Circular Dichroism of Ovine Interstitial Cell Stimulating Hormone and Its Subunits†

Thomas A. Bewley, M. R. Sairam, and Choh Hao Li*

ABSTRACT: The circular dichroism (CD) of ovine interstitial cell stimulating hormone, its subunits, and the reassociated molecule have been investigated. There is no evidence indicating the presence of α helix in any of these molecules. Both subunits appear to be essentially random coils, but some conformational change occurs on reassociation, leading to a more ordered structure. This reassociated molecule is conformationally identical with the native hormone. The CD spectra in the region of side-chain absorption indicate that the native and reassociated molecules contain one or more tyrosine residues in a highly nonpolar environment. These "buried" tyrosine(s) are not seen in the spectra of the isolated subunits. It is suggested that such residues become buried when the two subunits combine.

Ovine ICSH,[1] a glycoprotein hormone of the anterior pituitary gland, has been isolated as a homogeneous protein with a molecular weight of 30,000 (Squire and Li, 1958; 1959; Ward *et al.*, 1959). The dissociation of the hormone into smaller subunits was first demonstrated in acidic media by Li and Starman (1964). Following the suggestion that these might be two dissimilar subunits (Ward *et al.*, 1966), Papkoff and Samy (1967) succeeded in separating them by countercurrent distribution. While these subunits were essentially devoid of biological activity when assayed in the ovarian ascorbic acid depletion test (Papkoff and Samy, 1967), activity could be restored by recombination of the subunits under the proper conditions (Papkoff and Samy, 1968; De la Llosa and Jutisz, 1969).

Previous optical rotatory dispersion studies on native ICSH (Jirgensons, 1960, 1969) indicated the absence of any appreciable amounts of α-helical structure. This was confirmed by a more recent investigation of the CD spectra of the native molecule (Jirgensons and Ward, 1970) in which the authors also conclude that no major conformational change occurs on dissociation of the protein. It will be seen that our results are not in complete agreement with those of Jirgensons and Ward. Further, our methods of calculation and interpretation of the CD spectra differ from these authors.[2]

Materials and Methods

ICSH was isolated from fresh sheep pituitary glands by procedures previously described (Papkoff *et al.*, 1965). The α and β subunits were prepared using a countercurrent distribution procedure (Papkoff and Samy, 1967) and further purified by gel filtration (Papkoff and Gan, 1970; Sairam *et al.*, 1971). The reassociated molecule was prepared as described elsewhere (Sairam *et al.*, 1971). Guanidine hydrochloride was twice recrystallized from 95% ethanol using Norit decolorizing carbon. All other chemicals were of reagent grade and were used without further purification.

Determination of Protein Concentration. In all cases, protein concentrations were determined spectrophotometrically. The molar extinction coefficients of native ICSH and its subunits were arrived at as follows. A solution of the protein was prepared in deionized water. In the case of the β subunit, a small amount of acetic acid was added to ensure complete solubility. Equal aliquots (containing ≈ 3 mg of protein) of the solution were transferred to volumetric flasks (3.0 ml) and lyophilized. Each flask was then made up with one of three solvents: (a) 0.1 M Tris-phosphate buffer, pH 7.5, (b) 0.15 M KCl–5 M guanidine hydrochloride adjusted to pH 8.0, and (c) 0.15 M KCl–5 M guanidine hydrochloride adjusted to pH 12.9. For the β subunit, it was found necessary to add 5% dioxane (v/v) to solvent a in order to obtain clear solutions. Absorption spectra were taken of each solution from 360 to ≈ 245 nm on a Beckman DK-2A recording spectrophotometer, against the solvent as reference. All spectra were corrected for light scattering as described by Beavan and Holiday (1952). The difference in absorption at 295 nm, between the samples in solvents b and c, was assumed to represent the increase in molar absorptivity produced by essentially complete ionization of all the tyrosine residues in the sample. By using a value of 2390/mole of tyrosine for the increase in molar absorptivity at 295 nm in these solvents (Nozaki and Tanford, 1967; Bewley *et al.*, 1969), the molar concentration of tyrosine in each flask could be calculated. From this value and the tyrosine content (Papkoff *et al.*, 1971), the molar concentration of either ICSH or its subunits could be determined. In this manner, the protein concentration in solvent a, and hence the molar extinction coefficient in this solvent could be calculated. These values, as well as other properties of ICSH and its subunits are summarized in Table I.

