3790 © *1981 The Chemical Society of Japan* *Bull. Chem. Soc. Jpn.*, *54*, *3790—3793 (1981)* [Vol. 54, No. 12

The Interaction between α-Helical Poly-L-lysine and Thyroxine as Measured by Circular Dichroism

Nobuo Okabe,* Mariko Okamura, Ryoji Tokuoka, and Ken-ichi Tomita
Faculty of Pharmaceutical Sciences, Osaka University, Suita, Osaka 565

(Received October 24, 1980)

The interaction between α-helical poly-L-lysine (poly(L-Lys)) and the thyroid hormone, L-thyroxine (L-T_4), was investigated at pH 11 by means of circular dichroism (CD) measurements in the wavelength region of 200—380 nm. At least four CD bands were induced near 235, 250, 290, and 343 nm. This indicates that these induced Cotton effects occurred as the result of the binding of L-T_4 to poly(L-Lys), probably due to the electrostatic interaction. The wavelengths of the induced CD bands corresponded to those of the UV absorption bands of L-T_4 in the poly(L-Lys) : L-T_4 mixture, although small red-shifts of the CD bands were observed at the shortest and the longest wavelengths (235 nm and 343 nm). These CD bands might be interpreted by the fixed intramolecular orientation around an asymmetric α-carbon atom of the bound L-T_4 or by the intermolecular interaction between the peptide backbone of poly(L-Lys) and the bound L-T_4.

In previous papers,[2–4] the interaction between bovine serum albumin (BSA) or human serum albumin (HSA) and a thyroid hormone, L-thyroxine (L-T_4, Fig. 1), was investigated by means of CD measurements. The negative CD band was induced at the longest UV absorption maximum of L-T_4, suggesting a specific interaction between proteins and L-T_4. However, the binding mechanism has remained obscure. For this reason, as a model study to obtain much information about the binding mechanism of L-T_4 to a protein, the interaction between α-helical poly-L-lysine and L-T_4 was investigated by means of the CD method at pH 11. This synthetic polypeptide was chosen as a suitable model compound for this study because the lysine residue plays an important role in the binding of L-T_4 to human serum albumin;[5] the residue also sometimes appears either at the surface or at the helical region of globular proteins.

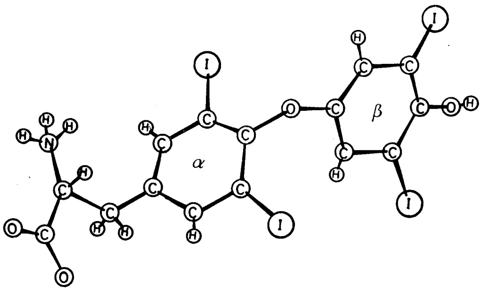


Fig. 1. Perspective drawing of L-T_4 molecule determined by X-ray analysis (Ref. 1). The dihedral angle between the planes of the α-ring and β-ring and the interring C–O–C angle are 79° and 120°±3°, respectively.

Experimental

Materials. Poly-L-lysine HBr (poly(L-Lys)) with a molecular weight of 46500 and a degree of polymerization (DP) of 222 (Lot No. LY-185) was purchased from Miles-Yeda, Ltd., U.S.A., and L-thyroxine sodium salt (L-T_4), from Nakarai Chemicals Co., Kyoto, Japan.

Procedure. Poly(L-Lys) was dissolved in distilled and deionized water, and the pH was adjusted to 11 by adding 1 mol dm^{-3} of NaOH. About 3×10^{-3} mol dm^{-3} of L-T_4 was dissolved in 0.01 mol dm^{-3} of the NaOH solution to serve as a stock solution. To prepared the mixture of poly(L-Lys) and L-T_4, a small amount of L-T_4 was gradually added to 2 cm^3 of the poly(L-Lys) solution under continuous stirring until the appropriate molar ratio was reached. The pH change in the poly(L-Lys) solution upon the addition of L-T_4 was negligible under those experimental conditions. The CD measurements were carried out on a JASCO J-40A spectropolarimeter equipped with a data-processor system, J-DPZ, at the wavelength region of 200—380 nm. CD cells with light-path lengths of 1 and 10 mm were used for the measurement in the wavelength regions of 200—260 nm and 260—380 nm respectively. The optical rotatory dispersion (ORD) measurements were performed with a JASCO J-20 spectropolarimeter. A cell with a light-path length of 2 mm was used in the wavelentgth region of 210—300 nm. The ultraviolet (UV) absorption spectra were obtained with a Hitachi 323 recording spectrophotometer. The pH adjustment was done by means of a Hitachi Horiba D-5 pH meter. The CD ellipticity and the ORD rotation were represented as a unit of degree cm^2 $decimol^{-1}$ on the basis of the concentration of the lysine residues of poly(L-Lys) or L-T_4. All the measurements except one noted in the text were carried out at 22 °C.

