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Fluorescence Studies with Tryptophyl Peptides*

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ABSTRACT: The influence of amino and phenolic ionization on the fluorescence intensity of several series of tryptophan peptides has been evaluated. The effect of amino ionization falls off rather gradually in the series $(Gly)_{0-3}$-Trp. In the series, $NH_2(CH_2)_{2-8}CO$-Trp, fluorescence is quenched in the smaller molecules and augmented in the larger molecules with protonation of the amino group. Cyclization has been suggested to occur in the larger molecules in order to account for the reversal.

Quenching was observed in the series Trp-$(Gly)_{0-4}$-Tyr with ionization of the phenolic group. The efficiency of quenching decreases with increasing molecular size. Quenching is attributed to radiationless energy transfer between the tryptophan and ionized tyrosine residues.

Since the pioneering studies of Weber and his coworkers on the ultraviolet fluorescence of the aromatic amino acids (Weber, 1961; Teale and Weber, 1957; White, 1959), there has been evidence from his and other laboratories (Teale, 1960; Brand *et al.*, 1962; Steiner and Edelhoch, 1961, 1962, 1963a,b; Gally and Edelman 1964), that denaturation of proteins is usually accompanied by changes in the fluorescence parameters of tryptophan and/or tyrosine.

The indications are that a variety of functional groups in proteins may modify quantum yields of indole or phenol fluorescence. Of particular interest are the effects of amines, carboxyl groups (White, 1959; Cowgill, 1964; Weber, 1961), and the phenolate ion (Edelhoch *et al.*, 1963; Cowgill, 1963b).

In this report the influence of these functional groups on the fluorescence of indole in a number of simple model compounds will be described. It is hoped that these and future observations will make it possible to provide more definitive interpretations of the fluorescence changes observed when proteins undergo conformational or allosteric changes.

Methods

Computations

Apparent Equilibrium Constants of Phenolic Ionization. The apparent equilibrium constant (K_a) of the dissociation of the phenolic group in the L-tryptophyl-L-tyrosine series of compounds was obtained from a spectrophotometric titration curve. The data were plotted according to the law of mass action in the

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form, pH = pK_a + log $\alpha/(1 - \alpha)$ where α is the degree of dissociation. The pK_a is defined as the pH when $\alpha = 0.50$. This value was determined from a straight line drawn through the experimental points with a slope of one. In water and in 35% ethyl alcohol solutions, the absorbance was measured at 242 mμ. In 9 M urea, absorbance measurements were obtained at 300 mμ, since the absorbance of the solvent changed significantly with pH at 242 mμ. The pK_a values are reported in Table I. The values in parentheses have not been measured. They have been chosen to accord with their neighbors since they were necessary for computations of the quenching efficiencies of the phenolate ion in this series of tryptophan compounds. The precision of the pK_a values appearing in Table I is about 0.05 pH unit.

TABLE I: Fluorescence Studies on Tryptophyl-(glycyl)$_n$-tyrosine.

n	pK_a Amine[b]	% Q[a]	pK_a Carboxyl	% Q[a]	pK_a Phenol[c,d]	% Q
			Solvent = H_2O			
0	7.6 est	52	3.2	22	10.22 sp	82
1	7.7	48	—	5	10.22 sp	62
2	7.6	55			10.33 fl	52
3	7.5	45			(10.33)	46
4	7.6	47			10.30 sp	42
			Solvent = 35% Ethanol			
0	7.4 est	55	3.9	15	11.17 sp	89
1	7.4 est	56	—	0	11.17 sp	69
2	7.5 est	67			11.13 sp	63
3	7.2	58			(11.15)	43
4	7.2	58			(11.15)	48
			Solvent = 9 M Urea			
0	7.6	52	4.1	19	11.00 sp	85
1	7.9	48	—	1.5	11.22 fl	64
2	7.8 est	63			11.23 sp	57
3	7.7	51			11.12 sp	42
4	7.7 est	52			(11.10)	43

[a] The degree of quenching (% Q) is based on the theoretical maximum values. Values shown in parentheses were assumed. [b] pK_a values (indicated by est) were determined from the pH value when $\alpha = 0.50$ of the fluorescence curve. [c] sp, determined by spectrophotometric titration. [d] fl, determined from analysis of fluorescence curve.

Apparent Equilibrium Constants of Ionizable Groups from Fluorescence Intensity Measurements. Changes in indole fluorescence produced by ionizable groups follow the ionization curves of the various charged groups. It is, therefore, possible to determine the pK_a values from fluorometric–pH curves. The influence of the carboxyl and phenolic groups, however, extends into the regions of acid and base quenching, respectively. It is possible nevertheless to estimate the fluorescence intensity of the form of the molecule in which the carboxyl group is un-ionized, since an inflection usually occurs in the fluorescence–pH curve which separates the pH zones of quenching by the carboxyl group and by acid (Figure 1). Confirmation of this value was then found in the slope of the plot of pH against log $\alpha/(1 - \alpha)$, which must be one. In 35% ethyl alcohol and 9 M urea solutions, the pK_a values of the carboxyl groups are increased and a plateau is found in the acid region, thereby facilitating the analysis of the pK_a values for the carboxyl groups (Table I). pK_a values could be determined for L-tryptophyl-L-tyrosine only since, for the higher molecular weight compounds of this series, *i.e.*, L-tryptophyl(glycyl)$_n$-L-tyrosine, the carboxyl group was too far removed from the indole group to affect its fluorescence by more than a few per cent.

The quenching by phenolate ion overlaps that of hydroxyl. The two effects are readily separable, however, since the pK_a of the phenolic group can be determined independently by spectrophotometric titration. It is then possible to evaluate from the fluorescence data the degree of quenching due to the phenolate ion. Since pH − pK_a = $\alpha/(1 - \alpha)$, α is known at any pH. A plot of the fluorescence intensity against α can then be extrapolated to $\alpha = 0$ and 1.00 in order to obtain the fluorescence intensity of the molecule when the phenolic group is in the un-ionized and completely ionized forms, respectively (Figure 2). From these two values of the fluorescence intensity the degree of quenching due to phenolic ionization is readily computed.