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, 10, and 20 mm. No dilutions of the samples were made at any time during taking of the

† From the Hormone Research Laboratory, University of California, San Francisco, California 94122. *Received October 18, 1971.* This work is supported in part by the National Institute of Health (A-6097), the Geffen Foundation, and the Allen Foundation.

[1] Abbreviations used are: ICSH, interstitial cell stimulating hormone; CD, circular dichroism; ORD, optical rotatory dispersion.

[2] While this manuscript was in the hands of the editor, an additional report (Pernollet and Garnier, 1971) on the CD of ICSH and its subunits appeared. The far-uv spectra are very similar to those reported herein. The spectra in the region of side-chain absorption also indicate that the dissociation and reassociation of ICSH is attended by a conformational change.

spectra. Measurements were made from 325 to 205 nm, reducing the optical path whenever the dynode voltage reached 700 V with rescanning of an appropriate region at the lower voltage. With the exception of the region very near 205 nm, the results are all calculated from data obtained at dynode voltages below 500. Scanning speeds were 1 nm/min or less.

Since the carbohydrate portion of these glycoproteins is not expected to contribute significantly to the CD above 200 nm (see Discussion), all spectral data has been corrected for carbohydrate content as described below. For the region below 240 nm (Figure 1a) the results are expressed in the usual manner as mean residue molecular ellipticities $[\theta]_{MRW}$. In this calculation, the mean residue weights have been corrected for carbohydrate content by using molecular weights (calculated for the polypeptide portions only) and numbers of amino acid residues indicated by the appropriate primary structures (Papkoff *et al.*, 1971). These mean residue weights and the values from which they were computed are included in Table I. Protein concentrations (w/v) were determined spectrophotometrically using the $E^{0.1\%}_{1cm}$ values. These extinction coefficients provide the weight concentrations of *total* glycoprotein (polypeptide + carbohydrate). Each concentration value was then reduced to the corresponding weight concentration of polypeptide chromaphore by subtracting the appropriate carbohydrate content. The carbohydrate contents of these proteins are also included in Table I. Above 232 nm (Figure 1b), the results are expressed as molecular ellipticities $[\theta]_M$. The molecular weights used in these calculations represent only the polypeptide portions of the molecules.

TABLE I: Some Properties of Ovine ICSH and its Subunits.[a]

Property	ICSH	α	β
Molecular weight[b]	28,300	13,700	14,600
Molecular weight (polypeptide only)[b]	23,560	10,800	12,760
Number of amino acid residues[b]	216	96	120
Mean residue weight (polypeptide only)[b]	109	112	106
Tyrosine (residues/mole)[b]	7	5	2
Disulfide bonds/mole[b]	11	5	6
Per cent carbohydrate (w/w)	14.3	16.9	10.1
Hexosamine[c]	10.7	5.9	4.0
Fucose[c]	2.9	1.3	1.0
Hexose[c]	12.0	7.7	4.0
E_m[d]	12,420	8,030	4,390
$E^{0.1\%}_{1cm,276\,nm}$[d]	0.439	0.586	0.301

[a] Taken in part from Sairam *et al.* (1971). [b] Taken or calculated from the primary structures (Papkoff *et al.*, 1971). [c] Residues/30,000 for ICSH; residues/15,000 for the α and β subunits. [d] Measured as described in the text.

