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Fluorescence Studies on Poly-α-amino Acids: Models of Protein Conformation

IV. COPOLYMERS OF TRYPTOPHAN WITH GLUTAMIC ACID OR LYSINE*

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SUMMARY

From this study with a tryptophan model system, several conclusions may be drawn. (*a*) Both —COOH and —NH_2 are strong quenchers of tryptophan fluorescence. (*b*) Interaction of —COOH with tryptophan has a hypochromic effect on the tryptophanyl absorption which is similar to that noted in proteins. (*c*) There is little if any effect of α-helix formation on tryptophanyl fluorescence. (*d*) Tryptophanyl fluorescence is constant throughout the pH range 2.0 to 12.5. (*e*) Block copolymers containing tryptophan sequences have indole-indole residue interactions resulting in absorption and fluorescence emission shifts and low quantum yields (0.04). (*f*) The quantum yield of both (Glu:Trp) and (Lys:Trp) random copolymers lies between 0.121 and 0.145, and the emission spectra are identical with that of tryptophan in aqueous solution.

The fluorescence excitation and emission spectra of proteins that contain aromatic residues are predominantly those of the tryptophan residues (2). The presence of tyrosyl and phenylalanyl fluorescence in such proteins may be considered negligible. The fluorescence difference found for a protein in its native and denatured state may therefore be ascribed to differences in the tryptophanyl environment. This fluorescence difference has been used as an indication of protein structural changes (3–7). The quantum yield of tryptophan fluorescence varies about 10-fold in the proteins thus far studied (2). Little information is available which elucidates the factors which are responsible for the large variation in fluorescence yield among proteins and which cause altered fluorescence emission when proteins undergo conformational changes. Studies with model systems containing tryptophan have thus far been limited to small peptides (6, 7). Synthetic polypeptides containing tryptophan offer a model system suitable for the study of factors influencing tryptophan fluorescence. In the present work copoly (lysyl:tryptophanyl) and copoly (glutamyl:tryptophanyl) were investigated in a manner similar to that reported for tyrosyl copolymers (8–10). Conformational effects were investigated by use of the random chain ⟶ helix transitions of copoly (L-glutamyl:L-tryptophanyl) or copoly (L-lysyl:L-tryptophanyl) that are induced by the appropriate pH changes. Poly-L-lysine becomes helical over the pH range 8 to 11 (11). Poly-L-glutamic acid undergoes this transition over the pH range 7.0 to 4.0 (12–14). As these new copolymers have small amounts of tryptophan, it is assumed that their helix ⟶ random chain transitions are identical with those of the homopolymers. The helical conformation of the copoly (γ-benzyl-L-glutamate:L-tryptophan) in nonaqueous media has been established previously (15).

To verify this for aqueous solution, one of the copolymers was studied by optical rotatory dispersion. The random sequence copolymer of L-lysine and L-tryptophan, Copolymer V, exists in the helical conformation in aqueous media at pH 11.86, 0.2 M NaCl, as a b_0 value of −462, and $[m']_{233}$ of −9310 was found.[1] This value agrees well with previously published values of poly-L-lysine under similar conditions (11) and unpublished work of this laboratory.

The polymers synthesized had sequences of two types, random or block (Table I). The random sequence, *e.g.* copoly (L-Lys:L-Trp), was made by polymerizing a mixture of the appropriate *N*-carboxy-α-amino acid anhydrides. The block sequence, *e.g.* copoly (L-Lys) (L-Trp) (L-Lys), was made by first forming a glutamyl or lysyl chain, then adding a short sequence of tryptophan residues, and finally adding another sequence of lysyl or glutamyl residues, by the addition of the desired NCA[2] by

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[1] Polymer concentrations of 6.5 mg per ml were used. The wave length range of 455 to 294 mμ was studied in a 1-cm path length cell, and the spectral region of 286 to 222 mμ was studied in a 1-mm path length cell. The copolymer at pH 4.16, 0.2 M NaCl, had a $b_0 = 0$ and $[m']_{233}$ of −1900, indicating the random conformation.

[2] The abbreviation used is: NCA, *N*-carboxy-α-amino acid anhydride or anhydrides.

TABLE I

Estimated molecular weights of tryptophan copolymers and conditions of preparation

Polymer	Sample	Copolymer composition	Ratio of anhydride to initiator[a]	$[\eta]^b$	Degree of polymerization	Weight average molecular weight
I	GF-11-108-9	(L-Glu:L-Trp) (99:1)	50[c]	0.80[d]	529[e]	68,500[e]
II	GF-11-360-9	(DL-Glu:L-Trp) (99:1)	50[c]	0.203[d]	147[e]	19,050[e]
III	GF-11-366-28	(L-Glu) (L-Trp) (L-Glu) (95:5:95)	50[c]	0.74[d]	495[e]	64,000[e]
IV	GF-11-440-19	(DL-Glu) (L-Trp) (99:1)	50[c]	0.180[d]	114[e]	14,800[e]
V	GF-11-253-25	(L-Lys:L-Trp) (99:1)	25[f,g]	0.985[h]	527[i]	68,200[i]
VI	GF-11-373-27	(DL-Lys:L-Trp) (99:1)	25[g]	0.30[h]	185[i]	14,050[i]
VII	GF-11-438-23	(L-Lys) (L-Trp) (L-Lys) (99:1:99)	25[g]	1.64[h]	930[i]	120,000[i]
VIII	GF-11-437-27	(DL-Lys) (L-Trp) (99:1)	25[g]	0.38[h]	163[i]	21,000[i]
IX	GF-11-167-25	(L-Lys:L-Trp) (95:5)	25[g]	0.91[h]	465[i]	60,000[i]

[a] Concentration, 1%, initiated with 0.345 N $NaOCH_3$.
[b] Intrinsic viscosity of unblocked polymers.
[c] Polymerization in benzene.
[d] At pH 7.0, in 0.2 M NaCl.
[e] Estimated from the molecular weight calibration of Idelson and Blout (16).
[f] Initiated with 0.302 N hexylamine.
[g] In dioxane.
[h] At pH 4.0, in 1.0 M NaCl.
[i] Estimated from the molecular weight calibration of Applequist (17).

the method previously described (18). Copolymers were also made of DL-amino acids, *e.g.* copoly (DL-Lys:L-Trp). These copolymers are essentially random chains throughout the pH range studied. Such polymers have been shown to have a maximum helical content of 25% in the un-ionized form (18). Amino acid analyses were not performed as the exact composition of the copolymers is not of significance in this study.

