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Molecular Weight and Circular Dichroism Studies of Bovine and Ovine Pituitary Growth Hormones†

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ABSTRACT: The state of aggregation and the conformation of bovine and ovine pituitary growth hormones have been studied by means of exclusion chromatography, osmotic pressure, and circular dichroism measurements in acidic, slightly alkaline, and strongly alkaline solutions. The dimer of both proteins (mol wt 41,000–42,000) may be dissociated into the monomer form (mol wt ≈ 22,000) under either acidic (pH 3.6) or strongly alkaline (pH 11.5) conditions. In acidic solution, this dissociation is not accompanied by significant changes in the secondary structures of either protein, the α-helix contents being 45–50% in all cases. Some alterations in the tertiary structures does occur however, as evidenced by changes in the circular dichroic spectra in the region of side-chain absorption.

Sedimentation studies in the ultracentrifuge have previously indicated molecular weights of 46,000 for BGH[1] (Li and Pedersen, 1953) and 47,800 for SGH (Papkoff and Li, 1958). The possibility of a dissociation of BGH into smaller components in highly alkaline solutions was also strongly suggested by Li and Pedersen (1953). Subsequently, considerable evidence for the dissociation of BGH under various acidic, basic and denaturing conditions has appeared in the literature. These results have been summarized by Dellacha *et al.* (1968), who also present their own evidence indicating that BGH may be completely dissociated from the dimeric form (mol wt 40,000–45,000) in neutral or slightly alkaline solution, to the monomer (mol wt ≈ 21,000) in acidic solutions of low ionic strength.[2] This monomer weight for BGH is in excellent agreement with a minimum molecular weight of 20,846 calculated by Fellows and Rogol (1969) from the composition of fragments obtained by treatment of the hormone with cyanogen bromide.

The conformation of BGH has also been a subject of considerable interest. The results of a number of earlier investigations have been summarized by Aloj and Edelhoch (1970). There is some doubt as to the state of aggregation of the hormone in some of these earlier studies. With this in mind, Edelhoch and Lippoldt (1970) have presented a more recent conformational study of this protein based on circular dichroism measurements in solvents which might be expected to provide predominantly monomer or dimer forms of the hormone.

Although BGH and SGH have been shown to have a number of important chemical and immunochemical similarities (Papkoff and Li, 1958; Moudgal and Li, 1961; Hayashida, 1969), relatively little is known about the ovine hormone. In this study, we have reinvestigated the dissociation of BGH, both in strong alkali and under the acidic conditions described by Dellacha *et al.* (1968), with the goal of isolating and characterizing samples of the monomeric and dimeric forms of the protein. We have estimated the molecular weights of each form by exclusion chromatography and osmotic pressure. Circular dichroism measurements were then carried out on the purified monomer and dimer. Finally, par-

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[1] Abbreviations used are: BGH, bovine pituitary growth hormone; SGH, ovine pituitary growth hormone; HGH, human pituitary growth hormone; LTH, ovine pituitary lactogenic hormone; HCS, human chorionic somatomammotropin; CD, circular dichroism.

[2] 0.1 M glycine hydrochloride buffer, pH 2.2–3.6, μ = 0.045–0.01.

allel experiments were carried out on SGH and the results, in terms of the extent of dissociation, molecular wights, and the circular dichroic properties of the monomer and dimer forms are compared with those of BGH reported herein, as well as those obtained on BGH by others.

Materials and Methods

The methods of isolation and properties of the growth hormones from beef (Li, 1954) and sheep (Papkoff and Li, 1958) pituitaries have been previously described. Both products were further purified by exclusion chromatography on Sephadex G-100 in 0.01 M NH_4HCO_3 buffer (pH 8.4). Glycine and 2-amino-2-(hydroxymethyl)-1,3-propanediol (Tris buffer) were obtained from Eastman Organic Chemicals, Rochester, N. Y., and were used without further purification.

