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Spectrophotometric Studies of the Copolymer of L-Tryptophan and L-Glutamic Acid in an Aqueous Solution[1]

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The synthetic copolymer of L-tryptophan and L-glutamic acid exhibited two specific bands, around 292 mμ and 234 mμ, in the difference spectrum at pH 4.68 *vs.* pH 7.60 in a 0.1 M phosphate buffer. The former, that at 292 mμ, might be caused by the red shift of the ordinary absorption band around 280 mμ, and the latter, that at 234 mμ, by the red shift of the far-ultraviolet absorption band, around 220 mμ, of the tryptophanyl residue in the copolymer. This specific peak at 234 mμ in the difference spectrum of the tryptophan copolymer may be distinguished from that at 222 mμ, which is due to the hyperchromisity ($n\rightarrow\pi^*$ transition) of the amide group in the peptide bond reported with poly-L-glutamic acid. The peak at 230—235 mμ appearing in the difference spectrum between native and denaturated proteins may be due to the same red shift ($\pi\rightarrow\pi^*$ transition) of the far-ultraviolet absorption band of the tryptophanyl residue as that shown by the tryptophan copolymer. The difference in absorption at 234 mμ was five times larger than that at 292 mμ, and both of them increase proportionally with a lowering of the pH. The relation between the difference absorption and the pH was quite similar to that between the helical content and the pH. The relative fluorescence intensity of the tryptophanyl residue was also depressed with a lowering of the pH, and the profile of the pH-dependence curve on the fluorescence intensity was almost identical with the titration curve of the copolymer. Therefore, it may be said that the appearance of the difference spectrum at 234 mμ corresponds to the formation of the α-helix in polypeptide and that the protonation of the glutamyl residue induces the quenching of the fluorescence of the tryptophanyl residue.

The characteristic absorption spectrum of the tryptophanyl residue in protein has been widely used to study the structure and the biological activity of protein; *e. g.*, the enzyme-substrate complex was demonstrated spectrophotometrically by means of the concomitant change of the absorption spectrum of the tryptophanyl residue in the lysozyme molecule.[2] To elucidate the role and behavior of the tryptophanyl residue in protein, it may be instructive to ascertain spectrophotometrically basic information on the tryptophanyl residue using some simple tryptophan copolymers.

Tryptophan has two specific absorption bands in the near visible region (250—300 mμ) and the far-ultraviolet region (200—240 mμ);[3] the same absorption bands were observed in the tryptophan copolymer in these experiments. Recently, Fasman *et al.*[4] showed that the fluorescence of the trytophanyl residue in copoly(Glu,Trp) and copoly(Lys,Trp) was strongly quenched by the unionized carboxyl group of glutamyl or the unionized amino group of the lysyl residue in these copolymers, but the fluorescence was almost independent of the conformation of α-helix in the copolymers. They suggested also that the hyperchromisity on the absorption spectrum of the tryptophanyl residue might be affected by the dissociation of the carboxyl group of the glutamyl residue from the difference spectrum between pH 4.6 and pH 7.3 in the range of 250—320 mμ, using copoly(Glu,Trp 99 : 1). However, the difference spectrum seems to be somewhat ambiguous as a basis for a further, detailed discussion of the behavior of the tryptophanyl residue, because the copolymers contained so many amide bonds in relation to the tryptophanyl residue (99 : 1 molar ratio) that the absorption band of the tryptophanyl residue in the far-ultraviolet region overlapped with that of the amide bond with a high optical density. It is interest to study the behavior of a copolymer with a higher

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2) K. Hayashi, T. Imoto and M. Funatsu, *J. Biochem.*, **54**, 381 (1963).

3) D. B. Wetlaufer, "Advances in Protein Chemistry," Vol. 17, ed. by C. B. Anfinsen, Jr., Academic Press, New York and London (1962), p. 320.

4) G. D. Fasman, E. Bodenheimer and A. Pesce, *J. Biol. Chem.*, **241**, 916 (1966).

content of tryptophan, since the absorption spectrum of the tryptophanyl residue seems to be a good indicator of the molecular conformation of the tryptophan copolymer. The present work is concerned with a spectrophotometric study of copoly-(Glu,Trp 9 : 1) in an aqueous solution in relation to a study of NPA*2 hydrolysis[5] by means of the interaction between glutamyl and tryptophanyl residues.