EXPERIMENTAL PROCEDURE

L-Tryptophan, Grade A, was obtained from Calbiochem, and *N*-acetyl-L-tryptophan ethyl ester (H1537) was obtained from Mann. Dioxane was purified by the Fieser procedure (19); dimethyl sulfoxide was obtained from Crown Zellerbach and purified by distillation from KOH under reduced pressure (2 to 3 mm of Hg), b.p. 38°. Dialysis was performed with Visking tubing bags, size 32.

Absorbance Measurements—A Cary model 14 recording spectrophotometer was used to determine the optical densities of the solutions. Solutions of optical densities lower than 0.1, at 280 mμ, in a 1-cm cell were measured in a cell with a 5-cm path length.

Fluorescence Measurements—An Aminco-Bowman or Zeiss spectrofluorometer was used for fluorescence measurements in the manner previously described (10).

Cells—The fluorescence cells, 10- and 2-mm path lengths, were purchased from Pyrocell. The absorption cells, 10- and 50-mm path lengths, were purchased from Quaracell Products.

Solutions for Fluorescence Measurements—Solutions were made up in a manner similar to that previously described (10).

Preparation of Polymers

Random Copoly (L-Glu:L-Trp) (99:1)—γ-Benzyl-L-glutamate-NCA (20) (2.47 g, 9.42×10^{-3} mole) and L-tryptophan NCA (15) (0.022 g, 9.51×10^{-5} mole) were dissolved in dry distilled benzene (250 ml) by warming. The solution was cooled to room temperature, the polymerization was initiated by the addition of $NaOCH_3$ (0.51 ml of 0.373 N; ratio of anhydride to initiator, 50) with stirring, and the solution was allowed to stand for 5 days. A 15-ml aliquot of the slightly viscous solution was removed and slowly added to 5 times its volume of *n*-hexane, with stirring. The polymer, which separated out as a fibrous material, was dried in a vacuum (1 mm of Hg) at 80° for 2 hours; η_{sp}/c = 0.745 (0.2% in dichloroacetic acid). Anhydrous hydrogen chloride was bubbled through the remaining solution for 15 min. Precautions were taken to exclude moisture during this procedure and the following one. Anhydrous hydrogen bromide was then bubbled through the solution for 30 min, and the polymer began precipitating out. The solution was allowed to stand overnight, the supernatant was removed by decantation, and the excess solvent and HBr were removed by suction on a water aspirator.

The polymer was extracted for 2 hours with anhydrous ether (previously treated with $FeSO_4$ and distilled) while the ether was repeatedly changed. The polymer was dried in a vacuum (1 mm of Hg) at 40° for 2 hours, yielding a fine white powder, 1.02 g; yield, 86%. $[\eta]^{0.2\ M\ NaCl}_{pH\ 7.05} = 0.8$.

Random Copoly (DL-Glu:L-Trp) (99:1)—This copolymer was prepared in the same manner as copoly (L-Glu:L-Trp) (99:1), above (see Table I).

Block Copoly (DL-Glu):(L-Trp) (99:1)—γ-Benzyl-DL-glutamate NCA[3] (1.0 g, 3.798×10^{-3} mole) was dissolved in 100 ml of dry benzene (distilled from CaH_2) by warming. The solution was cooled to room temperature and the polymerization was initiated by adding $NaOCH_3$ (0.22 ml, 0.345 N; ratio of anhydride to initiator, 50) with stirring. After 24 hours, L-tryptophan NCA (8.9 mg, 3.865×10^{-4} mole), dissolved in dry dioxane (1 ml), was added with stirring to the polymerization mixture. After 24 hours, a small aliquot was removed, and the polymer was recovered as in the above preparation. The specific viscosity of this polymer was 0.14 (0.2% in dichloroacetic acid). Anhydrous hydrogen chloride was bubbled through the viscous solution for 10 min, with moisture being carefully excluded. Hydrogen bromide was then bubbled through the solution for 15 min, and the slightly colored polymer began to precipitate out. The solution was allowed to stand overnight. The excess

[3] Prepared as for the γ-benzyl-L-glutamate NCA.

HBr and the solvent were removed by suction on a water aspirator. The polymer was dissolved in a saturated $NaHCO_3$ solution (30 ml) at pH 12, adjusted with 2 N NaOH. The solution was extracted twice with anhydrous ether (previously treated with $FeSO_4$ and distilled), the pH was brought to 7 with 1 N HCl, and the solution was dialyzed against H_2O at pH 7. The solution was lyophilized, yielding a white spongy material. The polymer was dried in a vacuum (1 mm of Hg) at 90° for 2 hours. The weight of the resulting powder was 0.44 g; yield, 84%. $[\eta]^{0.2\ M\ NaCl}_{pH\ 7.2} = 0.18$.