Determination of Protein Concentration. A 100-mg sample of purified BGH dimer was prepared by exclusion chromatography at pH 8.4 as described above. This material was recovered by lyophilization after being exhaustively dialyzed against deionized water to remove any nonprotein nitrogen. Six aliquots (5–10 mg each) of this product were carefully weighed out and dissolved in sufficient 0.1 N acetic acid to give approximately 0.1% solutions. Spectra were taken of each solution from 360 to 245 nm on a Beckman DK-2A recording spectrophotometer against a reference of 0.1 N acetic acid. After correcting for light scattering as described by Beavan and Holiday (1952) an average value of 0.637 was calculated for the absorbance of a 0.1% solution of the lyophilized protein, with a standard error of ±0.006. Five other aliquots were found to contain an average of 14.53% nitrogen by the micro-Dumas procedure, with a standard error of ±0.13%. Using this nitrogen content of the lyophilized material, and Parcell's (1961) value of 16.70% for the nitrogen content of BGH (100% peptide) a peptide content of 87.1 ± 0.8% was obtained. From these results, the absorbance of BGH, corrected for light scattering and peptide content, in 0.1 N acetic acid, was calculated to be $E^{1\%}_{1\text{cm},\ 277\ \text{nm}} = 7.30$ with a standard error of ±0.14. This value was used to determine the concentration of both BGH and SGH solutions.[3]

Exclusion Chromatography. Exclusion chromatography was carried out at 25° on columns (3 × 50 cm) of Sephadex G-100 (Pharmacia Fine Chemicals Inc., Piscataway, N. J.) at pH 11.5 (0.1 M phosphate buffer), pH 8.2 (0.1 M Tris Buffer), and at pH 3.6 (0.1 M glycine hydrochloride buffer). Void volumes (V_0) were determined with Blue Dextran 2000 (Pharmacia). The column effluents were continuously monitored on a Beckman DB-G spectrophotometer equipped with a 5-in. strip chart recorder and a flow-through cell (cell capacity, 0.3 ml). A second flow-through cell containing only buffer was kept in the reference beam of the spectrophotometer. After passing through the spectrophotometer, the effluent was collected directly into a measuring cylinder so that the elution volumes (V_e) could be continuously measured. Small bore polyethylene tubing was used for all connections in order that the error between the volume indicated on the measuring cylinder and the elution volume of the material passing through the spectrophotometer would be less than 0.5 ml. Elution volumes were marked on the chart paper every 10 ml except in the vicinity of an elution peak, where they were marked every milliliter. Unless otherwise stated, all samples were applied to the columns within 1 hr after dissolving in the appropriate solvent. For estimation of the molecular weights of the various fractions from their V_e/V_0 ratios, the columns were each calibrated with HGH (mol wt 21,700) and LTH (mol wt 22,500) as described previously (Bewley and Li, 1971).

Osmotic Pressure. Measurements of osmotic pressure were made in a Melabs recording membrane osmometer, Model CSM-2 at 20.0 ± 0.002°. Monomer or dimer fractions, prepared by exclusion chromatography, were pooled for measurement of osmotic pressure by reference to the previously established elution patterns under each condition. The contents in those tubes near the peak were pooled and concentrated to ≈4 mg/ml on a Diaflo ultrafiltration apparatus. Four to six dilutions of each fraction were prepared covering the range from 1 to 4 mg per ml of protein. The osmotic pressure of each dilution was determined to ±0.02 cm of H_2O. Molecular weights were calculated from the osmotic pressure at zero protein concentration, obtained by least-squares extrapolation through the experimental points. The concentration of the solutions at pH 11.5 was determined spectrophotometrically on an aliquot after adjustment to pH 8.0–8.4.

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. No dilutions of the samples were made at any time during the taking of the spectra. Measurements were made from 325 nm to as close to 200 nm as possible, reducing the optical path whenever the dynode voltage reached 700 V. Scanning speeds were 1 nm/min or less. Mean residue molecular ellipticities $[\theta]_{MRW}$, were calculated using 115 for the mean residue weight in both proteins. Helical contents were estimated as previously described (Bewley *et al.*, 1969). Samples for CD measurement (≈7 ml) were prepared from separate exclusion chromatography experiments, using the same columns as in the osmotic pressure and V_e/V_0 determinations, but in which the eluate was collected in a fraction collector. Elution patterns were determined from the optical density in each tube and the two tubes representing the highest concentration of a particular molecular form were pooled for the CD sample. In order to compare our results with those of Edelhoch and Lippoldt (1970), CD spectra were also taken of BGH and SGH samples which had been dissolved in 0.01 M $(NH_4)_2CO_3$ and then titrated to pH 3.0 with HCl.