The phenolic pK_a is increased by approximately 1 unit in both 35% ethyl alcohol and 9 M urea solutions (Table I). In these two solvents the pK_a of phenolic dissociation is more than 3 pK units greater than that of the α-amino dissociation and consequently the two ionization curves do not overlap significantly. In aqueous solution, however, the difference in pK_a values is about 1 unit less, resulting in a small though significant overlap. The theoretical fluorescence maximum in water was obtained by determining the fluorescence when the phenolic group is un-ionized by the extrapolation procedure outlined in the previous paragraph. The degree of quenching by the amino group was obtained from this theoretical maximum and the plateau region that occurs between the carboxyl and amino ionization curves. The pK_a of the amino group can be determined also from the pH at the midpoint in the fluorescence curve between the plateau region and the theoretical maximum. The pK_a determined from $\alpha = 0.50$ in the fluorescence curve is affected by the overlap in amine and phenolic ionizations only at values of α much closer to one. The pK_a values listed as estimated (est) in Table I were determined in this way. The other values were determined from a plot of pH *vs.* log $\alpha/(1 - \alpha)$. In 35% ethyl alcohol and in 9 M urea the pH range of amino ionization is independ-

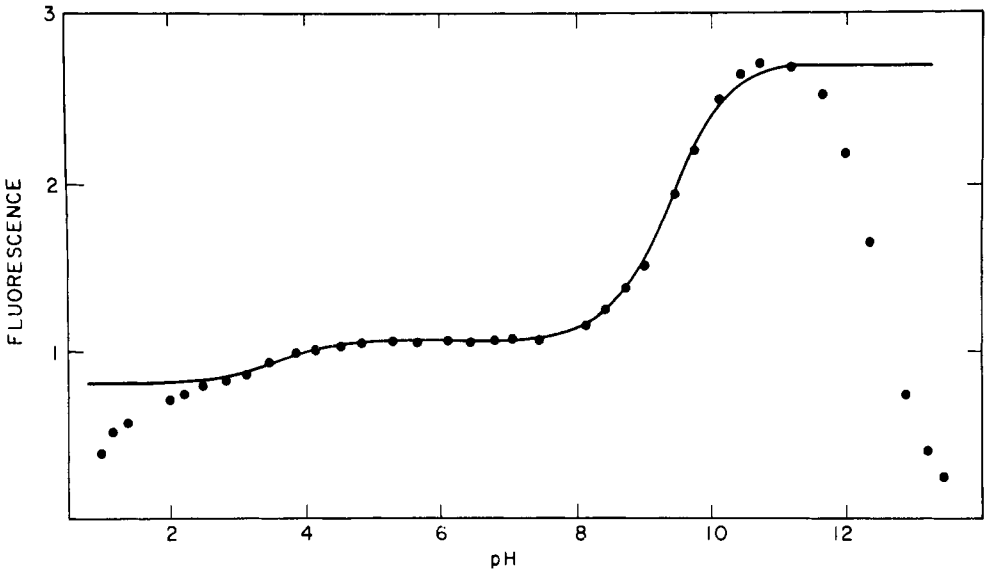

FIGURE 1: Effect of pH on the fluorescence of β-Ala-L-Trp. The line is the theoretical curve based on pK_a values of 9.35 and 3.45 and 61 and 10% quenching for the charged amine and uncharged carboxyl forms, respectively. The data are given by the points.

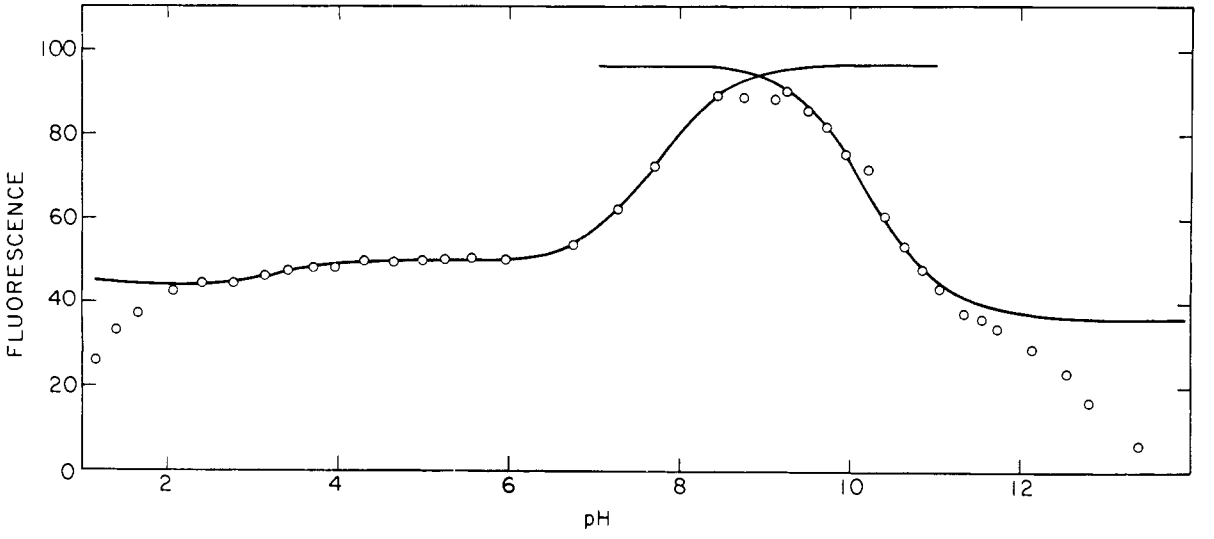

FIGURE 2: The pH dependence of the fluorescence of L-tryptophyl-glycyl-L-tyrosine in water. The lines are theoretical curves based on the following values of pK_a and degree of quenching: phenolic group 10.22, 62%; amine group 7.70, 48%; and carboxyl group 3.50, 5%. The points are experimental values. Below pH 2 and above pH 11 the quenching is by acid and base, respectively.

ent of either carboxyl or phenolic ionization and the pK_a is readily computed from the fluorescent data.

In the series $NH_2(CH_2)_nCO$-Trp, the quenching due to charging the amino group of β-Ala-Trp did not extend into the region of base quenching (Figure 1). Consequently the pK_a of the amino group and the degree of quenching were determined in a manner already described. In the longer chain length compounds in this series the pK_a was increased and overlap occurred between the quenching by uncharged amine and by base. Since an inflection occurs between the two quenching curves it is possible to estimate the fluorescence intensity representing the end point of the amine quenching. Verification of this estimate was found in a slope of one in the plot of pH *vs.* log $\alpha/(1-\alpha)$. The degree of quenching by the amino group

TABLE II: Blocked Peptides of L-Tryptophan.[d]

Peptide[a]	Yield (%)	$[\alpha]_D^5$	Mp (°C)	Formula	Calculated (%) C	H	N	Found (%) C	H	N
N-Z-$(Gly)_2$-L-Trp-OBz	72	+10[b]	85	$C_{30}H_{30}N_4O_6$	66.41	5.56	10.33	66.31	5.42	10.24
N-Z-$(Gly)_3$-L-Trp-OBz	78	−1[c]	115–116	$C_{32}H_{33}N_5O_7$	64.09	5.55	11.68	64.18	5.46	11.39
N-Z-4-A-But-L-Trp-OBz	73	−11[c]	75–76	$C_{30}H_{31}N_3O_5$	70.16	6.08	8.18	70.18	6.21	8.08
N-Z-5-A-Val-L-Trp-OBz	80	−7[c]	82–84	$C_{31}H_{33}N_3O_5$	70.57	6.30	7.97	70.52	6.22	7.75
N-Z-6-A-Cap-L-Trp-OBz	71	−10[c]	95–96	$C_{32}H_{35}N_3O_5$	70.96	6.51	7.76	70.92	6.46	7.85
N-Z-7-A-Hep-L-Trp-OBz	78	−14[c]	103–104	$C_{33}H_{37}N_3O_5$	71.33	6.71	7.56	71.50	6.62	7.41
N-Z-9-A-Non-L-Trp-OBz	75	−12[c]		$C_{35}H_{41}N_3O_5$	72.01	7.08	7.20	72.12	7.19	7.38
N-Z-11-A-Und-L-Trp-OBz	73	−13[c]	90–92	$C_{37}H_{45}N_3O_5$	72.64	7.41	6.87	72.50	7.31	6.59

[a] Abbreviations: Z = $C_6H_5CH_2OCO$; Bz = $C_6H_5CH_2$; A = amino; But = butyric acid; Val = valeric acid; Cap = caproic acid; Hep = heptanoic acid; Non = nonanoic acid; Und = undecanoic acid. [b] *c* 1, dimethylformamide. [c] *c* 1, methyl alcohol. [d] Recrystallized from ethyl acetate–ether.

could then be computed.