Further, the difference spectra between the native and denaturated states of proteins containing tryptophanyl residues presented by Glazer and Smith[6,7] are discussed in comparison with the results presented in this paper.

Experimental

Preparation of the Copolymer. The copolymer of L-tryptophan and L-glutamic acid was prepared from the *N*-carboxyanhydride (NCA) of each amino acid in a molar ratio of 9 : 1 as follows; 1.34 g (0.0058 mol) of L-tryptophan NCA,[8] after having been purified in a two-layer column (charcoal-silver oxide/activated charcoal),[9] and 13.8 g (0.053 mol) of γ-benzyl-L-glutamate NCA[10,11] were dissolved in 150 m*l* of dioxane; then 0.55 g (0.0049 mol) of triethylenediamine[9] was added to the mixture as an initiator. The solution was allowed to stand for 5 days at 40°C under stirring, and then the viscous solution was poured into a large amount of water in order to precipitate the copolymer. The precipitate was washed with water and ethanol, and dried *in vacuo*. To remove the benzyl group of the glutamyl residue, the copolymer was dissolved in 90 m*l* of dioxane, and then 0.2N aqueous alcoholic sodium hydroxide (0.9 g of sodium hydroxide, 22.5 m*l* of water, and 90.0 m*l* of ethanol)[12] was added. The mixture was allowed to stand for 10 hr at 15°C to complete the debenzylation of the glutamyl residues. The debenzylation of the copolymer was confirmed by the disappearance of the 1740 cm^{-1} absorption band of the ester. The sodium salt of the copolymer was precipitated with ethanol and washed several times with ethanol. It was colorless and easily soluble in water. Yield 7.5 g (85%). The tryptophanyl residue in the copolymer was estimated from the optical density at 280 mμ,[13] while the glutamyl residue was analysed with ninhydrin[14] by paper chromatography after acid hydrolysis. The molar ratio of glutamyl and tryptophanyl residues in this copolymer was 89.7 : 10.3. The intrinsic viscosity of the copolymer was 1.84 in 0.2M sodium chloride at pH 7.6 and 25°C. The molecular weight of the copolymer was calculated to be 85000 by applying the relation between the intrinsic viscosity and the molecular weight of poly-L-glutamic acid.[15]

Poly-L-glutamic Acid. Poly-L-glutamic acid was obtained by the saponification[12] of poly-γ-benzyl-L-glutamate prepared from γ-benzyl-L-glutamate NCA.[10,11] The intrinsic viscosity was 2.13 in 0.2M sodium chloride at pH 7.6 and 25°C; the molecular weight was calculated to be 97000 by the method described above.

Fractionation of Copolymer. The copolymer was fractionated by gel filtration through a column of Sephadex G-50, using a 0.1M phosphate buffer at pH 7.0 as an eluant.

Titration. Samples were titrated with 0.1N sodium hydroxide, using a Radiometer TTT-1 titrator with a SBR2-C titrigraph. Before titration, the solution of polyamino acid was treated by passing it through a mix-bed column of Amberite IR-120 and IRA-410 to remove the sodium ions; it was then adjusted to a 0.1M so ium chloride solution by the addition of 1.0M sodium chloride.[16]

Optical Rotatory Dispersion. The optical rotatory dispersion of the copolymer in a 0.1M phosphate buffer at various pH values was measured in the 300—600 mμ range with a Yanagimoto ORD-3 spectropolarimeter using a 50-mm quartz cell at 25°C (c=0.5), and the parameter, b_0, was calculated from the Moffitt-Yang equation.[17] The helical content from b_0 was corrected by applying the results of Fasman *et al.*[18]

Absorption Spectrum and Difference Spectrum. The ultraviolet absorption spectrum and the difference spectrum were studied with a Hitachi 124 spectrophotometer, using 10-mm quartz cells at 25°C. In the measurement of the difference spectrum, a copolymer solution at pH 7.60 was used as the reference.

Fluorescence Spectrum. The fluorescence spectra of the copolymer and tryptophan in 0.1M phosphate buffer were measured with a Hitachi MPF-2 spectrofluorophotometer, using a 10-mm quartz cell at 25°C.

Results

Fractionation. The results by gel filtration on a column of Sephadex G-50 are shown in Fig. 1.

*2 *p*-Nitrophenyl acetate.

5) J. Noguchi, S. Tokura, T. Komai, H. Yamamoto and A. Ohnishi, *J. Biochem.*, in press.