Tri-Block Copoly (L-Glu):(L-Trp):(L-Glu) (95:5:95)—γ-Benzyl-L-glutamate NCA (1 g, 0.38 × 10^{-2} mole) was dissolved in dry benzene (100 ml, 1% solution). The polymerization was initiated by the addition of $NaOCH_3$ (10.22 ml, 0.345 N; ratio of anhydride to initator, 50). After 24 hours, L-tryptophan NCA (0.046 g, 0.2 × 10^{-3} mole), dissolved in dry dioxane (5 ml), was added to the polymerization mixture. After 24 hours, γ-benzyl-L-glutamate NCA (1 g, 0.38 × 10^{-2} mole) dissolved in dry benzene (100 ml) was added to the viscous solution, and the polymerization was allowed to proceed for 24 hours. A small aliquot was removed, and the polymer was recovered as in the above preparations. The specific viscosity of this block polymer was 0.76 (concentration, 0.2% in dichloroacetic acid). The polymer was debenzylated and treated in the same manner as block copoly (L-Glu):(L-Trp) (99:1) above. The weight of the powder was 0.79 g; yield, 69.5%. $[\eta]^{0.2\ M\ NaCl}_{pH\ 7.2} = 0.77$.

Random Copoly (L-Lys:L-Trp) (99:1)—ε-Carbobenzyloxy-L-lysine NCA (21) (0.96 g, 3.14 × 10^{-3} mole) and L-tryptophan NCA (7.3 mg, 3.2 × 10^{-5} mole) were dissolved in dry dioxane (99.7 ml, 1% solution). *n*-Hexylamine (0.42 ml of a benzene solution, 0.302 N; ratio of anhydride to initiator, 25) was added with stirring to this mixture to initiate the polymerization, and the solution was allowed to stand for 1 day. Chloroform (reagent grade, 275 ml) was added to dilute the viscous opalescent solution, and anhydrous hydrogen chloride was bubbled through the solution for 30 min, with moisture being carefully excluded. Anhydrous hydrogen bromide was then bubbled through the solution for 45 min; the polymer began to precipitate out approximately 4 min after the HBr treatment was started. The reaction mixture was stirred for 90 min, nitrogen was bubbled through to remove excess HBr (90 min), and the supernatant was decanted.

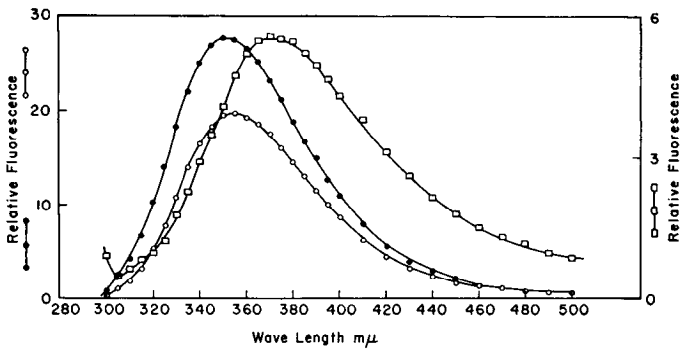

FIG. 1. Fluorescence emission spectra. ●——●, L-tryptophan in 0.01 M PO_4 buffer, pH 7.51; ○——○, (L-Glu:L-Trp) (99:1), pH 7.5, in 0.2 M NaCl; □——□, block (L-Glu) (L-Trp) (L-Glu) (95:5:95), pH 7.5, in 0.2 M NaCl; 1-cm path length; exciting wave length was 280 mμ; optical density at 280 mμ (1-cm path length) was 0.06. The three spectra have been normalized to the same optical density.

The polymer was dissolved in a saturated $NaHCO_3$ solution (40 ml) by stirring. The aqueous solution was adjusted to pH 3 with 3 N HCl; the clear solution was then extracted with anhydrous ether (previously treated with $FeSO_4$ and distilled) and dialyzed against 6 liters of 0.01 N HCl (Visking dialyzing tubing bag, size 32). The outer 0.01 N HCl solution was changed four times. The clear solution was lyophilized, yielding a white spongy polymer. The polymer was dried in a vacuum at 40° for 2 hours; weight, 0.35 g; yield, 67%. $[\eta]^{1\ M\ NaCl}_{pH\ 4.0} = 0.985$.

Random Copoly (L-Lys:L-Trp) (95:5)—This polymer was prepared as the above polymer, with mole ratios of 95:5, rather than 99:1. The initiator used was sodium methoxide (ratio of anhydride to initiator, 25) in place of *n*-hexylamine. The reaction mixture was treated in the same manner as above.

Random Copoly (DL-Lys:L-Trp) (99:1)—This copolymer was prepared in the same manner as copoly (L-Lys:L-Trp) (99:1), above. DL-Lysine-NCA was prepared as the L-lysine-NCA (21).

Tri-Block Copoly (L-Lys):(L-Trp):(L-Lys) (99:1:99)—ε-Carbobenzyloxy-L-lysine NCA (21) (1.0 g, 3.27 × 10^{-3} mole) was dissolved in dry dioxane (100 ml, 1% solution). The polymerization was initiated by the addition of $NaOCH_3$ (0.379 ml, 0.345 N $NaOCH_3$; ratio of anhydride to initiator, 25). After 24 hours L-tryptophan NCA (7.7 mg, 3.34 × 10^{-5} mole), dissolved in 1 ml of dioxane, was added to the polymerization mixture. After 24 hours, ε-carbobenzyloxy-L-lysine NCA (1 g, 3.27 × 10^{-3} mole) dissolved in dry dioxane (100 ml) was added to the viscous solution, and the polymerization was allowed to proceed for 24 hours. A small aliquot was removed, and the polymer was recovered as in the above preparations. The specific viscosity of this block polymer was 1.96 (0.2% in dichloroacetic acid).