Materials

An Amino-Bowman fluorometer was used to measure fluorescence. A wavelength of 275 mμ was selected for excitation since the molar extinction coefficient of the L-tryptophyl$(glycyl)_n$tyrosine series changed by less than 10% between 270 and 280 mμ with ionization of the phenolic group. All fluorescence measurements were made in solutions where the absorbance at 275 mμ was less than 0.10. The fluorometer was checked for linearity of response to absorbance to values of 0.10. Solutions were kept at room temperature (25°) which was the temperature of measurement.

pH values were obtained with a Radiometer Model TTT1 pH meter. Standard buffers of pH 4.00, 7.00, and 10.00 were used to calibrate the meter.

Absorption measurements were made either on a Beckman Model DK-1 or Cary Model 14 spectrophotometer. Ethyl alcohol was a spectroscopic grade. Urea was recrystallized once from ethyl alcohol.

Synthesis of Model Compounds

A. $NH_2(CH_2)_nCO$-L-Tryptophan. The following compounds were prepared according to the literature: L-tryptophan benzyl ester (Wilcheck and Patchornik, 1963); L-tryptophyl-L-tyrosine (Wilcheck and Patchornik, 1963); carbobenzoxyglycylglycine (Sheehan and Hess, 1955); L-tryptophyl-L-phenylalanine (Wilcheck and Patchornik, 1963); L-tryptophyl-L-tryptophan (Wilcheck and Patchornik, 1963); carbobenzoxyglycylglycylglycine (Wilcheck and Patchornik, 1963); carbobenzoxy-4-aminobutyric acid (Evans and Irreverre, 1959); carbobenzoxy-6-aminocaproic acid (Schwyzer *et al.*, 1960, 1962); glycyl-L-tryptophan (Rao *et al.*, 1952); carbobenzoxy-L-tryptophan (Smith, 1948); and carbobenzoxy-L-tryptophylglycine (Anderson and Callahan, 1958).

Carbobenzoxy-5-aminovaleric acid (mp 110°), carbobenzoxy-7-aminoheptanoic acid (mp 94°), carbobenzoxy-9-aminononanoic acid (mp 94–95°), and carbobenzoxy-11-aminoundecanoic acid (mp 96–97°) were prepared by the usual methods for carbobenzoxylation (Bergmann and Zervas, 1932). The long-chain amino acids were obtained from Fluka, Basel (Switzerland).

The protected tryptophan peptides were synthesized from the appropriate carbobenzoxy amino acid and tryptophan benzyl ester by the *N,N'*-dicyclohexylcarbodiimide method (Table II). The unblocked peptides were obtained by catalytic hydrogenation of the blocked peptides in the presence of palladium on charcoal (Table III). All the peptides were chromatographically homogeneous in at least two solvent systems. Electrophoresis at pH 3.5 in pyridine–acetate buffer and paper chromatography in butanol–acetic acid–water (25:6:25) gave single spots with all the tryptophan–tyrosines peptides when treated with ninhydrin, Ehrlich, or Pauly reagents for free amino, tryptophan, or tyrosine residues, respectively.

L-Tyrosine Benzyl Ester Hydrochloride. Dry phosgene was passed at 50° through a suspension of L-tyrosine (18 g) in anhydrous dioxane (720 ml) until a clear solution was obtained. Phosgene was removed by a stream of dry nitrogen and one-half of the solvent was distilled off *in vacuo* at 45°. Benzyl alcohol (50 ml) was added and the solution was saturated with 2–3 moles of dry hydrochloric acid. During this time the tyrosine-*N*-carboxyanhydride separates out; it was brought again into solution by heating in a steam bath, and the solution was left overnight at room temperature. The ester which separated was filtered off, washed with ether, and recrystallized from water; yield 85%, mp 210°, $[\alpha]_D^{25}$ −23.5° (*c* 1, 0.1 N HCl); lit. mp (Erlanger and Hall, 1954) 205°, $[\alpha]_D^{25}$ −23.3° (*c* 1, 0.1 N HCl).

Anal. Calcd for $C_{16}H_{18}ClNO_3$: C, 62.45; H, 5.86; Cl, 11.40; N, 4.56. Found: C, 62.53; H, 5.98; Cl, 11.55;

TABLE III: Free Peptides of L-Tryptophan.[d]

Peptide	Yield (%)	$[\alpha]_D^{25}$	Formula	Calculated (%) C	H	N	Found (%) C	H	N
$(Gly)_2$-L-Trp	85	+5[a]	$C_{15}H_{18}N_4O_4$	56.59	5.70	17.60	56.65	5.83	17.72
$(Gly)_3$-L-Trp	82	+15[b]	$C_{17}H_{21}N_5O_5$	54.39	5.64	18.66	54.61	5.38	18.48
β-Ala-L-Trp	72	+15[c]	$C_{14}H_{17}N_3O_3$	61.08	6.22	15.26	61.28	6.40	15.03
4-A-But-L-Trp	90	+12[b]	$C_{15}H_{19}N_3O_3$	62.26	6.62	14.52	62.50	6.51	14.73
5-A-Val-L-Trp	84	+12[b]	$C_{16}H_{21}N_3O_3$	63.35	6.98	13.85	63.18	7.04	13.67
6-A-Cap-L-Trp	87	+13[b]	$C_{17}H_{23}N_3O_3$	64.33	7.30	13.24	64.44	7.38	13.16
7-A-Hep-L-Trp	90	+12[b]	$C_{18}H_{25}N_3O_3$	65.23	7.60	12.68	65.08	7.46	12.48
9-A-Non-L-Trp	85	+13[b]	$C_{20}H_{29}N_3O_3$	66.82	8.13	11.69	66.58	8.31	11.37
11-A-Und-L-Trp	84	+13[b]	$C_{22}H_{33}N_3O_3$	68.18	8.58	10.84	68.32	8.70	10.53

[a] *c* 2, 5 N hydrochloric acid. [b] *c* 1, water. [c] *c* 2, 1 N hydrochloric acid. [d] All the compounds were recrystallized from water–ethanol.

N, 4.45.