Results and Discussion

Figure 2 shows the absorption spectra of L-T_4 in a 30% ethanol aqueous solution at various pH values. The peak around 300 nm (pH 3.2) was shifted to 330 nm with an increase in its height in the alkaline solution (pH 11.7), although a small shoulder remained at 290 nm. The isosbestic points appeared at 285 nm and 308 nm. This peak-height increment at 330 nm is due to the dissociation of the phenolic hydroxyl group of the β-ring of L-T_4 (Fig. 1). The p*K* value of this group was calculated as 6.32, coincident well with the values of 6.2 and/or 6.7 reported by Steiner[6] and Gemmill[7] respectively. This indicates that the hydroxyl group completely dissociates at pH 11. It is well known that poly(L-Lys) takes a α-helical conformation in the aqueous solution at pH 11. The ε-amino groups of poly(L-Lys) may be not completely uncharged at pH 11, since the p*K* of these groups is 10.8.[9] This suggests that the negatively charged hydroxyl and carboxyl groups of L-T_4 at pH 11 cause electrostatic interaction with some positively charged ε-amino groups of poly(L-Lys). The binding of L-T_4 to the helical poly(L-Lys) may induce some optical activities to arise from L-T_4, although a

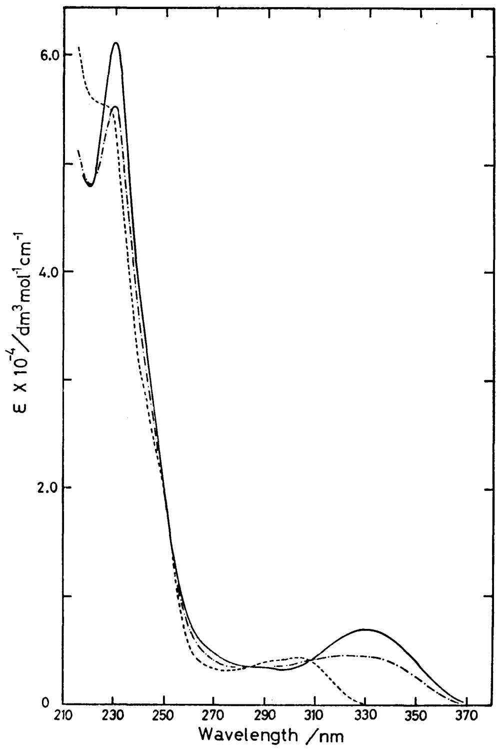


Fig. 2. The ultraviolet absorption spectra of L-T$_4$ as a function of pH in 30% ethanol aqueous solution. Curves are at pH 11.7 (——); at pH 6.6 (—•—); at pH 3.2 (------). L-T$_4$ concentration was 4.28 × 10^{-5} mol dm^{-3}. Temperature was 24 °C.

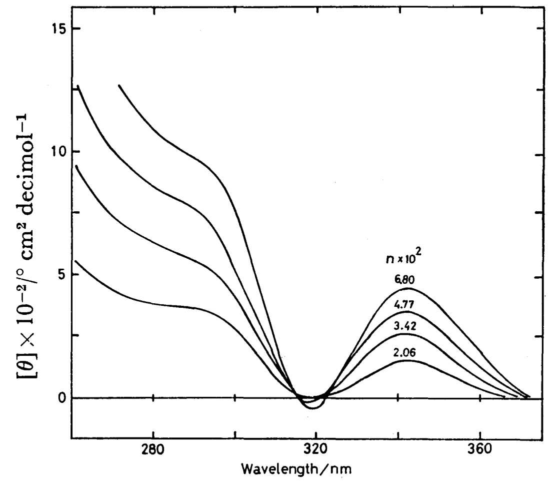


Fig. 3. CD spectra of poly(L-Lys) : L-T$_4$ at pH 11. The ratios of the L-T$_4$ molecules to lysine residues of poly(L-Lys), *n*, are cited in the figure. Poly(L-Lys) concentration was kept at 5.31 × 10^{-6} mol dm^{-3}. The residue ellipticity was calculated on the basis of poly(L-Lys) concentration (DP=222).