The polymer was debenzylated and treated in the same manner as copoly (DL-Lys:L-Trp) above. The weight of the powder was 0.893 g; yield, 76.5%. $[\eta]^{1\ M\ NaCl}_{pH\ 4.0} = 1.64$.

Block Copoly (DL-Lys):(L-Trp) (99:1)—This copolymer was prepared in the same manner as the block copoly (L-Lys:L-Trp) above.

In Table I are listed the various polymers prepared, the conditions of the polymerization, the mole ratios of the anhydrides used, the intrinsic viscosities, and estimated weight average molecular weights.

The approximate weight average molecular weights of the copolymers used in this study were determined from viscometry measurements. The copolymers containing L-amino acids were of a higher molecular weight than those containing DL-amino acids. This is in accordance with the results of other studies which have shown that DL polymers are of lower molecular weight than L polymers when formed under the same anhydride to initiator ratios and polymerization conditions.

RESULTS

Considerable degradation of tryptophan was originally experienced during the preparation of the tryptophan copolymers; however, this was circumvented by the experimental procedures developed. The criteria used to estimate whether tryptophan was intact in these polymers were quantum yield, absorption spectra, and color. The emission spectra of random sequence copoly (L- or DL-Glu:L-Trp) (Polymers I and II) or copoly (L-Lys:L-Trp) (Polymer V) copolymers are nearly identical with that of L-tryptophan (Fig. 1). At pH 7.5 in 0.2 M NaCl, copoly (L-Glu:L-Trp) (99:1) (I) and copoly (DL-Glu:L-Trp) (99:1) (II)

TABLE II

Quantum yields of L-tryptophan copolymers[a]

Polymer	Copolymer	pH[b]	Quantum yield
I	(L-Glu:L-Trp) (99:1)[c]	7.5	0.121
		4.3	0.064
II	(DL:Glu:L-Trp) (99:1)	7.5	0.145
		4.3	0.065
III	(L-Glu) (L-Trp) (L-Glu) (95:5:95)[d]	7.5	0.042
		4.5	0.036
IV	(DL-Glu) (L-Trp) (99:1)	7.5	0.040
		4.3	0.040
V	(L-Lys:L-Trp) (99:1)	7.5	0.128
		10.6	0.071
VI	(DL-Lys:L-Trp) (99:1)	7.5	0.128
		10.6	0.069
VII	(L-Lys) (L-Trp) (L-Lys) (99:1:99)	7.5	0.030
		10.6	0.030
VIII	(DL-Lys) (L-Trp) (99:1)	7.5	0.040
		10.6	0.040

[a] Fluorescence was measured in quartz cells of 1-cm path length; optical density of the solution was 0.06 to 0.10 at 280 mμ for 1-cm path length (measured in a 5-cm path length cell). Exciting wave length was 280 mμ. Maximum emission wave length was 350 mμ.

[b] In 0.2 M NaCl.

[c] Random copolymer nomenclature.

[d] Block copolymer nomenclature.

had quantum yields of about 0.12 to 0.14 (Table II). Under similar conditions copoly (L-Lys:L-Trp) (99:1) (V) had a quantum yield of 0.13. When the first copoly (DL-Glu:L-Trp) (IV) was synthesized, the quantum yield was 0.08, and a further decrease, to 0.04, in the quantum yield of the copolymer occurred with time. Copolymers with these low quantum yields (0.08 or less) usually were slightly colored. In addition, the absorption spectra of these degraded polymers were different in the 250 to 280 mμ region. The ratio of 280 mμ absorption to that at 260 mμ was much less than that found for tryptophan or proteins containing tryptophan. The low quantum yield was probably due to degradation of the indole nucleus and was attributed to incomplete neutralization of the HBr used as an unblocking reagent during polymer synthesis.

The effect of pH on the quantum emission of copoly (L-Lys:L-Trp) (99:1) (V) and copoly (L-Glu:L-Trp) (99:1) (I) was first determined in the absence of a conformational change (Fig. 2). For copoly (L-Glu:L-Trp) (I) this condition prevails above pH 7, while for copoly (L-Lys:L-Trp) (V) this was below pH 8.0. The quantum yield is constant over a broad pH range, pH 2 to 12.5, and drops above and below this range.

Glutamic Acid:Tryptophan Copolymers: Copoly (Glu: Trp)—The random chain → helix transition of copoly (L-Glu:L-Trp) (99:1) (I) was used to study the effect of a conformational change upon tryptophanyl fluorescence emission. The quantum yield of the polymer was measured at pH 7.5, where the molecule was in the random chain form, and at pH 4.3, where the molecule was in a helical conformation. Similar fluorescence measurements were made on copoly (DL-Glu:L-Trp) (99:1) (II) which was in the random chain conformation at both pH values. The fluorescence was quenched almost 2-fold in both copoly (L-Glu:L-Trp) (99:1) (I) and copoly (DL-Glu:L-Trp) (99:1) (II) at pH 4 compared to pH 7.5 (Table II). The fluorescence emission intensities of the copoly (DL-Glu:L-Trp) (99:1) (II) and copoly (L-Glu:L-Trp) (99:1) (I) at pH 4.3 were almost identical, although the two polymers exist in different conformations.

The conformational effect may be obscured at pH 4 because the polymers contain both ionized and un-ionized carboxyls. Therefore, another attempt to study the effect of conformation on tryptophanyl fluorescence was made by completely converting the polymers to the un-ionized form. Since it was not possible to attain this state in aqueous solution because the polymers became insoluble, a mixed solvent consisting of dimethyl sulfoxide and water, 1:1 (v/v), was used. The apparent pH of the mixed solvent reflected the aqueous component, *e.g.* 0.03 N HCl or 0.002 M phosphate (pH 7.0) (22). It was thus possible to compare the ionized polymer to its un-ionized form. The fluorescence yield of the polymers was higher in the mixed solvent than in water. The ratio of fluorescence of the un-ionized (helical) to ionized (random chain) forms of the copoly (L-Glu:L-Trp) (99:1) (I) was 0.55 (Table III). A comparable fluorescence ratio was also found for the un-ionized (random chain) to ionized (random chain) forms of copoly (DL-Glu:L-Trp) (99:1) (II).