N-Carbobenzoxy-L-tryptophylglycyl-L-tyrosine Benzyl Ester. Triethylamine (1.4 ml) was added to a solution of L-tyrosine benzyl ester hydrochloride (3.0 g) in 50 ml of chloroform. This was mixed with a solution of carbobenzoxy-L-tryptophylglycine (4.1 g) in 50 ml of chloroform at 0°. *N,N'*-Dicyclohexylcarbodiimide (2.1 g) was added and the solution was stirred overnight at room temperature. *N,N'*-Dicyclohexylurea was removed by filtration and the filtrate washed successively with 0.5 N hydrochloric acid, water, and 5% sodium bicarbonate solution, and finally dried over sodium sulfate. The solvent was evaporated *in vacuo;* upon adding petroleum ether (bp 30–40°), it precipitated in an apparently amorphous, but readily filtered form. The product was reprecipitated from ethyl acetate–petroleum ether; yield 81%, mp 90°, $[\alpha]_D^{25}$ −25° (*c* 1, dimethylformamide).

Anal. Calcd for $C_{37}H_{36}N_4O_7$: C, 68.50; H, 5.59; N, 8.64. Found: C, 68.45; H, 5.80; N, 8.83.

The procedure described for the synthesis of this compound was used for the preparation of the following compounds.

N-Carbobenzoxy-L-tryptophylglycylglycine ethyl ester was prepared from *N*-carbobenzoxy-L-tryptophan (Brand *et al.*, 1962) and glycylglycine ethyl ester in chloroform; recrystallized from ethyl acetate–ether; yield 75%, mp 163°, $[\alpha]_D^{25}$ −28° (*c* 1, dimethylformamide).

Anal. Calcd for $C_{25}H_{28}N_4O_6$: C, 62.50; H, 5.83; N, 11.66. Found: C, 62.62; H, 5.74; N, 11.79.

N-Carbobenzoxyglycylglycyl-L-tyrosine benzyl ester was prepared from *N*-carbobenzoxyglycylglycine and L-tyrosine benzyl ester in dimethylformamide; recrystallized from ethyl acetate or methanol; yield 93%, mp 167°.

Anal. Calcd for $C_{28}H_{29}N_3O_7$: C, 64.73; H, 5.63; N, 8.09. Found: C, 64.82; H, 5.90; N, 8.17.

N-Carbobenzoxytriglycyl-L-tyrosine benzyl ester was prepared from carbobenzoxyglycine and glycylglycyl-L-tyrosine benzyl ester hydrobromide in a mixture of dimethylformamide and chloroform. The product was amorphous and was recrystallized from ethyl acetate; yield 80%, mp 89–91°, $[\alpha]_D^{25}$ − 2.5° (*c* 2, dimethylformamide).

Anal. Calcd for $C_{30}H_{32}N_4O_8$: C, 62.49; H, 5.59; N, 9.72. Found: C, 62.45; H, 5.79; N, 10.00.

Glycylglycyl-L-tyrosine Benzyl Ester Hydrobromide (II). A solution of 10 g of *N*-carbobenzoxyglycylglycyl-L-tyrosine benzyl ester in 50 ml of warm glacial acetic acid was allowed to cool to room temperature. Hydrogen bromide (30 ml of 45%) in acetic acid and a few crystals of phenol were added. The solution was kept for 20 min at room temperature. The hydrobromide salt was precipitated by the addition of absolute ether. The oily residue was dissolved in about 10 ml of isopropyl alcohol. Upon adding ether, it crystallized. The compound was recrystallized from isopropyl alcohol; yield 74%, mp 146–148°, $[\alpha]_D^{25}$ +13° (*c* 1, methanol).

Anal. Calcd for $C_{20}H_{24}BrN_3O_5$: C, 51.50; H, 5.15; N, 9.01. Found: C, 51.32; H, 5.32; N, 9.00.

Triglycyl-L-tyrosine benzyl ester hydrobromide and tetraglycyl-L-tyrosine benzyl ester hydrobromide were prepared in the same manner from the corresponding carbobenzoxy derivatives with HBr in acetic acid.

N-Carbobenzoxy-L-tryptophylglycylglycyl-L-tyrosine Benzyl Ester (III). *N*-Carbobenzoxy-L-tryptophan (1.65 g) and triethylamine (0.71 ml) were dissolved in a mixture of purified dioxane and chloroform (20 ml). The solution was chilled and ethyl chloroformate (0.54 g) was added. After about 6 min, glycylglycyl-L-tyrosine benzyl ester hydrobromide (2.33 g) and triethylamine (0.71 ml) were added. After 1 hr the mixture was washed successively with 0.5 N hydrochloric acid, water, and 5% sodium bicarbonate, and then dried over sodium sulfate and evaporated. The product was dissolved in ethyl acetate; upon addition of petroleum ether, it precipitated in an amorphous form which melted at about 115–120°; yield 72%, $[\alpha]_D^{25}$ −22° (*c* 2, dimethylformamide).

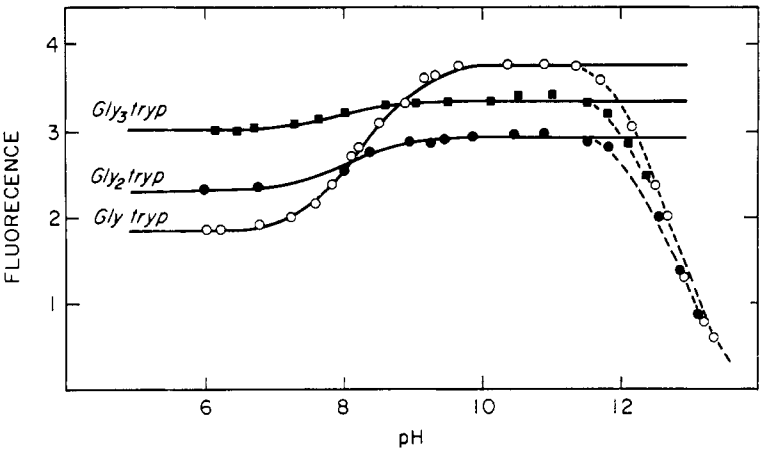

FIGURE 3: Relative fluorescence intensity of (glycyl)$_n$-L-tryptophan compounds as a function of pH. The continuous lines are theoretical curves based on the following values of pK_a and degree of quenching by the charged amine group. Glycyl-L-tryptophan, 8.20, 50%; (glycyl)$_2$-L-tryptophan, 8.00, 21%; (glycyl)$_3$-L-tryptophan, 8.00, 10%. Points are experimental values. The fluorescence intensities are based on solutions of equal absorbance at 280 mμ.

Anal. Calcd for $C_{39}H_{39}N_5O_8$: C, 66.37; H, 5.57; N, 9.92. Found: C, 66.36; H, 5.80; N, 9.98.

The procedure described for the synthesis of this compound was also used for the preparation of the following compounds.

*N-Carbobenzoxy-*L*-tryptophyltriglycyl-*L*-tyrosine benzyl ester* was prepared from carbobenzoxy-L-tryptophan and triglycyl-L-tyrosine benzyl ester hydrobromide in a dioxane–chloroform mixture in an amorphous form which melts at about 115°; yield 79%, $[\alpha]_D^{25}$ −20° (*c* 1, dimethylformamide).