6) A. N. Glazer and E. Smith, *J. Biol. Chem.*, **235**, PC43 (1960).

7) A. N. Glazer and E. Smith, *ibid.*, **236**, 2942 (1961).

8) A. Patchornik, M. Sela and E. Katchalski, *J. Am. Chem. Soc.*, **76**, 299 (1954).

9) J. Noguchi, N. Nishi, M. Itaya and S. Tokura, *Kogyo Kagaku Zasshi* (*J. Chem. Soc. Japan, Ind. Chem. Sect.*), **69**, 745 (1966).

10) H. Yuki, *Nippon Kagaku Zasshi* (*J. Chem. Soc. Japan, Pure Chem. Sect.*), **77**, 49 (1956).

11) E. R. Blout and R. H. Karlson, *J. Am. Chem. Soc.*, **78**, 941 (1956).

12) Y. Takeda, T. Okuda and S. Sakurai, Japanese Pat. 3290 (1965).

13) H. G. Beaven and E. R. Holiday, "Advances in Protein Chemistry," Vol. 7, ed. by M. L. Anson *et al.*, Academic Press, New York and London (1952), p. 320.

14) R. A. Boissonnas, *Helv. Chem. Acta*, **33**, 1975 (1950).

15) A. Wada, *Mol. Phys.*, **3**, 409 (1960).

16) K. Nitta and S. Sugai, *Nippon Kagaku Zasshi* (*J. Chem. Soc. Japan, Pure Chem. Sect.*), **88**, 412 (1967).

17) W. Moffitt and J. T. Yang, *Proc. Natl. Acad. Sci. U. S.*, **42**, 596 (1956).

18) G. D. Fasman, M. Landsberg and M. Buchwald, *Can. J. Chem.*, **43**, 1588 (1965).

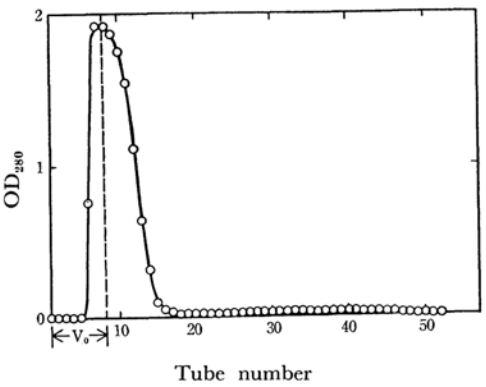

Fig. 1. Elution diagram of copoly(L-Glu, L-Trp 9 : 1) on Sephadex G-50, Fine.
Eluant: 0.1 M phosphate buffer at pH 7.0.
Bed dimensions: 2 cm×28 cm
Sample: 2 m*l* containing 10 mg of substance
Flow rate: 0.6 m*l*/min
V_0: void volume of the column which was determined with Blue Dextran 2000.

Since only one peak of the copolymer appears at the position of the void volume of the column, and since there is no other peak, the copolymer does not contain the fraction with a molecular weight less than 30000, because the effective fractionation with Sephadex G-50 for peptides is in the 1500—30000 range;*3 the molecular weight of 85000 obtained from the viscosity seems to support the above statement. It was shown also that no oxidation of the tryptophanyl residue occurred during the preparation of the copolymer, for normal absorption spectra of the tryptophanyl residue (250—300 mμ) were observed in each fraction and no absorption spectra of the oxidized tryptophanyl residue[19,20] were detected in any of the fractions.

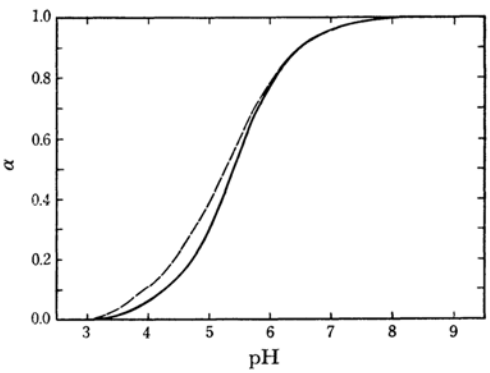

Fig. 2. Titration curve of copoly(L-Glu, L-Trp 9 : 1) (——) and poly-L-glutamic acid (------) at 20°C.
Sample: 10 m*l* of 0.1M NaCl aqueous solution containing 20 mg of substance
Titor: 0.1N NaOH aqueous solution

*3 The data is shown in the catalogue of Pharmacia Fine Chemicals, Uppsala, Sweden.