Results

Exclusion Chromatography and Osmotic Pressure. Figure 1 presents typical elution patterns obtained when samples of lyophilized BGH and SGH are submitted to exclusion chromatography. At pH 8.2 (Figure 1a) both proteins show very similar patterns indicating the presence of at least two major components. The first appears at the void volume of the column and represents highly aggregated material. The second peak, comprising the bulk of either protein appears at a V_e/V_0 ratio slightly above 1.60. A molecular weight around 41,000 may be estimated for this component of both proteins from their V_e/V_0 ratios. This value is in excellent agreement with that found by osmotic pressure measurements on the same components. These molecular weight values, and the cor-

[3] Difference spectra (T. A. Bewley and C. H. Li, unpublished results) indicate that at 277 nm, the absorbance of BGH in either the pH 3.6 or the pH 8.2 buffers differs by less than 3% from the value found in 0.1 N acetic acid. The ovine hormone was assumed to be equivalent to BGH in this regard.

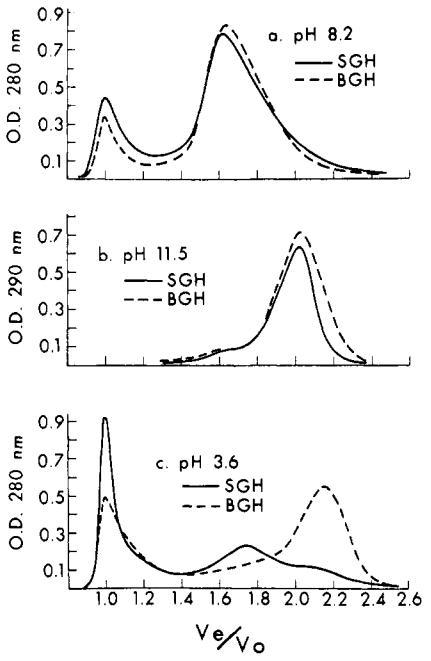

FIGURE 1: Exclusion chromatography of BGH and SGH on Sephadex G-100.

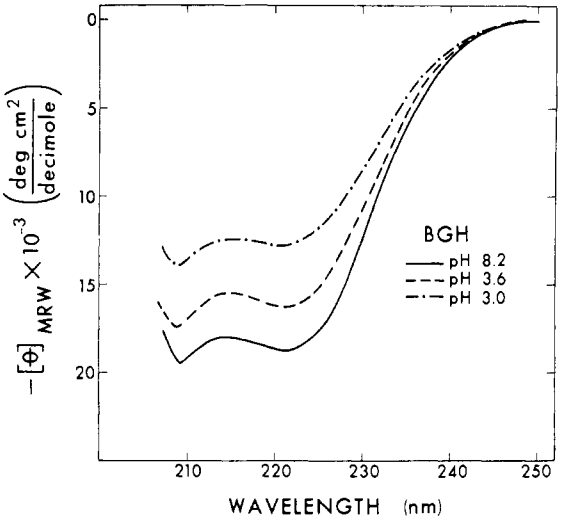

FIGURE 2: CD spectra of BGH in the region of amide bond absorption.

responding V_e/V_0 ratios are summarized in Table I. At pH 11.5 (Figure 1b) both proteins again behave in a very similar manner. Under this condition, however, there appears to be no highly aggregated material, with most of the sample eluting at a V_e/V_0 ratio slightly greater than 2.0. As shown in Table I, the molecular weight of these components, whether estimated from the V_e/V_0 ratios or measured by osmotic pressure, indicate values between 22,000 and 24,000. The presence of an unresolved shoulder in the elution pattern of both proteins around $V_e/V_0 = 1.6$ probably indicates the presence of a small amount of the dimer form. The elution patterns at pH 3.6 are shown in Figure 1c. Under this condition the two proteins do not behave identically, although both show appreciable amounts of highly aggregated material. BGH exhibits a major component ($V_e/V_0 = 2.13$) with a molecular weight of 22,000–25,000. This pattern also indicates the probable presence of a small amount of BGH dimer appearing between $V_e/V_0 = 1.6$–1.9. Under the same condition, SGH shows a much greater proportion of highly aggregated protein, with a second major component having an elution peak at $V_e/V_0 = 1.74$. A third component appears as an unresolved shoulder between $V_e/V_0 = 2.0$ and 2.5. As shown in Table I, the estimated elution volume of this component is in the range; $V_e/V_0 = 2.0$–2.15, corresponding to a molecular weight of 22,000. We were unable to obtain sufficient quantities of either of these forms of SGH on which to make osmotic pressure measurements. Although not shown in Figure 1c, experiments in which BGH, dissolved in the pH 3.6 buffer, was allowed to stand at 25° for 72 hr before applying to the column produced elution patterns which were very similar to that shown in Figure 1c for SGH, with the major component eluting at $V_e/V_0 = 1.70$ and a shoulder extending somewhat beyond $V_e/V_0 = 2.2$.