Anal. Calcd for $C_{41}H_{42}N_6O_9$: C, 64.55; H, 5.55; N, 11.02. Found: C, 64.36; H, 5.72; N, 11.23.

*N-Carbobenzoxytetraglycyl-*L*-tyrosine benzyl ester* was prepared from carbobenzoxyglycylglycine and glycylglycyl-L-tyrosine benzyl ester hydrobromide in dimethylformamide solution. It was recrystallized from ethyl acetate or dimethylformamide–ether; yield 81%, mp 153–155°.

Anal. Calcd for $C_{32}H_{35}N_5O_9$: C, 60.66; H, 5.52; N, 11.05. Found: C, 60.69; H, 5.32; N, 11.34.

*N-Carbobenzoxy-*L*-tryptophyltetraglycyl-*L*-tyrosine benzyl ester* was prepared from *N*-carbobenzoxy-L-tryptophan and tetraglycyl-L-tyrosine benzyl ester hydrobromide in dimethylformamide–chloroform mixture. The product was amorphous; yield 61%, mp 133–135°, $[\alpha]_D^{25}$ −12° (*c* 1, dimethylformamide).

Anal. Calcd for $C_{43}H_{45}N_7O_{10}$: C, 62.99; H, 5.53; N, 11.96. Found: C, 62.77; H, 5.81; N, 11.73.

L-*Tryptophylglycyl-*L*-tyrosine.* A solution of carbobenzoxy-L-tryptophylglycyl-L-tyrosine benzyl ester in 80% methanol and 1 drop of acetic acid was hydrogenated in the presence of 0.5 g of 10% palladium–charcoal for 4–6 hr. The catalyst was removed by filtration. The filtrate was evaporated *in vacuo* and the product was recrystallized from methanol–ethyl acetate; yield 80%, $[\alpha]_{545}^{25}$ +78° (*c* 1, acetate buffer, pH 5).

Anal. Calcd for $C_{22}H_{24}N_4O_5$: C, 62.25; H, 5.70; N, 13.20. Found: C, 62.07; H, 5.90; N, 13.03.

The other free peptides were similarly made.

L-*Tryptophyldiglycyl-*L*-tyrosine* showed: yield, 75%, $[\alpha]_{545}^{25}$ +74°, $[\alpha]_{576}^{25}$ +60° (*c* 1, acetate buffer, pH 5).

Anal. Calcd for $C_{24}H_{27}N_5O_6 \cdot 2H_2O$: C, 55.69; H, 6.04; N, 13.53. Found: C, 55.42; H, 6.05; N, 13.53.

L-*Tryptophyltriglycyl-*L*-tyrosine* showed: yield 80%, $[\alpha]_{545}^{25}$ +54°, $[\alpha]_{576}^{25}$ +42° (*c* 0.5, acetate buffer, pH 5).

Anal. Calcd for $C_{26}H_{30}N_6O_7 \cdot 2H_2O$: C, 54.35; H, 5.96; N, 14.63. Found: C, 54.51; H, 5.78; N, 14.32.

L-*Tryptophyltetraglycyl-*L*-tyrosine* showed: yield 77%, $[\alpha]_{545}^{25}$ +49°, $[\alpha]_{576}^{25}$ +40° (*c* 0.65, acetate buffer, pH 5).

Anal. Calcd for $C_{28}H_{33}N_7O_8 \cdot 2H_2O$: C, 53.24; H, 5.86; N, 15.53. Found: C, 53.43; H, 6.02; N, 15.41.

Results

The Influence of α-Amine Ionization on the Fluorescence Intensity of (Glycyl)$_n$tryptophan. It has previously been shown that the quantum yield of tryptophan fluorescence is strongly pH dependent between pH 10 and 6, the ionization region of the α-amine group (Weber, 1961). The influence of the charged state of the α-amine is also evident in the absorption spectrum of tryptophan (Donovan *et al.*, 1961). However, in the latter case only a small shift in spectrum occurs which results in rather small changes in absorption. Donovan *et al.* (1961) have shown that when the distance between the α-amine and the indole chromophore is increased, as in glycyltryptophan, the influence of amine ionization is decreased by an order of magnitude and becomes almost negligible. In contrast, data present by White (1959) and by Cowgill (1963b) show that the effect of amine ionization on the quantum yield of fluorescence remains considerable in this dipeptide.

The results of an analysis of the pH dependence

of the series (glycyl)$_n$tryptophan, where n = 1, 2, or 3, are indicated in Figure 3 and Table IV. The fluorescence intensity of glycyl-L-tryptophan was observed to be constant between pH 4.5 and 6.5 and between pH 9.5 and 11. The change in fluorescence intensity between pH 6.5 and 9.5 was related *to* and could be used *for* the determination of the apparent dissociation constant (pK_a) of the α-amine group. In the case of glycylglycyl-L-tryptophan and glycylglycylglycyl-L-tryptophan the pK_a was estimated as described under Methods.

TABLE IV: Influence of Ionization on Fluorescence of Tryptophan Peptides.

Compd	Solvent	pK Amine	% Q[a] by Amine	% Q by Carboxyl	pH of 50% Q by Alkali
Gly-Trp	H_2O	8.20	50	5	12.70
Gly-Trp	1.0 M KCl	8.20	43		
Gly_2-Trp	H_2O	8.00	21	10	12.75
Gly_2-Trp	35% ethanol		28		
Gly_3-Trp	H_2O	8.00	10		12.65
Trp-Gly	H_2O	7.85	67	13	12.15
Trp-Gly	35% ethanol	7.75	74	6	12.70
Trp-Phe	H_2O	7.70	57	12	12.15
Trp-Trp	H_2O	7.45	50	12	12.50

[a] % Q = per cent quenching.

In water the pK_a of the α-amine dissociation of glycyl-L-tryptophan is 8.2 and the fluorescence intensity (*i.e.*, relative quantum yield) is reduced 50% as the amine is converted from the neutral to the charged form.

The fluorometric titration curve of this dipeptide was also carried out in 1.0 M KCl. The curve was very similar to that observed in water. The pK_a remained the same; however, the degree of quenching was slightly reduced (Table IV). This indicates the possibility of ionic shielding of the amine group.

The decrease in fluorescence between pH 4.5 and 2.5 (carboxyl ionization) relative to the fluorescence at pH 10 is also indicated in Table IV. The quenching by carboxyl as contrasted to carboxylate has previously been observed with tryptophan (White, 1959). As compared to the effect of the α-amine, the α-carboxyl has only a small effect on the fluorescence intensity of the indole chromophore.

Below pH 2 and above pH 11 the fluorescence intensity of glycyl-L-tryptophan and the other indole compounds discussed in this report falls off rapidly. The pH of 50% quenching by alkali in the case of the (glycyl)$_n$-L-tryptophan series is given in Table IV. Weber (1961) has suggested mechanisms for the strong acid and strong base quenching of indole derivatives.