Titration. The titration curves of the tryptophan copolymer and poly-L-glutamic acid are shown in Fig. 2. The apparent pK values of both polymers were 5.34 and 5.26 respectively in 0.1M sodium chloride.

Optical Rotatory Dispersion. Figure 3 shows

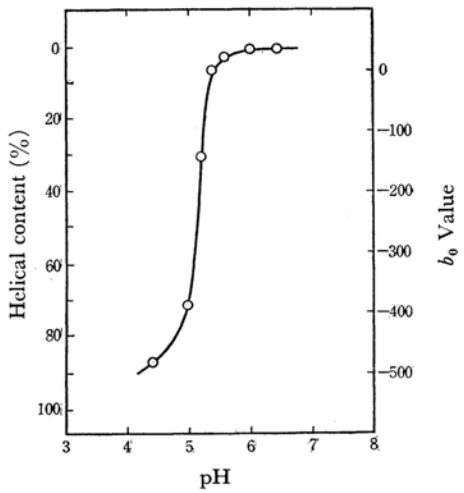

Fig. 3. Relationship between pH and helical content of copoly(L-Glu, L-Trp 9 : 1). b_0 Value are calculated from the ORD measurement in 0.1M phosphate buffer at 25°C.

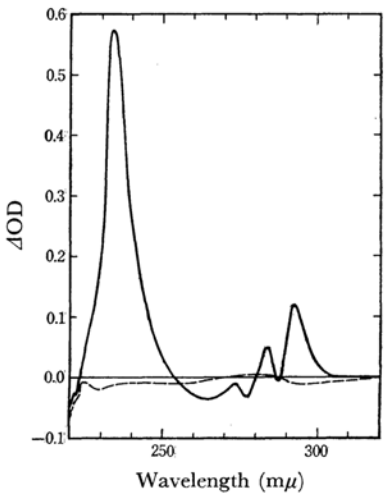

Fig. 4. Ultraviolet difference spectra of copoly(L-Glu, L-Trp 9 : 1) (——) and L-tryptophan (------) in 0.1M phosphate buffer.
Reference: pH 7.60 Sample: pH 4.68
Optical densities at 280 mμ of both substances are 1.5 at pH 7.60 and 25°C.

19) C. E. Dalgliesh, *J. Chem. Soc.*, **1952**, 137.
20) A. Previero, M.-A. Coletti-Previero and P. Jollès, *J. Mol. Biol.*, **24**, 261 (1967).

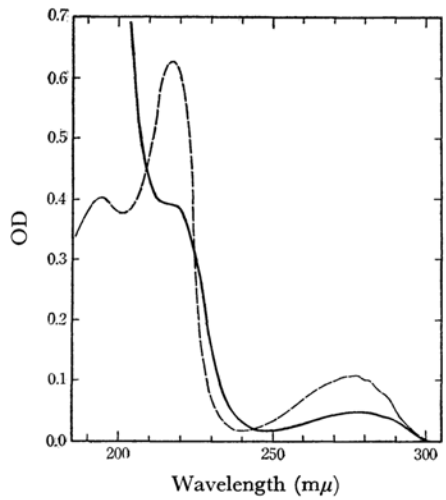

Fig. 5. Ultraviolet absorption spectra of copoly(L-Glu, L-Trp 9 : 1) (——) and L-tryptophan (------) in 0.1M phosphate buffer at pH 7.60 and 25°C.

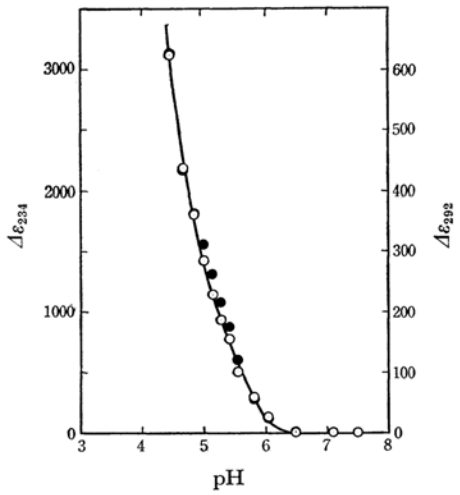

Fig. 6. Relationship between pH and difference absorption at 234 mμ (○) and 292 mμ (●) of copoly-(L-Glu, L-Trp 9 : 1).

the relation between the pH and the helical content of the copolymer. The inflection point of the curve was at pH 5.2, and a helical content of 0% was shown above pH 5.5, where the copolymer was in the random state.

