichroism in HGH and LTH may be assigned to the tryptophan residues.

Both proteins show an asymmetric positive peak in this region. This asymmetry is probably due to overlapping with the negative bands below 286 nm. The spectrum of LTH suggests that the positive tryptophan band in this protein may overlap an additional negative band not exhibited by HGH. Simple graphical subtraction of the HGH spectrum from that of LTH between 288 and 310 nm produces a weak negative band with a maximum at 291–292 nm, $[\theta]_{291} = -20°$. Since this "hidden" band lies above 290 nm we may tentatively assign it also to tryptophan. The existence of this tryptophan band in LTH is further supported by the finding (T. A. Bewley and C. H. Li, manuscript submitted for publication) of a band with analogous position (292 nm), and amplitude ($[\theta]_{292} = -30°$) in BGH dissolved in 0.1 M glycine hydrochloride buffer of pH 3.6. This protein contains only one tryptophan residue (Dellacha *et al.*, 1968) and shows no positive dichroism at all in this buffer. These findings are consistent with the reported amino acid compositions and sequences of HGH and LTH (Li *et al.*, 1969a,b), HGH containing a single tryptophan residue while LTH contains two. Furthermore, the amino acid sequence around the single tryptophan in HGH has been shown to be homologous with one of the tryptophan residues in LTH and nonhomologous with the other (Bewley and Li, 1970a). We may postulate that the immediate environments of these two homologous residues (HGH-$Trp_{25}$ and LTH-$Trp_{90}$) are very similar and that it is these two which give rise to the two positive bands in the circular dichroism spectra. The hidden negative band in LTH may then be tentatively assigned to LTH-$Trp_{149}$. The differences in sign for the circular dichroism of these two tryptophan residues in LTH are not inconsistent with the reported sensitivity of this chromophore's optical activity to its immediate environment.

The effects of the three perturbing solvents on side-chain optical activity in these proteins are similar to their effects on the amide bond dichroism. In all cases, changes in dichroism indicating conformational alterations were essentially reversible on removal of the perturbants. It is interesting to note that the positive tryptophan band in HGH is still present at pH 3.6 (though somewhat diminished in intensity) and entirely gone in 50% acetic acid. This is in agreement with the reported chemical reactivity of this residue (Brovetto-Cruz and Li, 1969) in which only partial reaction could be effected at pH 4.0 (0.2 N acetic acid) while full reactivity could be achieved in 50% acetic acid. It can also be seen from Figures 2a,b and 3a,b that the conformation of LTH is somewhat less stable than that of HGH, in agreement with the findings of Aloj and Edelhoch (1970) for the relative stability of BGH and LTH.

In conclusion, the studies presented above do indicate that the homologies in the amino acid sequence of these two peptides are reflected in demonstrable similarities in their three-dimensional structures. While the importance of these similarities to an eventual understanding of the modes of action of these two hormones cannot be doubted, such an understanding must result from giving equal importance to the nature of their structural differences, some of which are also demonstrated herein.

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