free L-T$_4$ has no detectable optical activity in the wavelength region of 200—400 nm, even at a high concentration of 10^{-4} mol dm^{-3} (these data are not shown here). Therefore, the CD spectra of the poly(L-Lys) : L-T$_4$ system was measured in the wavelength region of 200—400 nm at pH 11 with a variation in the *n* ratio, which is defined as the ratio of L-T$_4$ molecules to the lysine residues of poly(L-Lys).

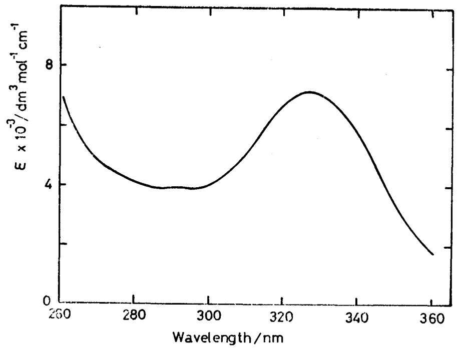


Fig. 4. The ultraviolet absorption spectra of poly(L-Lys) : L-T$_4$ at pH 11.0. The spectra were the same for the ratios of L-T$_4$ molecules to lysine residues of poly(L-Lys) between 2.58 × 10^{-2} and 7.75 × 10^{-2}. Poly(L-Lys) concentration was kept at 5.31 × 10^{-6} mol dm^{-3}.

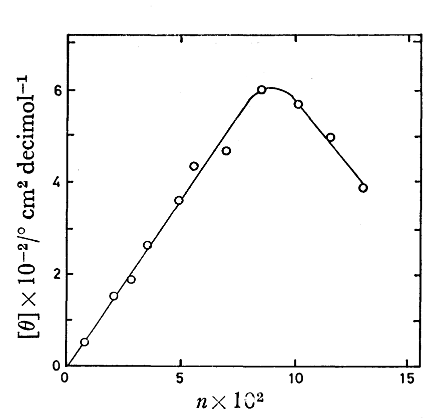


Fig. 5. Plot of the ellipticities of poly(L-Lys) : L-T$_4$ at 343 nm against the ratio of the L-T$_4$ molecules to lysine residues of poly(L-Lys), *n*, and pH 11.0. Poly(L-Lys) concentration was kept at 5.31 × 10^{-6} mol dm^{-3}. The residue ellipticity was calculated on the basis of poly(L-Lys) concentration (DP=222).

The CD spectra and the absorption spectra of the poly(L-Lys) : L-T$_4$ mixture at various *n* values in the wavelength region of 260—380 nm are shown in Figs. 3 and 4 respectively. The positive CD bands with a peak near 343 nm and a shoulder around 270—300 nm were induced. The peak near 343 nm showed a red-shift by about 13 nm from the longest absorption maximum at 330 nm. The residue ellipticities at 343 nm are plotted against *n* in Fig. 5. They increased linearly with the increase in *n* up to about $n=9\times10^{-2}$, but thereafter they decreased. At *n* values of over 9 × 10^{-2} it was impossible to obtain reliable CD data because of the appreciable turbidity in the sample solution due to the aggregation of the poly(L-Lys) : L-T$_4$ complex. These results suggest the binding of L-T$_4$ to poly(L-Lys). The aggregation of poly(L-Lys) : L-T$_4$ might be a result of the neutralization of positively charged ε-amino groups of poly(L-Lys) by the binding of negatively charged hydroxyl and carboxyl groups of L-T$_4$, followed by an increase in the hydrophobic interaction between poly(L-Lys) : L-T$_4$ complexes.