The ultraviolet absorption spectra of the random sequence copoly (L-Glu:L-Trp) (I) is similar (except for a small red shift)

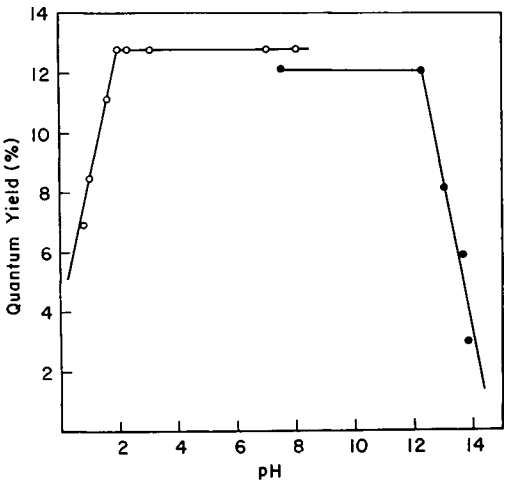

FIG. 2. Quantum yield as a function of pH. ○——○, (L-Lys:L-Trp) (99:1); ●——●, (L-Glu:L-Trp) (99:1). In 0.2 M NaCl.

TABLE III

Relative fluorescence of copoly (glutamic acid:tryptophan) (99:1) in dimethyl sulfoxide-H_2O (1:1)[a]

Polymer	Copolymer	pH	Conformation	Fluorescence ratio[b]
I	(L-Glu:L-Trp)	Acid[c]	Helix	0.55
I	(L-Glu:L-Trp)	Neutral[d]	Random	
II	(DL-Glu:L-Trp)	Acid[c]	Random	0.53
II	(DL-Glu:L-Trp)	Neutral[d]	Random	

[a] Fluorescence was measured in quartz cells of 1.0-cm path length. Excitation wave length was 280 mμ; maximum emission wave length was 350 mμ; optical density of the solution at 280 mμ used was ≃1.0 for 1-cm path length.

[b] Ratio of fluorescence intensity (acid) to fluorescence intensity (neutral).

[c] HCl, 0.03 N.

[d] Phosphate, 0.004 M, pH 7.0 (22).

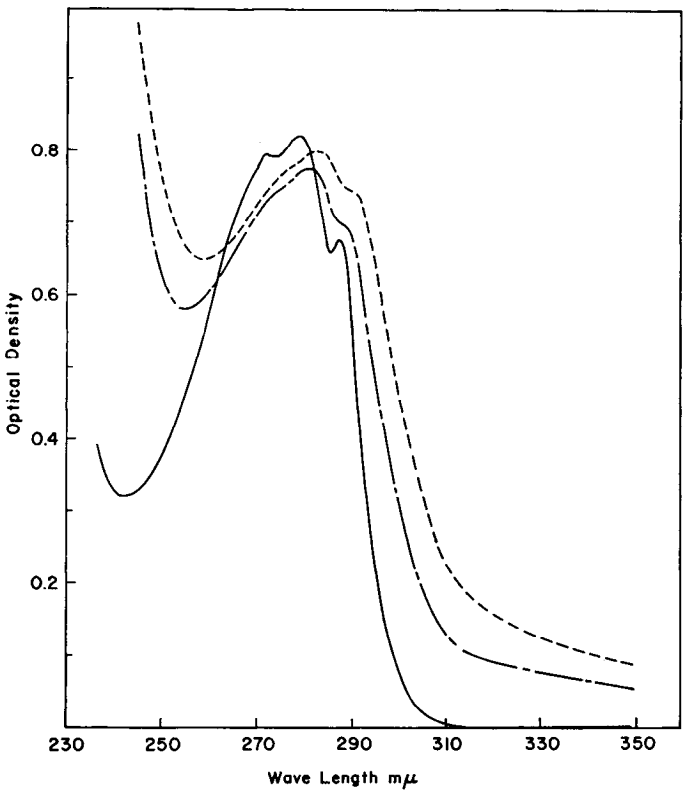

FIG. 3. Absorption spectra. ——, L-Trp in 0.01 M PO_4 buffer (22), pH 7.5; - — -, (L-Glu:L-Trp) (95:5), pH 7.5, in 0.2 M NaCl; – – –, block (L-Glu) (L-Trp) (L-Glu) (95:5:95), pH 7.5, in 0.2 M NaCl. Optical densities measured in a quartz cell of 5-cm path length. Concentrations of tryptophanyl residues are approximately equal.

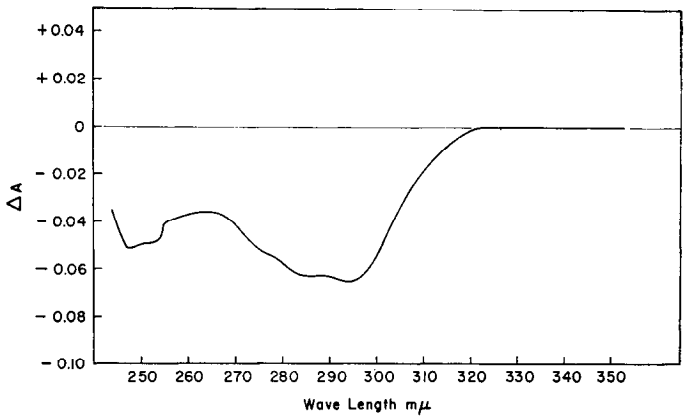

FIG. 4. Ultraviolet difference spectrum of (L-Glu:L-Trp) (99:1) in 0.2 M NaCl. Reference, pH 4.6. Sample, pH 7.3. Optical densities were measured in a quartz cell of 5-cm path length. Optical density at 280 mμ and pH 7.5 was 1.3.