Results

During the initial part of this investigation, a series of CD spectra of native ICSH, the α subunit and the reassociated molecule were taken in 0.1 M phosphate buffer of pH 6.0. However, it was found that this buffer was unsuitable for the β subunit due to insolubility and aggregation of the protein which resulted in slightly opalescent solutions; in the absorption spectrum of the β subunit, nearly 60% of the optical density at 276–277 nm was due to light scattering, even following prolonged centrifugation.[3] In addition, no absorption peak was evident in these spectra. Although CD spectra of these solutions could be taken in the near-ultraviolet region, it was impossible to accurately determine the protein concentrations. A solvent composed of 0.1 M Tris-phosphate buffer of pH 7.5, containing 5% dioxane was found to give clear solutions of the β subunit with minimal light scattering (less than 15% at 275 nm) and a distinct absorption peak at 275–276 nm. In order to allow meaningful comparisons to be made, the CD of all the proteins were retaken in this solvent and are shown in Figures 1a,b along with the original spectra taken in 0.1 M phosphate buffer of pH 6.0.

Circular Dichroism below 240 nm. The CD spectra of native ICSH, its subunits, and the reassociated molecule in the region of amide bond absorption are shown in Figure 1a. In the 0.1 M phosphate buffer of pH 6.0, the native and reassociated molecules give nearly identical spectra with a weak positive peak near 235–237 nm and a more intense negative peak at 210 nm. The spectra of these same two proteins in the Tris-phosphate–dioxane solvent, show essentially the same pattern but with slightly reduced negative ellipticities. Within experimental error, the α subunit gave the same spectrum in both solvents, showing a positive peak at 233 nm and a negative shoulder between 210 and 220 nm, superimposed on a stronger negative band centered below 205 nm. The β subunit exhibits no positive dichroism at all, but does indicate the presence of a strong negative band below 205 nm. Due to unfavorable signal-to-noise ratios, we have been consistently unable to obtain spectra of these proteins below 205 nm which were sufficiently accurate to be interpreted meaningfully.

Circular Dichroism above 232 nm. The CD spectra of these same proteins in the region of side-chain absorption are shown in Figure 1b. The native and reassociated molecules appear to be identical with each other in either of the two solvents used, although there is a definite decrease in the negative dichroism exhibited by either molecule in the Tris-phosphate–dioxane system. However, the basic pattern is the same in either solvent, with both exhibiting negative dichroism from about 325 nm, including a distinct shoulder near 287–288 nm and a peak at 281 nm. In addition, these spectra show a weak negative peak between 243 and 245 nm closely followed by a much stronger positive one between 235 and 237 nm. The exact positions and amplitudes of these last two features are more uncertain than those above 250 nm due to unfavorable signal-to-noise ratios and the rapidly

[3] Typical light-scattering corrections for the other molecules amounted to no more than 10% of the total OD at 275–277 nm. In order to get some indication of whether or not light scattering artifacts and/or aggregation effects were altering the CD spectra of the β subunit, the *shape* of the spectrum taken in phosphate buffer of pH 6.0 was compared with that taken in the Tris-phosphate–dioxane buffer by adjusting, in the calculation, the apparent concentration in phosphate to give the same ellipticity at the 278-nm peak as calculated in the better solvent. The entire spectrum was then recalculated using this "apparent concentration." The shape of the two curves between 230 and 300 nm was found to be equivalent to within ±4%. Below ≈220 nm the spectrum with the higher light scattering had the same shape as the other but showed consistently less negative ellipticity values, approaching 10% deviations at 208 nm. From this, we conclude that no significant artifact is present in our spectra.