Circular Dichroism (below 250 nm). The CD spectra of BGH at pH 8.2 (dimer) and pH 3.6 (monomer) in the region of amide bond absorption are presented in Figure 2. In both cases the protein exhibits two strong bands with negative maxima at 221 and 209 nm. The spectrum at pH 3.6 is essentially the same as that at pH 8.2, but with slightly decreased negative ellipticity. Mean residue molecular ellipticities and α-helix contents, calculated from these two spectra, are presented in Table II. Under both conditions the hormone appears to contain about 50% α helix. We have also included our own spectrum of BGH taken in the pH 3.0 solvent described by Edelhoch and Lippoldt (1970). A somewhat larger decrease in negative ellipticity is shown in this solvent, the α-helix content being about 35%.

CD spectra of SGH in the amide bond absorption region are presented in Figure 3; the spectra at pH 8.2 and 3.6 (both monomer and dimer) do not differ significantly from each other. Ellipticities and α-helix contents for SGH, presented in Table II, are also very similar to those of BGH under the same conditions. As in the case of BGH, the pH 3.0 solvent brings about a more pronounced change in the secondary structure of the protein, the α-helix content being reduced to 30–35%. It should be noted that for SGH at pH 3.6, spectra

TABLE I: Molecular Weights of BGH and SGH from Exclusion Chromatography and Osmotic Pressure Measurements.

Protein[a]	pH[b]	V_e/V_0[c]	Molecular Weight: From V_e/V_0	Molecular Weight: From Osmotic Pressure
BGH	3.6	2.13 ± 0.03 (6)	22,100	24,600
	8.2	1.65 ± 0.01 (3)	40,000	41,100
	11.5	2.03 ± 0.02 (2)	21,700	22,900
SGH	3.6	2.10–2.15 (3)	22,200–22,000	
	8.2	1.61 ± 0.01 (4)	41,400	41,500
	11.5	2.02 (1)	21,700	24,300

[a] The value of V_e/V_0 for HGH at pH 11.5 was found to be 2.02 ± 0.03. [b] pH 3.6, 0.1 M glycine hydrochloride; pH 8.2, 0.1 M Tris buffer; pH 11.5, 0.1 M phosphate buffer. [c] Mean ± standard error. Number of determinations in parentheses.

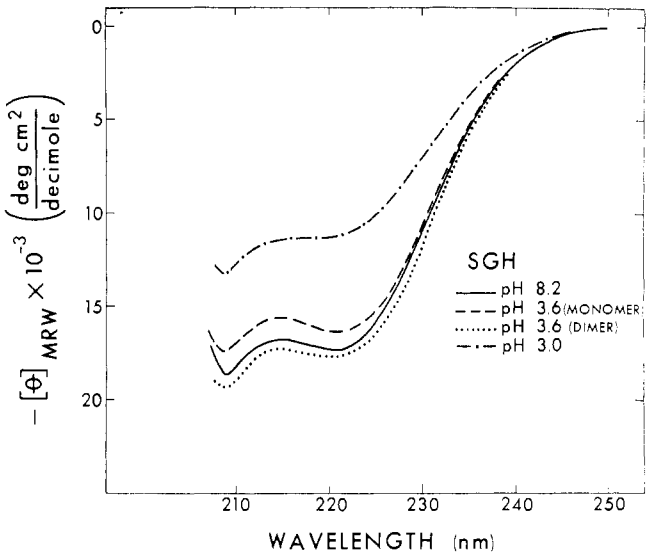

FIGURE 3: CD spectra of SGH in the region of amide bond absorption.

are presented for both the monomer (V_e/V_0 = 2.1–2.2) and the dimer (V_e/V_0 = 170–1.74) forms.