to L-tryptophan in the 250 to 290 mμ region (Fig. 3). A change in the absorbance of copoly (L-Glu:L-Trp) (99:1) (I) was observed between pH 7.3 and 4.6 in aqueous solution. The absorption at 280 mμ was 2.8% lower when the polymer was in the un-ionized form (Fig. 4). The difference spectrum exhibits a minimum in the 290 mμ region, and a maximum in the 260 mμ region. The curve below 250 mμ is in question because of the stray light present at extremely high optical densities. A similar hypochromicity was observed for copoly (DL-Glu:L-Trp) (99:1) (II). However, a block copoly (L-Glu) (L-Trp) (L-Glu) (95:5:95) (III) did not display hypochromicity under the same conditions. An ultraviolet difference spectrum, ΔA_{280}, with respect to pH of the copoly (L-Glu:L-Trp) (99:1) (I), is seen in Fig. 5. The difference spectrum was greatest at pH 4.3. At the lowest pH measured, pH 4.0, the polymer started to precipitate out of solution, and opalescence was observed. This might explain the slight increase in absorbance at pH 4.0. The spectral change was tentatively thought to be caused by an interaction of the indole nucleus with the COOH moiety of the glutamic acid residues. To test this hypothesis further, copolymers were exposed to a high concentration of carboxyl groups. When copoly (L-Lys:L-Trp) (99:1) (V) was placed in 1 M acetic acid, pH 3.06, the absorbance of the tryptophan was lowered about 5% relative to similar solutions minus the acetic acid.

Lysyl:Tryptophanyl Copolymers: Copoly (Lys:Trp)—The helix → random chain transition of the L-lysyl copolymer occurs in the pH range 9.5 to 10.5. The ionized (random chain) form at pH 7.5 had a fluorescence yield over 2-fold greater than the partially ionized (helical) form at pH 10.8 (Table IV).

As illustrated in Fig. 2 and Table V, the fluorescence of L-Trp in Copolymer V was not quenched until OH^- concentrations exceeded 3×10^{-2} M (pH 12.5). Thus OH^- does not have any

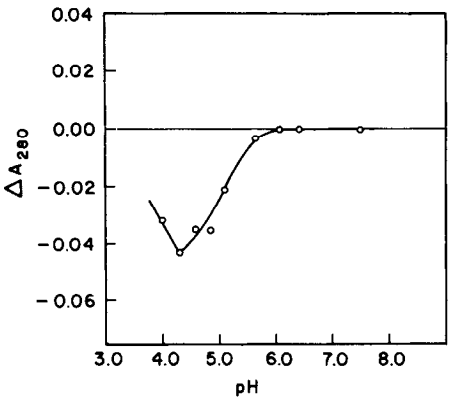

FIG. 5. Ultraviolet difference spectrum at 280 mμ, as a function of pH. (L-Glu:L-Trp) (99:1) in 0.2 M NaCl. Reference sample, pH 7.50. Sample was titrated from pH 7.5 to 3.99 by the addition of HCl; measured in a quartz cell of 5-cm path length. Optical density at 280 mμ and pH 7.5 was 1.07.

TABLE IV

Fluorescence quenching of random copolymers of L-lysine and L-tryptophan as function of pH in aqueous solution

pH[a]	Conformation	Relative fluorescence[b] [c]
7.5[d]	Random coil	1.00
10.0[d]	Partially helical	0.72
10.8[e]	Helical	0.46

[a] NaCl, 0.2 M.

[b] Ratio of fluorescence intensity at pH stated to fluorescence intensity at pH 7.5. Exciting wave length was 280 mμ. Maximum emission wave length was 360 mμ. Fluorescence was measured in quartz cells of 1-cm path length.

[c] Optical density of solutions at 280 mμ was 0.04 to 0.06 in 1-cm cell.

[d] (L-Lys:L-Trp) (99:1) (I).

[e] (L-Lys:L-Trp) (95:5) (IX).

effect upon tryptophanyl fluorescence in the pH range of the helix → random chain transition of the lysyl polymer. However during this transition —NH_2 is formed, and it may be responsible for the quenching observed.

A water-soluble amine was studied to determine the effect of —NH_2 upon the fluorescence of copoly (L-Glu:Trp) (99:1) (I). Ethanolamine was added to aqueous solutions of this polymer under conditions where the amine group was ionized and un-ionized. Ethanolamine quenches the tryptophan fluorescence above pH 10, where the amine moiety was un-ionized (pK_a, 9.50) (23).

Another model compound, *N*-acetyltryptophanyl ethyl ester, was selected for the study of the amine quenching of tryptophan fluorescence. The tryptophan derivative had a constant fluorescence yield up to pH 10.5 (Table VI). In alkaline solution the compound hydrolyzed slowly, and extrapolation to zero time was necessary. To study the effect of amine quenching on *N*-acetyltryptophanyl ethyl ester, nonaqueous solvents were selected, because these obviated the effect of hydrolysis of the ester. Varying concentrations of ethanolamine were added to this tryptophanyl derivative in two nonaqueous solvents (Table VI). The amounts added were comparable to those added to the glutamyl:tryptophanyl copolymer. In these experiments, at least 50% quenching of fluorescence ensued upon addition of 2% ethanolamine (Table VI). An even lower fluorescence resulted when the model compound was dissolved in pure ethanolamine. Clearly, it is seen that —NH_2 is an effective quencher of tryptophanyl fluorescence. As the dielectric constant of the medium is known to have an effect on fluorescence (24), these values are included in Table VI. In this system there does not appear to be a correlation between fluorescence and dielectric constant.