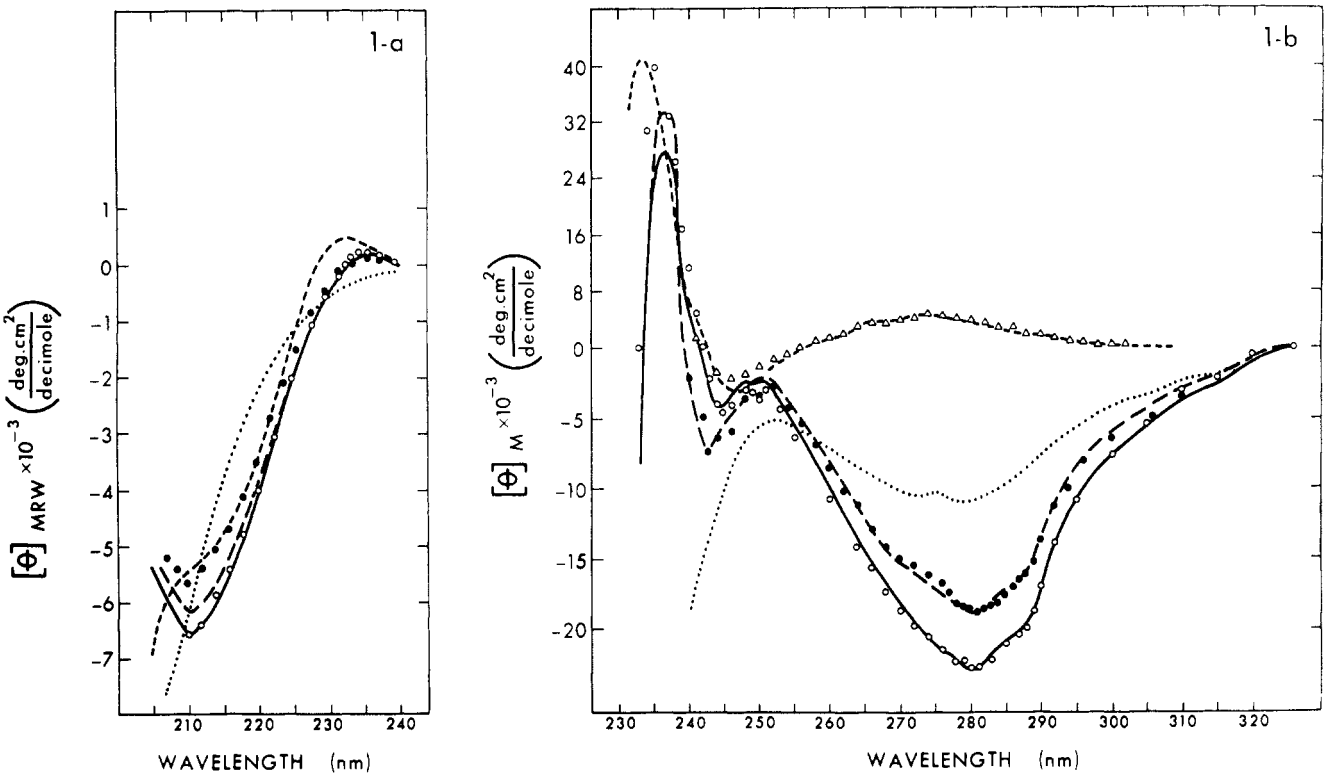

FIGURE 1a: Amide bond circular dichroism spectra of ICSH (——) and the reassociated molecule (○) in 0.1 M phosphate buffer, pH 6.0; ICSH (— —), the reassociated molecule (●), the α subunit (- - -), and the β subunit (· ·) in 0.1 M Tris-phosphate–5% dioxane buffer (pH 7.5). Protein concentrations were between 0.5 and 1.0 mg per ml. Data are expressed as mean residue ellipticities. (b) Side-chain circular dichroism spectra of ICSH(—), the reassociated molecule (○), and the α subunit (△) in 0.1 M phosphate buffer, pH 6.0; ICSH (— —), the reassociated molecule (●), the α subunit (- - -) and the β subunit (· ·) in 0.1 M Tris-phosphate–5% dioxane buffer (pH 7.5). Protein concentrations were between 0.5 and 1.0 mg per ml. Data are expressed as molecular ellipticities.

changing dichroism pattern in this region. The α subunit displays no dichroism at all above 298 nm. Below this point, a broad, weakly positive spectrum is seen with poorly resolved but reproducible maxima at 274 and 265 nm. Below 250 nm there is a weak negative peak at 247 nm and a strong positive one at 233 nm. Within experimental error, the spectrum of the α subunit is the same in both solvent systems. The β subunit exhibits only negative dichroism, with the spectrum appearing as a broad and nearly featureless band between 325 and 250–252 nm; two reproducible but weak negative maxima do appear at 278–279 and 273 nm. Below 250 nm, overlapping with the amide bond dichroism causes the spectrum to become strongly negative.