In view of the strong influence of the α-amine on the fluorescent properties of glycyl-L-tryptophan the effect of further removing the amine from the indole ring is of particular interest. Fluorometric titration of (glycyl)$_2$-L-tryptophan and (glycyl)$_3$-L-tryptophan (Figure 3) revealed that the pK_a of the amine remained about the same while the degree of quenching as the amine went from the neutral to the charged form fell to 21 and 10% for (glycyl)$_2$-L-tryptophan and (glycyl)$_3$-L-tryptophan, respectively. Although each glycine residue introduces three additional atoms between the amine and the indole, the influence of the amine on the fluorescence intensity falls off only gradually.

In contrast to the compounds to be described in the next section no unusual structural modifications appear to be involved in the fluorescence changes observed with this series of compounds. This is indicated by the uniform decline in the quenching by the charged amine group. In addition, the relative fluorescence intensities of the di-, tri-, and tetrapeptides are quite similar at pH 10 (Figure 3).

The pH dependence of several tryptophan dipeptides (tryptophylphenylalanine, tryptophyltryptophan, and tryptophylglycine) was measured in order to determine whether the second functional group has a significant effect on the α-amine–indole interaction. A quenching of 67% was found with tryptophylglycine and 57% for tryptophylphenylalanine which are comparable to that of tryptophan. With tryptophyltryptophan less quenching (about 50%) was found. The lower value presumably results in part from the fact that the amine group is now α to one indole but one peptide group removed from the other. Since emission from both indoles is observed, less quenching is to be expected. No new excitation or emission peaks are observed, in agreement with the findings of Shifrin (1961) who was unable to observe any interactions between indole residues in polytryptophan. In all three dipeptides, protonation of the carboxyl group resulted in approximately the same degree of quenching relative to the maximum yield observed at pH 10 (Table IV).

The Influence of α-Amine Ionization on the Fluorescence Intensity of $NH_2(CH_2)_nCO$-Trp. The hypothesis that the polypeptide backbone of proteins could act as a semiconductor permitting the flow of electrons has been advanced both on theoretical and experimental grounds (Evans and Gergely, 1949; Szent-Györgi, 1941). In order to exclude this possibility as an explanation of the long-range influence of the amine group in the (glycyl)$_n$-L-tryptophan peptides discussed in the preceding section, a series of compounds were synthesized where the repeating unit between the amine and the indole groups is a methylene group. (The number of methylenes is indicated by n.) It is unreasonable to postulate the possibility of an electron flux through two or more methylene groups.

The fluorescence intensity of β-alanyl-L-tryptophan (n = 2) decreased by 61% when the amine group was charged (Figure 1). Since the degree of quenching

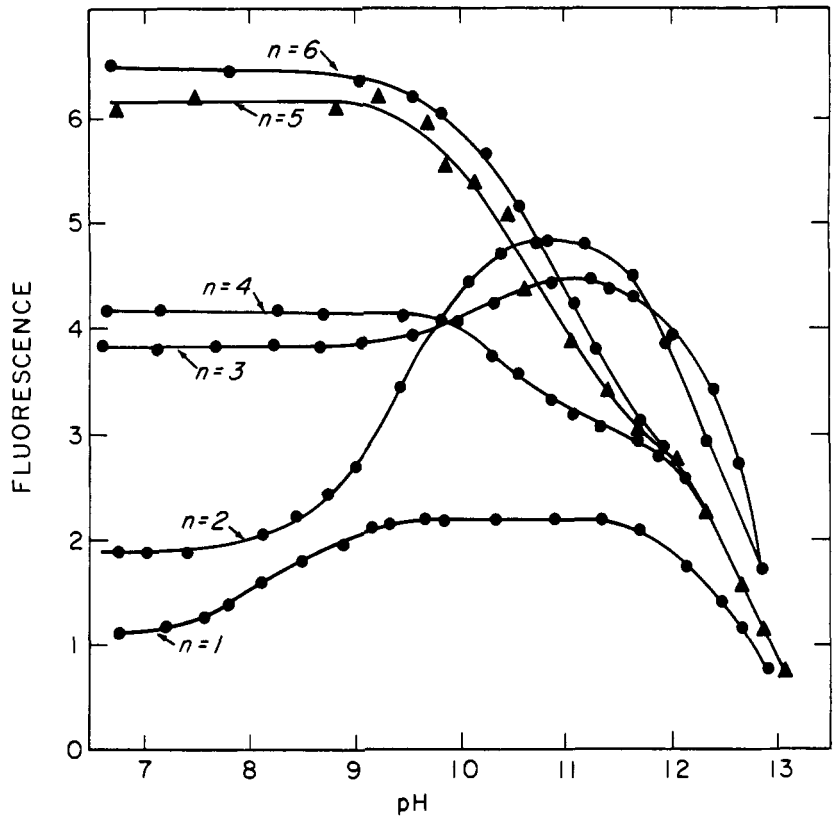

FIGURE 4: Effect of pH on the relative fluorescence intensities of the series $NH_2(CH_2)_n$-CO-L-Trp, n = 1–6. The fluorescence intensities are based on solutions of equal absorbance at 280 mμ.

is greater in this compound than in glycyl-L-tryptophan (n = 1), a conduction mechanism appears to be unlikely. The pK_a of the amine group, determined from the fluorescence data, was 9.35. The increase in pK_a of over one unit from that in glycyl-L-tryptophan reflects the greater separation between the amine and peptide groups.

In the next member of this series, γ-aminobutyryl-L-tryptophan (n = 3), the degree of quenching resulting from charging the amino group was markedly diminished compared to β-alanyl-L-tryptophan (Figure 4). The next higher analog, δ-aminovaleryl-L-tryptophan (n = 4), not only *failed* to show any quenching but revealed a significant *increase* in fluorescence in the same pH region (Table V). With n = 5 and with n = 6 the rise in fluorescence was about twofold, whereas in the compound with n = 8 an increase of only ~50% was observed (Table V). The pK_a values of the higher analogs were very similar (~10.7) and near the values of aliphatic amines, as measured by potentiometric titration.

In Figure 4 are illustrated the fluorimetric–pH titration curves for glycyl-L-tryptophan through ω-aminoheptanoyl-L-tryptophan (n = 6). The ordinate scale represents the relative fluorescence intensity of solutions of *similar* absorbance which have been normalized to equal absorbance. Absorbances were measured at 280 mμ at pH 5.5 in 0.01 M sodium acetate. It was established that solutions of glycine-L-tryptophan and ω-aminoheptanoyl-L-tryptophan (n = 6) conformed to Beer's law up to absorbancies of one. The relative intensities shown in Figure 4 should, therefore, approximately represent their relative quantum yields since the absorption spectra of this series of compounds should be very similar.

Figure 5 depicts the dependence of the relative fluorescence of the series of tryptophan compounds as a function of the number of methylenes (n) situated between the indole chromophore and the ionizable amine group at two different pH values. At pH 7.0 the amine groups of all the compounds are completely charged except for glycyl-L-tryptophan which is slightly uncharged (25%). At pH 11.5 the amino groups are largely uncharged. A higher pH value could not be used for comparative purpose since alkaline quenching effects become significant above pH 11.5.