Copolymers with Tryptophan Blocks—Polymers with tryptophan block sequences were made with either lysine or glutamic acid to examine the case where the tryptophan residues were all in one consecutive sequence and possibly interacting. An indication of interaction between residues is the shift of the emission maximum of about 20 mμ and the extension of the emission to longer wave lengths (Fig. 1). The quantum yield of these polymers, whether DL- or L-glutamic acid or L-lysine, is very similar in neutral solution (Table II). The quantum yield is about one-fourth (0.03 to 0.04) that of the comparable random polymer. This large decrease in fluorescence yield has also been found for block copolymers of tyrosine (9, 10). The quantum yields of block copoly (Glu) (Trp) (III) and (IV) were unaffected when the pH of the solution was changed from 7 to 4. The conformation of the tryptophan block is also unchanged during this pH alteration. This is in sharp contrast to the 2-fold decrease observed for the random sequence polymers.

The block copoly (L-Glu) (L-Trp) (III) was examined at pH 7.5 and 4.5 for hypochromicity by the same procedure used for the random copolymers. A zero difference spectrum was found. However, when the block copoly (DL-Glu) (L-Trp) (IV) was examined in acidic solution (pH 4.3), it was hypochromic as compared to pH 7.0. The difference was 2.3% at 280 mμ, and was comparable to that found for the random copolymers. Clearly, there must be interactions of the DL copolymer-carboxyl groups with the tryptophanyl residues, while these interactions do not exist in the block copoly (L-Glu) (L-Trp).

The absorption spectra of the block tryptophanyl copolymers are very similar to those of the random copolymers at pH 7.5 (Fig. 3). The block copolymers have a more pronounced absorption tail than the random copolymers. This end absorption decreases slowly to zero between 300 and 500 mμ. The absorption minimum found for L-tryptophan at 242 mμ was shifted to higher wave lengths and was less pronounced in the copolymers.

TABLE V

Amine quenching of fluorescence of copoly (L-glutamic acid: L-tryptophan) (99:1) (V) in random conformation

pH[a]	Ethanolamine	Relative fluorescence[b,c]
	%	
7.5	0	1.00
10.0	0	1.00
12.5	0	1.00
10.5	1	0.92
10.9	10	0.73

[a] NaCl, 0.2 M.

[b] Ratio of fluorescence intensity at pH stated to fluorescence intensity at pH 7.5, measured in 1-cm path length cell. Excitation wave length was 280 mμ. Maximum emission wave length was 350 mμ.

[c] Optical density of solutions at 285 mμ was 0.04 to 0.06, in 1-cm cell.

TABLE VI

Dielectric constant and amine effect on relative fluorescence of N-acetyl-L-tryptophanyl ethyl ester

Dielectric constants: H_2O, 80; ethanolamine, 37.7; ethanol, 24.3; dioxane, 2.21; hexane, 1.89.

Solvent	Ethanolamine	Relative fluorescence[a]
	%	
H_2O, pH 7.5	0	1.00
H_2O, pH 10.5	0	1.00
Dioxane	0	1.80
Dioxane	2[b]	0.86
Hexane	0	0.95
Hexane	2[b]	0.40
Ethanolamine	100	0.22
Ethanol	0	1.50

[a] Ratio of fluorescence intensity in solvent indicated to fluorescence intensity in H_2O, pH 7.5. Fluorescence was measured in a 1-cm path length cell. Optical density of solutions at 280 mμ was 0.06 to 0.08. Excitation wave length was 280 mμ. Emission was read at 350 mμ.

[b] By volume.

DISCUSSION

Glutamic Acid:Tryptophan Copolymers

Helix-Random Chain Transition—For random sequence copoly (L-Glu:L-Trp) there is no conformational effect, due to the random chain ⟶ helix transition, on the fluorescence quantum yield, nor is there any change in the fluorescence spectrum. This is true as both copoly (L-Glu:L-Trp) (I) and copoly (DL-Glu:L-Trp) (II) have the same F ratio under conditions where the L polymer changes conformation, while the DL polymer remains a random chain. This is in marked contrast to copoly (L-Glu:L-Tyr), where 25% of the increase in fluorescence could be ascribed to a helical environment around the tyrosyl residues (9). Hydrogen ion concentration has no effect on tryptophanyl fluorescence over the range of the glutamyl copolymer helix ⟶ random

chain transition (pH 4.0 to 7), although below pH 2 quenching is observed. White (6) reported similar quenching, below pH 3, for L-tryptophan and *N*-acetyl-L-tryptophan. Therefore, the large change in fluorescence yield observed upon lowering the pH is attributed to quenching by —COOH which resulted from neutralizing the negative charge of the glutamyl residues. The fluorescence profile of L-tryptophan with pH also has shown that a decrease in fluorescence occurs below pH 4.0 (6). This decrease in fluorescence could be interpreted as following the appearance of the un-ionized carboxyl group.