Discussion

In view of the present knowledge concerning their composition and structure, ovine ICSH and its subunits provide a unique opportunity for evaluating the CD spectra, both of a glycoprotein, and in addition, a protein whose structure contains two nonidentical subunits. We have approached this problem by first attempting to estimate to what extent the carbohydrate portion of these molecules might function as an optically active chromophore in the wavelength region we have studied. From an ORD study of a number of sugars and oligosaccharides, Beychok and Kabat (1965) concluded that simple saccharides do not display Cotton effects above 200 nm and hence would not be expected to contribute significantly to CD spectra above this wavelength. However, they did find a distinctive Cotton effect associated with an n–π* transition of the amide bonds of 2-acetamido-2-deoxy sugars and their oligosaccharides. These findings were confirmed and extended by the CD studies of Lloyd *et al.* (1967) on oligosaccharides derived from blood group substances. They observed the same amido sugar Cotton effect as a narrow CD band with negative maximum at 210–212 nm. The intensity of this band was relatively low but highly dependent on the configuration as well as the type and extent of substitutions on the individual amido sugar monosaccharide units. The highest intensities were found for nonterminal *N*-acetylhexosamine residues which were substituted on C_4 by fucose residues. This is of special interest because of the possibility of such oligosaccharide arrangements being present in ICSH. As shown in Table I, ICSH contains 10–11 residues of hexosamine and 3 residues of fucose per mole. There is also strong evidence indicating that these hexosamine residues are N-acetylated (Ward *et al.*, 1961; Ward and Coffey, 1964). Using the molecular ellipticities reported by Lloyd *et al.* (1967) and assuming that the monosaccharide units in ICSH are linked in a combination giving rise to the most intense CD band, we estimate the maximum total molecular ellipticity, contributed by the carbohydrate to be between 50,000 and 75,000 deg·cm²·dmole⁻¹ at 210–212 nm. Since the molecular ellipticity[4] of ICSH at this wavelength is close to 1.5×10^6 deg·cm²·dmole⁻¹, it would appear that the carbohydrate portion is not likely to contribute more than

[4] This value is computed on the basis of the polypeptide molecular weight alone. It is obtained by multiplying the mean residue ellipticity (from Figure 1a) by the number of amino acid residues in the protein.

5% to the dichroism between 205 and 215 nm, and essentially nothing at all above 220 nm. For this reason we have chosen to neglect any such contribution and to consider the polypeptide portion of the molecules as the only significant source of optically active chromophores in the spectral region under consideration. In computing chromophore concentrations, mean residue weights and molecular weights, we have used values which entirely eliminate the carbohydrate from entering into the subsequent calculations of dichroic strength. Since the polysaccharide is not evenly distributed between the two subunits (see Table I), this calculation procedure should give a more realistic representation of the CD properties of the proteins under investigation.

Circular Dichroism below 240 nm. While it is probably true that in most proteins the circular dichroism exhibited between 200 and 240 nm originates primarily from the amide bond transitions, an unequivocal interpretation of these patterns in terms of the polypeptide backbone conformation may not always be possible because of complex overlapping with the far-ultraviolet CD bands of aromatic residues and/or disulfide bonds (Beychok, 1966). This difficulty may be particularly true of the amide bond CD of ICSH and its subunits due to the apparent lack of any high degree of ordered secondary structures and the presence of an unusually high content of cystine. Nevertheless, the lack of a negative maximum or even a shoulder near 220–222 nm, in the spectra of these proteins, almost certainly eliminates the presence of any significant amounts of α helix. Both the native hormone and the reassociated molecule exhibit identical spectra, with a definite negative maximum at 210 nm, indicating that these two proteins are conformationally equivalent. Our spectrum of the native protein also agrees fairly well with that reported by Jirgensons and Ward (1970). These authors have suggested that the 210-nm band may be due in part to some type of β structure, but at the present time this cannot be clearly defined because of the band's potentially composite nature.