It is apparent from Figure 5 that both curves are irregular. Although the fluorescence increases with n in the pH 7.0 curve, it does not change uniformly with n between 1 and 5. Above n = 6, the fluorescence increases only slightly. Based on a quantum yield of ~5.5% for glycyl-L-tryptophan at pH 7 (White, 1959; Cowgill, 1963a), the maximum quantum yield reaches a value of about 33% for 9-aminononanoyl-L-trypto-

phan (n = 8).

The pH 11.5 curve shows both a maximum and minimum at n values near 2 and 5, respectively. The pH 7.0 and 11.5 curves intersect at an n value between 3 and 4, indicating that a transition occurs at this chain length, from a quenching to an enhancement in fluorescence, with protonation of the amine group.

In order to explore the nature of the reversal of the influence of the charged state of the amino group on the fluorescence efficiency of the indole group, the effect of several solutes and solvents, known to be effective in breaking noncovalent interactions, has been evaluated. In 1.0 M KCl the fluorescent properties of ε-aminocaproyl-L-tryptophan (n = 5) did not change significantly from that in water (Table V). Concentrated urea (and guanidine) solutions are better solvents than water for peptides containing aromatic amine acids (Nozaki and Tanford, 1963). In 9.0 M urea and 5.0 M guanidine, only a slightly smaller enhancement in fluorescence occurred with ω-aminocaproyl-L-tryptophan (n = 5) compared with water. Moreover, the pK_a values remained approximately the same (Table V).

TABLE V: Influence of Amine Ionization on Fluorescence of $NH_2(CH_2)_nC(=O)$-Trp.

Compd (n)	Solvent	pK_a	% Q
			By Charged Amine
2	H_2O	9.35	61
	35% C_2H_5OH	9.20	66
	5.0 M guanidine	9.25	44
3	H_2O	10.2	15[a]
			By Uncharged Amine
4	H_2O	10.60	31
5	H_2O	10.60	50
	1.0 M KCl	10.65	55
	9.0 M urea	10.75	43
	5.0 M guanidine	10.50	42
	35% C_2H_5OH	11.20	26
	47% C_2H_5OH	—	0
6	H_2O	10.75	52
8	H_2O	10.85	35
	20% dioxane	~10.3	27
	50% dioxane	~9.6	15

[a] Minimum value.

The effect of ethyl alcohol, which might be expected to weaken hydrophobic interactions, was also tested. In 35% ethanol, the twofold enhancement in fluorescence observed with ω-aminocaproyl-L-tryptophan (n = 5) in water was reduced by 60%. In 47% ethanol the effect on fluorescence of charging the amine group was eliminated completely (Figure 6).

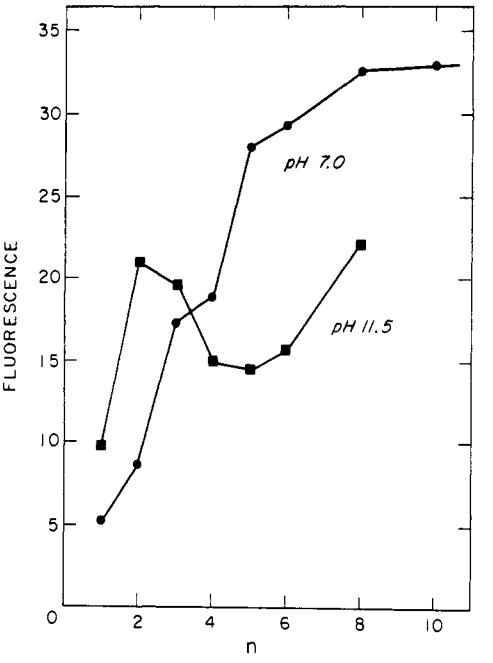

FIGURE 5: The influence of the number of methylene groups on the relative fluorescence in the series $NH_2(CH_2)_nCO$-L-Trp in the charged (pH 7.0 = NH_3^+) and uncharged (pH 11.5 = NH_2) forms of the amine groups.

In several compounds in which the *charged* amine group quenches indole fluorescence strongly (*i.e.*, β-alanyltryptophan, $(glycyl)_2$-L-tryptophan, and L-tryptophylglycine), 35% ethanol had no significant influence either on the pK_a of the amine or on the degree of quenching from that observed in water (Tables IV and V).

The fluorescence of 9-aminononanoyl-L-tryptophan (n = 8) increased by 53% with titration of the amine to its charged state using water as solvent. In 50% (v/v) dioxane this enhancement was reduced to 15% (Table V).

The Influence of Phenol Ionization on the Indole Fluorescence of L-*Tryptophyl*$(glycyl)_n$-L-*tyrosine.* We turn now from the effect of amines to the effect of phenol on tryptophan fluorescence. It has already been shown that the ionization of tyrosine affects the quantum yields of tryptophan fluorescence (Steiner and Edelhoch, 1963b). In the case of those proteins already studied the degree of quenching of tryptophan fluorescence by ionized tyrosine is greater in the native than in the unfolded forms of the same protein. It has already been reported that the phenolate ion causes a similar quenching in the simple dipeptide tryptophyltyrosine (Edelhoch *et al.*, 1963; Cowgill, 1963b). In order to determine the dependence of quenching efficiency on the distance between the two chromophores, a series