The effect of L-T$_4$ binding on the helical poly(L-Lys) was also investigated by measuring CD and ORD in the far-ultraviolet region, 200—260 nm for CD and 210—

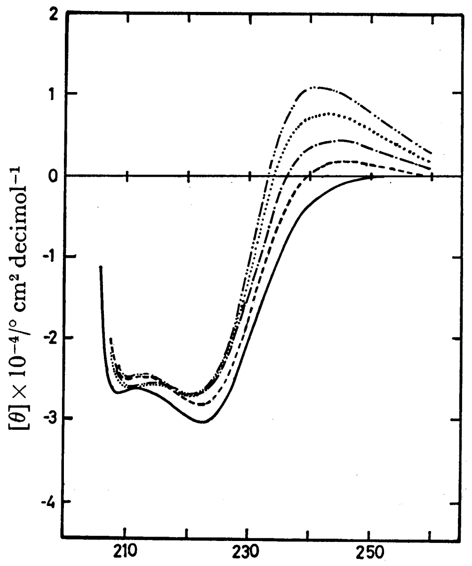


Fig. 6. The far ultraviolet CD spectra of poly(L-Lys) : L-T_4 at pH 11.0. The ratios of the L-T_4 molecule to lysine residues of poly (L-Lys) mol are, poly(L-Lys) alone, (——); 2.25×10^{-2}, (------); 4.50×10^{-2}, (—·—); 6.75×10^{-2}, (······); 9.00×10^{-2}, (-··-). Poly(L-Lys) concentration was kept at 6.13×10^{-6} mol dm^{-3}. The residue ellipticity was calculated on the basis of poly(L-Lys) concentration (DP=222).

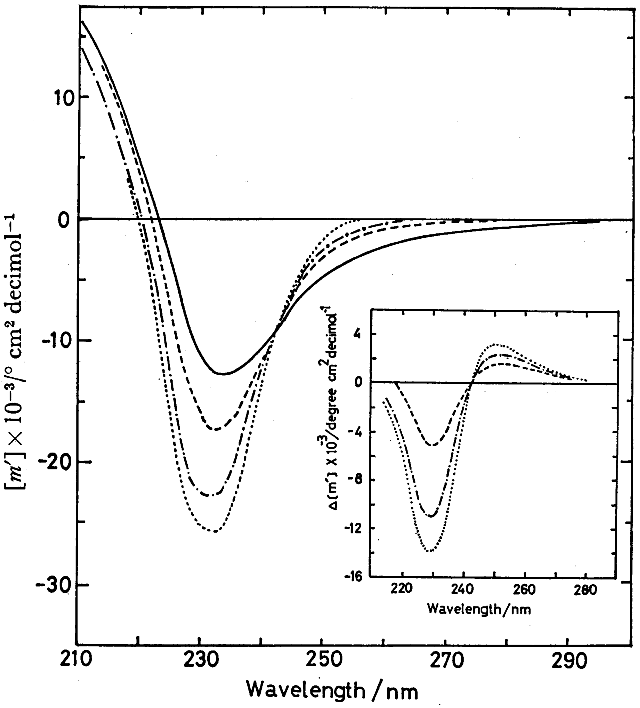


Fig. 7. ORD spectra of poly(L-Lys): L-T_4 at pH 11.0. The ratios of the L-T_4 molecules to lysine residues of poly(L-Lys) are, poly(L-Lys) alone, (——); 2.25×10^{-2}, (----); 4.50×10^{-2}, (-·-); 6.75×10^{-2}, (······). Poly(L-Lys) concentration was kept at 6.50×10^{-6} mol dm^{-3}. The residue rotation was calculated on the basis of poly(L-Lys) concentration (DP=222). The difference rotation is also shown in the same figure.

300 nm for ORD. Figure 6 shows the CD spectra of poly(L-Lys) in the presence of various amounts of L-T_4. The CD spectrum of a free poly(L-Lys) at pH 11 is characteristic of the α-helical conformation, having the CD maxima both at 208 nm and 222 nm; these maxima are assigned to the π-π^* and n-π^* transitions of the peptide groups respectively, as has been reported by Greenfield and Fasman.[8] The percent helix of poly(L-Lys) was calculated by means of the equation:[8]

$$\% \text{ helix} = ([\theta]_{208} - 4000)/(33000 - 4000), \quad (1)$$

where $[\theta]_{208}$ is the residue ellipticities at 208 nm. The helix content of poly(L-Lys) in the absence of L-T_4 was 78%. In the presence of L-T_4, a new CD band appeared at around 240 nm, although the characteristic CD bands at 208 nm and 222 nm still remained. Figure 7 shows the ORD spectra of poly(L-Lys) in the presence of various amounts of L-T_4. The ORD spectrum of a free poly(L-Lys) is characteristic of the α-helical conformation, as has been reported by Davidson *et al.*[10] The negative ORD band of poly(L-Lys) increased at 233 nm, and decreased around 250 nm, in the presence of L-T_4. The difference rotation, $\Delta[m']$, was obtained by subtracting the residue rotation of a free poly(L-Lys) from that of a poly(L-Lys) : L-T_4 mixture according to the following equation and inserted into Fig. 7:

$$\Delta[m'] = [m'](\text{poly(L-Lys)}: \text{L-}T_4) - [m'](\text{poly(L-Lys)}). \quad (2)$$