The spectra of the α and β subunits indicate that neither contains any appreciable amount of ordered structure. The possibility that the strongly negative band at 196–197 nm, characteristic of the unordered state (Greenfield and Fasman, 1969), is the dominant feature in the spectra of both subunits is strongly indicated. This 197-nm band is clearly shown in the spectrum of the α subunit[5] reported by Jirgensons and Ward (1970). Their α subunit spectrum also shows a definite peak at 214 nm, where ours shows only a shoulder. A more serious discrepancy between our results and those of Jirgensons and Ward (1970) concerns the β subunit. Their spectrum shows a shoulder around 215 nm, a distinct peak near 205 nm, and the complete absence of the random coil band at 197 nm. From this pattern, they conclude that the β subunit contains most of the ordered structure in the intact molecule. Although we have not been able to obtain completely satisfactory spectra below 205 nm, we see no indication of a shoulder near 215 nm, nor any evidence of a peak down to 205 nm. Thus, we conclude that there is no significant amount of ordered structure in our β subunit.[6]

Circular Dichroism above 232 nm. The side-chain CD spectra of these proteins are presented herein as molecular ellipticities (corrected for carbohydrate content) rather than as mean residue ellipticities. Accordingly, the spectrum of ICSH *should* be the graphical sum of the spectra of the α and β subunits, provided that upon dissociation or reassociation there are no conformational changes, or other effects leading to altered environments of the chromophoric groups. This is not the case when the data are presented as mean residue ellipticities since the mean residue weights do not indicate the proper relationship between the molecular weights (and hence the dichroic strengths) of the individual proteins. This difference may be readily seen by comparing our results with those of Jirgensons and Ward (1970) which are presented as mean residue ellipticities. Two important observations may be made immediately from the spectra in Figure 1b: native ICSH and the reassociated molecule exhibit identical spectra in either solvent system used, and the spectrum of either intact molecule is definitely *not* the sum of the spectra of the two subunits. From these observations we may conclude that some structural changes *do* occur upon dissociation of the hormone, producing alterations in the environments of the aromatic residues and/or the disulfide bonds. In addition, it is also clear that these alterations may be completely reversed upon reassociation of the two subunits.

Since these molecules contain no tryptophan (Papkoff *et al.*, 1971) and the CD shows none of the peaks typical of phenylalanine residues (Horwitz *et al.*, 1969), we may assume that the chromophores giving rise to these spectra are primarily the tyrosine residues and disulfide bonds. The spectrum of the α subunit from 298 to 250 nm has the broad featureless character and spectral position usually attributed to bands arising from disulfide bonds (Beychok, 1966; Horwitz *et al.*, 1970). The disulfide content of this subunit and the relative weakness of this band, suggests that it may be the sum of several separate bands, of both positive and negative sign. The strong positive band at 233 nm may be tentatively assigned to tyrosine (Beychok, 1966).

Since tyrosine residues do not show dichroism above 300 nm (Horwitz *et al.*, 1970), we propose that the β subunit spectra from 300 to 325 nm represents the "leading edge" of the disulfide bond contribution in this molecule. From a crude extrapolation of this leading edge, and assuming that the disulfide contribution can be represented as a single band, roughly Gaussian in shape and centered near 270 nm, it would appear that most of the side chain dichroism in the β subunit may also be due to the disulfide bonds. However, the two negative maxima at 278–279 and 273 nm, probably arise from tyrosine residue(s) whose 0–0 transition lies near 285–286 nm (Horwitz *et al.*, 1970).

The most striking features in the spectrum of the native protein are the pronounced shoulder at 287–288 nm and the negative maximum at 281 nm. Neither of these bands are seen in the spectra of the subunits. The 287- to 288-nm band may be assigned with considerable confidence to the 0–0 transition of one or more tyrosine residues occupying positions with nonpolar environments and essentially completely shielded from the external aqueous media. A very similar band at 288.5 nm has been assigned to the most "buried" of the six tyrosine residues in ribonuclease A (Horwitz *et al.*, 1970). The 281-nm peak is then assigned (at least in part) to the $0 + 800$ cm^{-1} transition corresponding to the 287- to 288-nm 0–0 transition. Unfortunately, on the basis of the CD spectra alone, we are unable to determine the number of tyrosine residues undergoing this reversible "burying" process, or to indicate whether residues in only one or both

[5] In their paper, Jirgensons and Ward (1970) used the older designation; C-I and C-II for the α and β subunits, respectively.