Circular Dichroism (above 250 nm). The CD spectra of BGH at pH 8.2 and 3.6, in the region of side-chain absorption, are presented in Figure 4a. At pH 8.2, the BGH dimer exhibits two almost identical negative bands at 291.5 and 286 nm. Below 275 nm, as interpreted by Edelhoch and Lippoldt (1970), there are two positive bands at 271.5 and 265 nm and a negative band at 261.5 nm. However, these bands might be better interpreted as two negative bands at 268.5 and 261.5 nm, superimposed on a broad, somewhat stronger positive band centered near 265–270 nm. Two additional, very weak negative bands appear only as changes in curvature around 278 and 255–256 nm. At pH 3.6, the monomer form of BGH exhibits only negative dichroism, with maxima at 291, 285.5, 279, 268.5, and 261.5 nm. An additional very weak band appears again as a change in curvature near 255–256 nm. The

TABLE II: Mean Residue Molecular Ellipticities and α-Helix Contents of BGH and SGH.

Protein	pH[a]	λ (nm)	$-[\theta]_\lambda$	% Excess Right-Hand α Helix[b]
BGH	3.6	221	16,300	45
		209	17,400	50
	8.2	221	18,700	50
		209	19,400	50
SGH	8.2	221	17,300	45
		209	18,700	40
	3.6	221	16,300	45
	Monomer	209	17,400	40
	3.6	221	17,500	50
	Dimer	209	19,300	45

[a] pH 3.6, 0.1 M glycine hydrochloride; pH 8.2. 0.1 M Tris buffer. [b] Calculated to the nearest 5%.

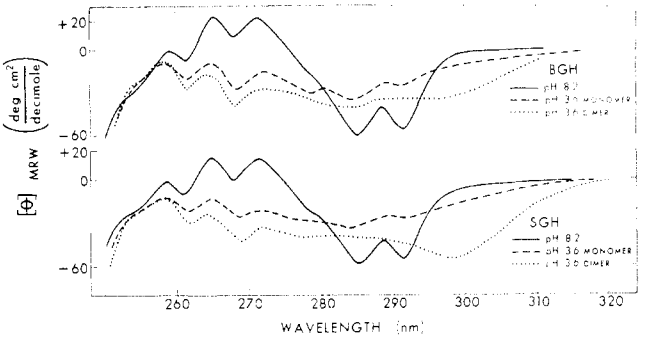

FIGURE 4: CD spectra of BGH and SGH in the region of side-chain absorption.

dimer of BGH, produced at pH 3.6 by "aging" the protein at 25° before exclusion chromatography, also shows only negative dichroism. This spectrum is less distinct in terms of clearly defined band maxima than either of the other two forms, but there is clearly an increase in the negative dichroism above 295 nm. Two distinct negative maxima are again seen at 268 and 261.5 nm.

Equivalent spectra for SGH are shown in Figure 4b. At pH 8.2, the dimer exhibits a spectra virtually identical with that of BGH. Similarly, the monomeric form at pH 3.6 is nearly identical to the BGH monomer in the same solvent. However, the SGH dimer isolated at pH 3.6 is significantly different from the corresponding "aged" BGH dimer. The SGH dimer exhibits an intense and well-defined negative band at 298 nm and a broad featureless region between 288 and 276 nm. Two additional negative maxima at 269 and 262 nm complete the decription of this spectrum.

Figure 5 shows the near-ultraviolet CD spectra of BGH and SGH dissolved in the pH 3.0 solvent. Both proteins show only negative dichroism and are essentially equivalent below 280 nm. However, above 280 nm the two spectra appear to be very close to the spectra in Figure 4a,b of the dimers of these hormones at pH 3.6. The well-defined negative maximum at 298–299 nm and the nearly flat region from 288 to 276 nm, exhibited by the SGH sample, should be especially noted.

Discussion

The elution volumes and osmotic pressure data (Figure 1a and Table I) clearly indicate that both BGH and SGH exist predominantly as dimers in slightly alkaline solution, with essentially identical molecular weights close to 41,000. This value would indicate monomer molecular weights for both proteins of 20,500 which is in excellent agreement with the minimum molecular weight of 20,846 calculated by Fellows and Rogol (1969) for BGH. We have not been able to determine whether the asymmetry in the elution patterns shown in

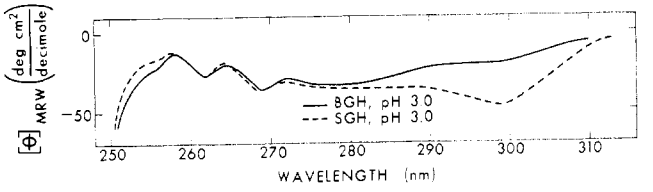

FIGURE 5: CD spectra of BGH and SGH in the region of side-chain absorption. In both cases the protein was dissolved in 0.01 M $(NH_4)_2CO_3$ and then titrated to pH 3.0 with HCl.