Difference Spectrum and Absorption Spectrum. The difference spectrum of the tryptophan copolymer and that of tryptophan itself are shown in Fig. 4. The difference spectrum of the tryptophan copolymer at pH 4.68 *vs.* pH 7.60 (shown by the solid line in Fig. 4) shows two specific bands, though that of tryptophan itself under the same conditions (shown by the dashed line) has no detectable absorption. The absorption spectrum of the tryptophan copolymer and that of tryptophan

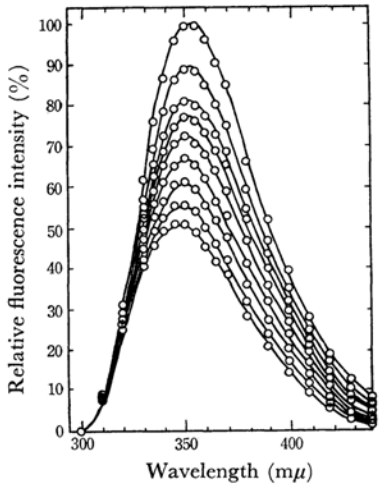

Fig. 7. Fluorescence emission spectra of copoly(L-Glu, L-Trp 9 : 1) in 0.1M phosphate buffer at various pH, pH 4.95—pH 7.40, at 25°C.
Exciting wavelength is 280 mμ.
Optical density at 280 mμ is 1.5 at pH 7.40.

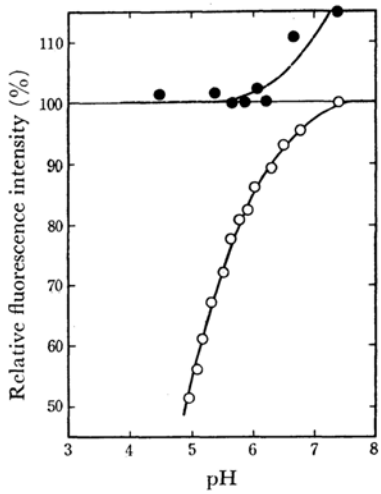

Fig. 8. Relationship between pH and the relative fluorescence intensity of copoly(L-Glu, L-Trp 9 : 1) (—○—) and L-tryptophan (--●--).

itself are shown in Fig. 5. The absorption shoulder at 220 mμ of the copolymer seems to correspond with the absorption peak at 218 mμ (ε=35000) of tryptophan.

The relationships between the pH and the difference absorption at 234 and 292 mμ respectively are shown in Fig. 6.

Fluorescence Spectrum. The fluorescence spectra of copolymers excited with a 280 mμ wavelength at various pH values are shown in Fig. 7. The fluorescence intensities of the copolymer and tryptophan at various pH values are shown in Fig. 8, in which the intensity at pH 7.40 is put as 100%. The pH-dependence of the

relative fluorescence intensity of the copolymer is quite different from that of tryptophan itself.

Discussion

The peaks at 273, 283, and 292 mμ of the difference spectrum at pH 4.68 *vs.* pH 7.60 in Fig. 4 seem to be due to the red shift ($\pi \rightarrow \pi^*$ transition) of the ordinary absorption band in the 250—300 mμ region on the tryptophanyl residue. Donovan *et al.*[21] reported that, in the difference spectrum of tryptophan at pH 1.7, 2.6, 6.4, and 8.7 *vs.* pH 1.2 at a high concentration ($OD_{280}=6$), the peak at 293 mμ decreased with a lowering of the pH; this suggested a blue shift of the absorption band around 280 mμ with a lowering of the pH. On the contrary, in the difference spectrum of the tryptophan copolymer at the low concentration of $OD_{280}=1.5$, the peak at 292 mμ increased with a lowering of the pH and showed a red shift of the absorption band around 280 mμ based on the tryptophanyl residue.

As is shown by the dashed line in Fig. 4, such a specific difference spectrum of tryptophan itself as was shown by Donovan *et al.*[21] was not observed in a dilute solution, in which tryptophan was dissolved at the same concentration as the tryptophanyl residue in the copolymer ($OD_{280}=1.5$). The difference spectrum of tryptophan itself[21] and that of the tryptophan copolymer were reversed in sign; also, the absolute value of the latter was much larger than that of the former. This might show that the behavior of the tryptophanyl residue in the copolymer is quite different from that of tryptophan itself upon a change in the pH in a solution.