The difference ORD curves show a positive Cotton effect around 240 nm. These results of both CD and ORD measurements suggest that the Cotton effect observed in the 230—250 nm region was induced by L-T_4 bound to poly(L-Lys). From the data in Fig. 6, the difference ellipticity, $\Delta[\theta]$, was calculated in the same manner as in Eq. 2 by means of the following equation, and $\Delta[\theta]$ at 235 nm was plotted against n in Fig. 8:

$$\Delta[\theta] = [\theta](\text{poly(L-Lys)}: \text{L-}T_4) - [\theta](\text{poly(L-Lys)}). \quad (3)$$

It increased linearly with the increase of n up to about $n = 9 \times 10^{-2}$, as was observed at 343 nm (Fig. 5). This indicates that the induced optical activity at around 235 nm as well as that at 343 nm is attributable to the binding of L-T_4 to poly(L-Lys). In this study, therefore, assuming that no conformational changes in the α-helical poly(L-Lys) were induced by the binding of L-T_4 at low n values, the difference CD spectrum was obtained by using Eq. 3 from the CD spectra measured at the same concentration of poly(L-Lys) at the wavelength region of 200—380 nm, while the difference molar ellipticity for L-T_4 was calculated on the basis of the L-T_4 concentration. The difference CD spectrum at $n = 2.25 \times 10^{-2}$ (Fig. 9(a)) had a large positive peak near 235 nm, a small one at 343 nm, and two shoulders around 250 nm and 290 nm. The UV absorption spectra of the L-T_4 and poly(L-Lys) : L-T_4 mixture were also measured at the same n value ($n = 2.25 \times 10^{-2}$) (Fig. 9(b)). In the case of the poly(L-Lys) : L-T_4 mixture, the UV maximum at 228 nm decreased and a new shoulder appeared near 250 nm, and the small peaks at both 290 nm and 330 nm increased as compared with a free L-T_4. The molar extinction coefficient of 7.25×10^3 at 330 nm for L-T_4 in the poly(L-Lys) : L-T_4 system is slightly larger than the values of 6.27×10^3

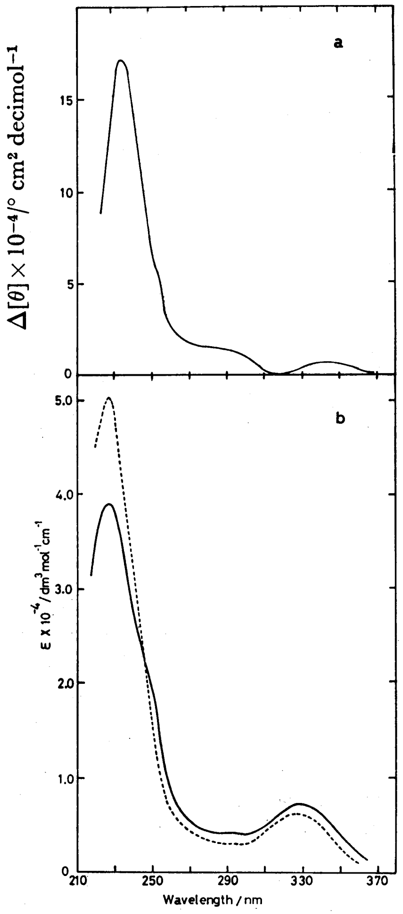
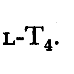


Fig. 9. (a): Difference CD spectrum of poly(L-Lys): L-T_4 obtained by subtracting the CD spectrum of poly(L-Lys) alone from that of poly(L-Lys): L-T_4. The concentration of poly(L-Lys) and L-T_4 were 1.13×10^{-5} mol dm^{-3} and 5.67×10^{-5} mol dm^{-3}, respectively. pH was 11.0. The difference ellipticity was calculated on the basis of L-T_4 concentration.
(b): The ultraviolet absorption spectra of poly(L-Lys): L-T_4, (——) and L-T_4, (······) at pH 11.0. The concentration of poly(L-Lys) and L-T_4 were 1.03×10^{-5} mol dm^{-3} and 5.10×10^{-5} mol dm^{-3}, respectively.

for the free L-T_4 at 325 nm, which is in good agreement with the 6.21×10^3 at 325 nm reported by Gemmill.[7] The induced CD bands appearing in the poly(L-Lys) : L-T_4 complex (Fig. 9(a)) coincided with the corresponding absorption maxima (Fig. 9(b)) except for the small red-shifts at 235 nm and 343 nm.