Figure 1a is due to a slight concentration dependence of V_e or to the presence of small amounts of monomer at $V_e/V_0 > 1.9$. Rechromatography of lyophilized dimer fractions indicates that the highly aggregated material appearing at V_0 is largely a result of lyophilization of the proteins. Nearly complete dissociation of both BGH and SGH dimers into the monomer form at pH 11.5 is also evident from the elution data and osmotic pressure measurements, confirming the original observation of Li and Pedersen (1953) and extending the observation to include the ovine hormone. It is apparent from Figure 1b that strong alkali is even capable of dissociating the highly aggregated protein. The dissociation of the BGH dimer at pH 3.6 is exactly as expected from the studies of Dellacha *et al.* (1968). The time dependent formation of higher molecular weight forms of BGH, when solutions of this protein are allowed to "age" in the pH 3.6 buffer before chromatography, is also strongly suggested by the work of these authors. Although we have not made osmotic pressure measurements on the product formed in our aging experiments, the $V_e/V_0 = 1.70$ is consistent with this being a dimer of BGH. Unlike the bovine hormone, SGH is not completely dissociated in the pH 3.6 buffer, differing markedly from BGH in this regard.

Circular dichroism measurements in the region of amide bond absorption indicate that both hormones contain 45–50% α helix. This helix content is the same (45–55%) for three other closely related proteins: HGH (Bewley *et al.*, 1969), HCS (Bewley and Li, 1971), and LTH (Bewley and Li, 1972). It is also in agreement with the value reported for BGH in 0.01 M $(NH_4)_2CO_3$ of pH 9.0 by Edelhoch and Lippoldt (1970). There is little or no change in the secondary structures of these two hormones as they undergo dissociation of the dimer into the monomer form.

The CD spectra of BGH and SGH dimers at pH 8.2, in the region of side chain absorption, are essentially identical. The negative band at 291.5 nm can be assigned to a ^1L_b transition of tryptophan (Strickland *et al.*, 1969) and corresponds closely to similar tryptophan bands found in HCS (Bewley and Li, 1971) and LTH (Bewley and Li, 1972). The band at 285 nm is probably a composite of contributions from both tyrosine and tryptophan residues (Horwitz *et al.*, 1970; Strickland *et al.*, 1969). In view of the model compound studies of phenylalanine and its derivatives, described by Horwitz *et al.* (1969), we prefer to interpret the spectra below 278 nm as two negative bands at 268 and 261 nm, assigned to phenylalanine residues, superimposed on a broad, somewhat stronger positive band centered around 265–270 nm, which might possibly be due to a ^1L_a transition of tryptophan (Strickland *et al.*, 1970). This is in contrast to the interpretation of a very similar spectrum of BGH at pH 9.0 reported by Edelhoch and Lippoldt (1970), in which they assign positive dichroism bands at 264 and 258 nm to phenylalanine residues and a positive band at 270 nm to tyrosine residues.

The monomers of these two hormones at pH 3.6 also exhibit nearly identical CD spectra. In both cases, the tryptophan band at 291.5 nm has been greatly reduced in intensity, and the phenylalanine residues are clearly giving negative bands at 268–269.0 and 261.5 nm. Surprisingly, the dimers at pH 3.6 do not show the same degree of similarity. While the BGH dimer shows some increase in negative dichroism above 295 nm (possibly due to a ^1L_a transition of tryptophan residues contained within the dimer in a strongly hydrophobic environment), no definite maximum is seen and the ellipticity is much less intense than that of the BGH dimer at pH 8.2. The SGH dimer at pH 3.6 exhibits an intense and well-defined tryptophan band at 298 nm. This spectrum is very similar to that reported for BGH at pH 3.0 by Edelhoch and Lippoldt (1970). In order to better compare our results, we have taken spectra of both BGH and SGH in the same pH 3.0 solvent (Figure 5). Although we have not determined the state of aggregation of the two proteins in this solvent, the spectra clearly indicate that both are predominantly dimers. Moreover, the spectra show no time dependence, being unchanged over a 48-hr period. The SGH spectrum is almost identical with that reported for BGH in the same solvent by Edelhoch and Lippoldt (1970), while the BGH spectrum reported herein is considerably different.

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