Difference Spectrum—The difference spectrum seen in Fig. 4 is distinct from that of tryptophan at pH 2.6 compared to pH 6.4 (25), which was attributed to an inductive effect. It is also dissimilar to that of glycyltryptophan at pH 6.7 compared to pH 3.5 (25). Both of these reported difference spectra have sharp transitions at 290 mμ which are not observed in copoly (Glu:Trp) (I and II). *N*-Acetyltryptophan ethyl ester exhibited no difference spectrum as a function of pH (25), and the polymers might be expected to behave in a similar fashion; however, they did not. The difference spectra of the copoly (Glu:Trp) (I and II) between pH 4.0 and 7.5 are quite distinct from the above models and are most probably the result of a perturbation of the tryptophanyl residues by the —COOH moiety. A similar interpretation based on a carboxyl-indole interaction for the difference spectrum of lysozyme caused by a group with an intrinsic pK of 4.2 was advanced by Donovan, Laskowski, and Scheraga (25). It must be noted that the difference spectrum of lysozyme at pH 1.5 and 6.4, as reported by these authors, is different from that of copoly (Glu:Trp). The reason for this difference must be that the tryptophans in the protein are affected by additional factors not found in the copolymer. Our results show that it is not necessary to have an anomalous carboxyl for the observation of a difference spectrum as previously suggested (25). However, the fact that the block copoly (L-Glu) (L-Trp) (III) does not exhibit a difference spectrum and the block copoly (DL-Glu) (L-Trp) (IV) does shows that the carboxyls must be in close proximity to the indole residues. In the helical block copoly (L-Glu) (L-Trp) (III) the carboxyls are held away from the tryptophan because the polymer may be considered as a rigid rod. In the random copoly (DL-Glu) (L-Trp) (IV), they can approach the indole nucleus, because the chains can fold back on themselves. This might imply, in the latter case, a preferred COOH-indole interaction. Therefore, in the interpretation of a pH titration curve of a protein, measured by a difference spectrum, one must consider this perturbation. Part of the difference spectrum may be due to this interaction as well as to an environmental change about the tryptophanyl residues.

Lysine:Tryptophan Copolymers

Amine Quenching—The copoly (L-Lys:L-Trp) (V) had a decreased fluorescence yield when converted to the helical conformation in alkaline solution. Several factors could be responsible for this reduced fluorescence: α-helix formation, $—NH_2$ formation, and/or increased OH^- concentration. The $—NH_2$ species was formed when the charge on the polymer was neutralized by the addition of base which was necessary to bring about the random chain → helix transition between pH 8.0 and 11. Differentiation between OH^- and $—NH_2$ quenching was accomplished by the use of the copoly (L-Glu:L-Trp) (I). This polymer does not change its conformation above pH 7.5. Quenching of the polymer fluorescence was not observed up to pH 12.5. Therefore, OH^- may be eliminated as a quenching species in the pH transition range. This is in accord with the data of Cowgill (7) for *N*-acetyltryptophan ethyl ester, and both these models behave quite differently than tryptophan (6). The quenching of fluorescence at pH 10.5, observed with tryptophan, does not occur with α-amino-substituted tryptophan derivatives and may be regarded as specific for the free amino acid. Therefore, it is not important in the study of *tryptophanyl* fluorescence in proteins.

The copoly (L-Glu:L-Trp) (I) was effectively quenched at pH 10.9 by the addition of 10% ethanolamine. Also the model compound, *N*-acetyltryptophan ethyl ester, was subject to the same amine quenching. Although the amount of ethanolamine added might be considered excessive, the local concentration of amine groups in a lysyl polymer, or a protein, is as large or many fold larger. Perhaps, the effect of ethanolamine on fluorescence can be used to distinguish between exposed and buried tryptophanyl residues in a manner analogous to solvent perturbation (26). Thus, the formation of $—NH_2$ during the random chain → helix transition of the copoly (L-Lys:L-Trp) (V) resulted in a lower fluorescence yield, and it was not possible to determine the effect of the conformational change alone. The quenching of tryptophan fluorescence in proteins at alkaline conditions must, in part, be due to this quenching by the lysyl ε-amino group that is formed under these conditions.

Quantum Yield and Emission Spectrum of Tryptophanyl Residues

Random Sequence—Copoly (Glu:Trp) (I, II) and Copoly (Lys:Trp) (V, VI) have comparable fluorescence yields (0.13) at pH 7.5, where they exist as random chains. This fluorescence efficiency may be considered that of tryptophan in a random chain polypeptide with little if any quenching interactions with neighboring groups.

The emission spectra of these various tryptophanyl polymers are identical and similar to that of tryptophan in water. One may therefore assume that in these copolymers the environment of the tryptophan moiety is an aqueous one. The difference in the quantum yield of the free amino acid and the tryptophanyl residue may be ascribed to the dipolar character of the former. The maximum decrease of fluorescence by either of the two quenching species, —COOH and $—NH_2$, was about 2-fold; however, no fluorescence spectral shift occurred. Therefore, the range of the fluorescence quantum yield of the tryptophanyl moiety, as measured for the copolymers, is between 0.08 and 0.13. Proteins have quantum yields from 0.05 to 0.48 (2). Thus the present model system includes only a limited segment of the range of values found for native proteins. Clearly, there are other factors not considered in this study that influence tryptophan quantum yield as well as the fluorescence spectra. However, in one respect, the model system is similar to proteins. In the denatured state of many proteins in 8.0 M urea (2, 27) the quantum yield is altered and is more closely related to the model system of this study. A similar observation is true for the emission spectra.

Block Sequence—The block sequence tryptophan copolymers have a lower quantum yield than the random copolymers. Their emission spectrum is also shifted toward longer wave lengths. Lower quantum yields are also observed with tyrosyl block copolymers (9, 10). All the data are consistent with a model that stacks the tryptophanyl moieties around an α-helical peptide backbone. This helix would be energetically favored, owing to the hydrophobic nature of the indole nucleus. The low quantum

yield must be in large part attributed to interactions among the tryptophan moieties. Evidence for interactions between residues is (*a*) the shift of the maximum of the absorption spectrum and (*b*) shift of the maximum of the emission fluorescence spectrum and extension of the emission to longer wave length. Other interactions not yet fully explored in this system may account for the remaining loss of fluorescence. Similar interactions between aromatic residues, or with coenzymes, may possibly be responsible for the lower quantum yields in proteins.

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