[6] Our experience would indicate that in neutral or slightly acidic phosphate buffers, the β subunit exists in an aggregated form rather than as freely soluble monomers. The ordered structure noted by Jirgensons and Ward in their β subunit might be the result of aggregation of the protein under their conditions (0.01 M phosphate buffer of pH 6.1–7.7).

subunits are involved. However, an assessment of the availability of tyrosines in native ICSH to reaction with tetranitromethane suggests that at least two residues are unreactive and that each subunit contains one such tyrosine.[7]

References

Beavan, G. H., and Holiday, E. R. (1952), *Advan. Protein Chem. 7*, 319.

Bewley, T. A., Brovetto-Cruz, J., and Li, C. H. (1969), *Biochemistry 8*, 4701.

Beychok, S. (1966), *Science 154*, 1288.

Beychok, S., and Kabat, E. A. (1965), *Biochemistry 4*, 2565.

De la Llosa, P., and Jutisz, M. (1969), *Biochim. Biophys. Acta 181*, 426.

Greenfield, N., and Fasman, G. D. (1969), *Biochemistry 8*, 4108.

Horwitz, J., Strickland, E. H., and Billups, C. (1969), *J. Amer. Chem. Soc. 91*, 184.

Horwitz, J., Strickland, E. H., and Billups, C. (1970), *J. Amer. Chem. Soc. 92*, 2119.

Jirgensons, B. (1960), *Arch. Biochem. Biophys. 91*, 123.

Jirgensons, B. (1969), Optical Rotatory Dispersion of Proteins and Other Macromolecules, Berlin, Springer-Verlag, pp 86 and 129.

Jirgensons, B., and Ward, D. N. (1970), *Texas Rept. Biol. Med. 28*, 553.

[7] M. R. Sairam, unpublished results.

Li, C. H., and Starman, B. (1964), *Nature (London) 202*, 291.

Lloyd, K. O., Beychok, S., and Kabat, E. A. (1967), *Biochemistry 6*, 1448.

Nozaki, Y., and Tanford, C. (1967), *J. Amer. Chem. Soc. 89*, 742.

Papkoff, H., and Gan, J. (1970), *Arch. Biochem. Biophys. 136*, 522.

Papkoff, H., Gospodarowicz, D., Candiotti, A., and Li, C. H. (1965), *Arch. Biochem. Biophys. 111*, 431.

Papkoff, H., and Samy, T. S. A. (1967), *Biochim. Biophys. Acta 147*, 175.

Papkoff, H., and Samy, T. S. A. (1968), *Fed. Proc., Fed. Amer. Soc. Exp. Biol. 27*, 371.

Papkoff, H., Sairam, M. R., and Li, C. H. (1971), *J. Amer. Chem. Soc. 93*, 1531.

Pernollet, J. C., and Garnier, J. (1971), *FEBS (Fed. Eur. Biochem. Soc.) Lett. 18*, 189.

Sairam, M. R., Papkoff, H., and Li, C. H. (1971), *in* Gonadotropins, Saxena, B. B., Beling, C. G., and Gaudy, H. M., Ed., New York, N. Y., Wiley and Sons (in press).

Squire, P. G., and Li, C. H. (1958), *Science 127*, 32.

Squire, P. G., and Li, C. H. (1959), *J. Biol. Chem. 230*, 524.

Ward, D. N., and Coffey, J. A. (1964), *Biochemistry 3*, 1575.

Ward, D. N., Fujino, M., and Lamkin, W. M. (1966), *Fed. Proc., Fed. Amer. Soc. Exp. Biol. 25*, 349.

Ward, D. N., McGregor, R. F., and Griffin, A. C. (1959), *Biochim. Biophys. Acta 32*, 305.

Ward, D. N., Walborg, E. F., and Adams-Mayne, M. (1961), *Biochim. Biophys. Acta 50*, 224.