The absorption peak of tryptophan itself at 218 mμ seems to correspond to the characteristic absorption shoulder in the 220 mμ region of the tryptophanyl residue in the copolymer, as is shown in Fig. 5; the red shift of the latter seems to give a peak at 234 mμ in the difference spectrum of the copolymer in Fig. 4. Therefore, from these results, it may be concluded that the sharp and large peak at 234 mμ in the difference spectrum of the copolymer was caused by the red shift ($\pi \rightarrow \pi^*$ transition) of the ultraviolet absorption band in the 220 mμ region of the tryptophanyl residue in the copolymer.

On the other hand, it has been explained that a large peak at 230—235 mμ in the difference spectrum between native and denaturated proteins, such as bovine albumin (234 mμ),[6] papain (235 mμ),[7] ovalbumin (232 mμ),[7] and lysozyme (231 mμ),[7] would be due to the hyperchromisity ($n \rightarrow \pi^*$ transition) of the amide group in the peptide bond, for in the case of poly-L-glutamic acid the hyperchromisity of the amide group was observed at 222 mμ in the difference spectrum between the α-helix and the random coil.[22] However, the difference spectrum at 230—235 mμ in proteins seems to be different from that of poly-L-glutamic acid at 222 mμ, since it is too big a shift to have the identical source ($n \rightarrow \pi^*$ transition). The hyperchromisity at 222 mμ due to the $n \rightarrow \pi^*$ transition of the amide group, as reported with poly-L-glutamic acid, was undetectable with the tryptophan copolymer (Fig. 4), for the intensity of transmitted light below 225 mμ was extremely weakened because of the high optical density of the tryptophanyl residue. The difference spectra of proteins are quite similar to that of the tryptophan copolymer (Fig. 4). Therefore, the difference absorptions at 234 mμ observed with the tryptophan copolymer and at 230—235 mμ with proteins must be due to the red shift ($\pi \rightarrow \pi^*$ transition) of the far-ultraviolet absorption band of the tryptophanyl residue, and not to the $n \rightarrow \pi^*$ transition of the amide group in peptide bond.

When we compare Fig. 6 with Fig. 3, it is obvious that the peaks of the difference absorption at both 234 mμ and 292 mμ vary proportionally to the helical content of the copolymer and that these difference absorptions are closely related to the secondary structure of polypeptides. Also, the difference absorption at 234 mμ in the tryptophan copolymer was completely proportional to that at 292 mμ; the former was five times of the latter. Therefore, it would be more accurate to confirm the conformation of a tryptophan copolymer or a tryptophan-containing protein by the difference absorption at 324 mμ instead of that at 292 mμ.

The fluorescence emission spectra of the tryptophan copolymer at various pH values (Fig. 7) are similar to that of the tyrosine copolymer, copoly(Glu,Tyr 95 : 5), reported by Pesce and Fasman.[23]

In view of the titration curve of the tryptophan copolymer (Fig. 2), the relative intensity of fluorescence of the tryptophanyl residue at 350 mμ against the pH (Fig. 8) is in good agreement with the extent of the protonation of the carboxyl group in the copolymer against the pH. The unionized carboxyl group seems to act as a quencher of the fluorescence of the tryptophanyl residue. These results support Fasman's suggestion[4] that the fluorescence intensity of the tryptophanyl residue depends on the degree of ionization of the carboxyl group in copoly(Glu,Trp 99 : 1).

The spectrophotometric behavior of the tryptophanyl residue in polypeptide is quite different from that of tryptophan itself. The appearance of

21) J. W. Donovan, M. Laskowski, Jr., and H. A. Scheraga, *J. Am. Chem. Soc.*, **83**, 2686 (1961).

22) K. Imahori and J. Tanaka, *J. Mol. Biol.*, **1**, 359 (1959).

23) A. Pesce and G. D. Fasman, *J. Am. Chem. Soc.*, **86**, 5669 (1964).

the difference spectrum seems to correspond closely to the formation of the α-helix in the polypeptide molecule, and the protonation of carboxyl groups in polypeptide seems to induce the quenching of the fluorescence of the tryptophanyl residue. It is not yet clear whether the spectrophotometrical results are related to the catalytic activity[5] of the tryptophan copolymer.

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