The induced CD bands observed in this study may be interpreted by the following mechanisms: (1) a certain fixed intramolecular orientation between the carboxyl group and the aromatic ring of L-T_4 occurs upon binding to poly(L-Lys), and (2) intermolecular interaction occurs between the peptide backbone of poly(L-Lys)  $(\text{-}\overset{\text{O}}{\overset{\|}{\text{C}}}\text{-}\underset{\text{H}}{\underset{|}{\text{N}}}\text{-})$ and the bound L-T_4. At present, however, it is unclear why the CD maxima at the shortest and the longest wavelengths (235 nm and 343 nm) shifted to wavelengths a little longer from their UV absorption maxima at 230 nm and 330 nm.

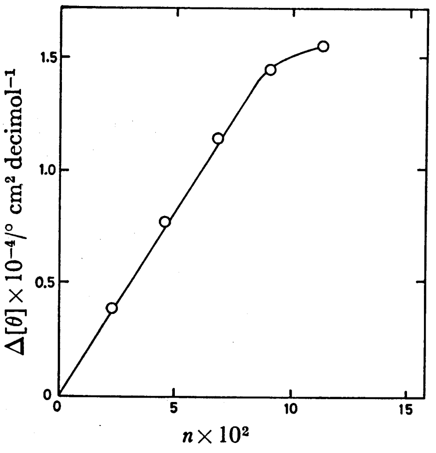


Fig. 8. Plot of the difference ellipticities of poly(L-Lys): L-T_4 at 235 nm against n and pH 11. Poly(L-Lys) concentration was kept at 6.13×10^{-6} mol dm^{-3}. The difference ellipticity was calculated on the basis of poly(L-Lys) concentration (DP=222).

Conclusion

The possibility of the binding of the thyroid hormone, L-T_4, to the α-helical poly(L-Lys) was strongly suggested by the facts that optical activity arising from L-T_4 was newly induced by mixing L-T_4 with α-helical poly(L-Lys) at pH 11 and that the induced ellipticity increased linearly with an increase in the ratio of L-T_4 molecules to lysine residues of poly(L-Lys). The results of this study suggest that L-T_4 may bind to the helical region of non-specific proteins as well as of specific ones for L-T_4 containing lysine residues in their α-helical regions. The CD peaks observed in this study seem to be usable in investigating the interaction between L-T_4 and specific or non-specific proteins for L-T_4 or other synthetic polypeptides by means of CD measurements.

References

1) N. Camerman and A. Camerman, *Proc. Natl. Acad. Sci. U.S.A.*, **69**, 2130 (1972).
2) N. Okabe, N. Manabe, R. Tokuoka, and K. Tomita, *J. Biochem.*, **77**, 181 (1975).
3) N. Okabe, N. Manabe, R. Tokuoka, and K. Tomita, *J. Biochem.*, **80**, 455 (1976).
4) R. Tokuoka, N. Okabe, and K. Tomita, *J. Biochem.*, **87**, 1729 (1980).
5) S. Y. Cheng, H. J. Cahman, M. Wilchek, and R. N. Ferguson, *Biochemistry*, **14**, 4132 (1975).
6) R. F. Steiner, J. Roth, and J. Robbins, *J. Biol. Chem.*, **241**, 560 (1966).
7) G. L. Gemmill, *Arch. Biochem. Biophys.*, **54**, 359 (1955).
8) N. Greenfield and G. D. Fasman, *Biochemistry*, **8**, 4108 (1969).
9) J. T. Edsall and J. Wyman, "Biophysical Chemistry," Academic Press, New York, N. Y. (1958), Vol. I, p. 465.
10) B. Davidson, N. Tooney, and G. D. Fasman, *Biochem. Biophys. Res. Commun.*, **23**, 156 (1966).