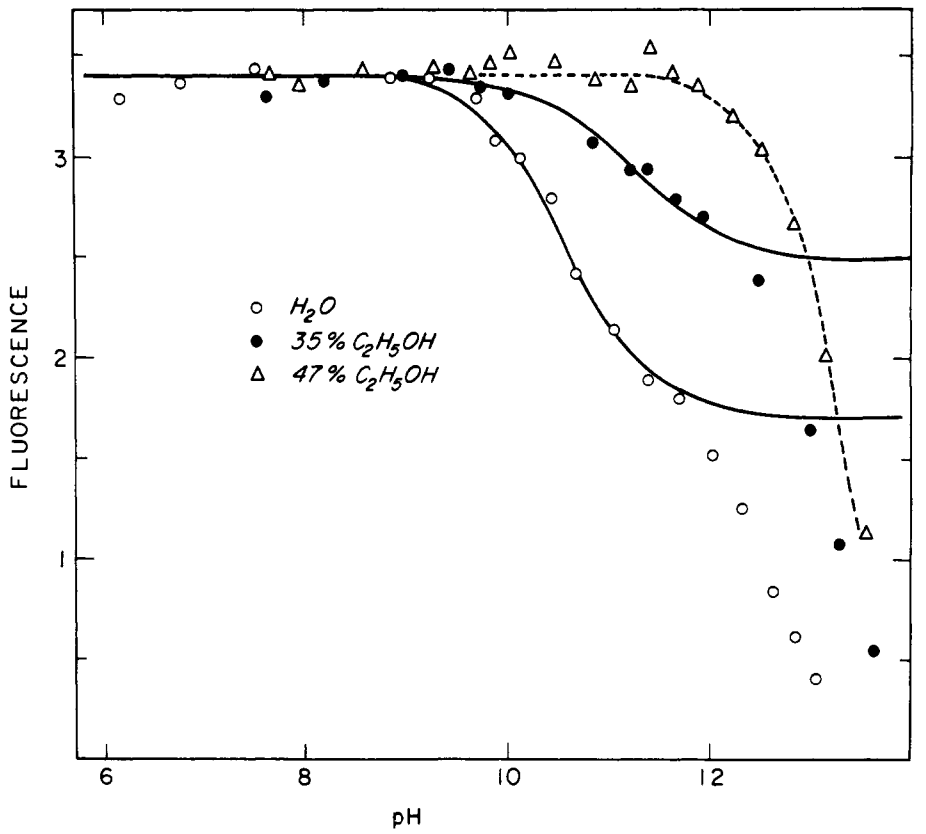

FIGURE 6: The effect of ethyl alcohol on the pH dependence of the fluorescence of ω-aminocaproyl-L-tryptophan (n = 5). The continuous lines are theoretical curves based on the following values of pK_a and degree of quenching of the amine group: H_2O 10.60, 50%; 35% ethyl alcohol 11.20, 26%; and 47% ethyl alcohol 0%. The fluorescence intensities have been normalized to give the same value at pH 7.0 in the three solvents.

of peptides containing tryptophan and tyrosine at the N- and C-terminal ends, respectively, were prepared.

The fluorescence intensity of L-tryptophylglycyl-L-tyrosine as a function of pH is shown in Figure 2. The fluorescence intensity of the indole emission depends on the ionic form of the molecule. Of particular interest is the inflection between pH 9 and 11 which is absent in the case of L-tryptophylphenylalanine. This quenching follows the ionization of the phenolic group. Since the pK_a of the tyrosyl ionization can be determined spectrophotometrically, it is possible to completely resolve the quenching due to phenolate from that due to alkali which overlaps the former. It has been computed that the fluorescence intensity of L-tryptophylglycyltyrosine is quenched by 62% by the ionization of the phenol group (see Methods). The ionization of the α-amine group also changes the fluorescence intensity of the indole. As expected the effect is comparable to that observed with other tryptophan peptides where the amine is α to the indole residue (Table I). Ionization of the carboxyl group can also be detected by its effect on the indole fluorescence.

The data for the pK_a values of the three groups and their respective effects on indole emission are given in Table I. The pK_a values of some of the phenolic groups were determined by spectrophotometric titration.

The fluorescence of the five compounds in this series containing from zero to four glycyl residues between the tryptophyl and tyrosyl residues was measured as a function of pH, and their pK_a values and quenching efficiencies are reported in Table I. A plot of the quenching efficiency of the phenolate ion as a function of the number of glycyl residues is shown in Figure 7. It can be seen that the quenching falls off rather gradually with increasing length of the chain. The influence of the α-amino group, however, remained constant near 50% (Table I). This is not unexpected since the distance between the α-amino and indole groups remains constant in this series of compounds. Introducing one glycyl group into Trp-Typ reduced the degree of quenching produced by the carboxyl group from 22 to 5%. In higher analogs, carboxyl ionization had no effect on emission intensity.

It should be noted that the influence of the carboxyl group disappears very rapidly as the length of the polypeptide chain increases, while that owing to the phenolate group shows a more gradual decline. Since the ratio of the distances of these two groups to the

indole ring should be approximately constant in any particular compound, it is likely that different mechanisms are operative in their quenching processes.

The fluorescence properties of this series were also investigated in two mixed solvent systems, both of which alter the structure of proteins though they change the dielectric properties of water in opposite directions. The pK_a values and quenching efficiencies of the ionizable groups in 35% ethyl alcohol–water (v/v) and 9 M urea solutions are summarized in Table I. The quantum yields increased in both solvent systems about twofold, the yields being slightly greater in ethyl alcohol solutions. The pK_a values of the phenolic groups in all the compounds increased by about one unit in both 35% ethyl alcohol and 9 M urea from their values in water. However, the degree of quenching produced by phenolic ionization was indistinguishable in the mixed solvents from that in water.

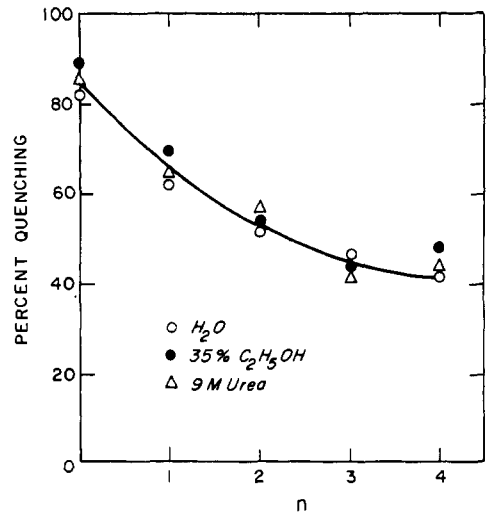

FIGURE 7: The effect of glycyl residues on the efficiency of quenching (by the phenolate group) in the series Trp-$(Gly)_{0-4}$-Tyr in water, 35% ethyl alcohol, and 9 M urea.

Discussion

The interaction of chromophores in proteins with radiant energy is susceptible to modification by the physical environment, *e.g.*, temperature and pressure, or by the chemical environment, *e.g.*, changes in solvent composition or in specific functional groups near the chromophore. Chemical effects can change the probability of transition to an excited state or the energy required for the transition. These can be observed as changes in the oscillator strength (*i.e.*, extinction coefficient) and in shifts in absorption spectra. Minor displacements in absorption spectra resulting from amine and carboxyl ionizations have been observed in model indole and phenol compounds (Scheraga, 1961).

In the excited state, chromophores are generally more reactive than in the ground state and thus neighboring groups or solvent molecules usually have a greater influence on the fluorescence than on the absorption. The quantum yield of fluorescence is particularly sensitive to environmental influences. The quantum yield may be defined as $q = \lambda/(\lambda + k_i + \Sigma k)$, where λ is the rate constant of emission of light as the excited state returns to the ground state; k_i is the rate constant of internal conversion which is responsible for the low yield of indole emission in the absence of any chemical quenching reaction; and Σk are the rate constants for other processes contributing to depopulation of the excited state by radiationless transitions. The various interactions involved in quenching or enhancement of fluorescence observed in the model compounds described in this paper fit into this last category.

The quenching of indole fluorescence by phenolate has been observed in proteins (Steiner and Edelhoch, 1963a,b). There can be no doubt on the basis of the data presented that the quenching of fluorescence is due to the conversion of phenol to phenolate; however, there are a number of mechanisms that might be responsible.

Energy transfer may occur from the excited indole to the phenolate anion which would be manifested by quenching of the indole fluorescence, since the phenolate anion is known to have a very low quantum yield (Cornog and Adams, 1963). Energy-transfer processes could be of three main types: (1) bond formation between the indole and phenolate either in their ground states or when indole is in an excited state, (2) "trivial energy transfer" or inner filter effect where the emitted radiation from the indole group is absorbed by phenolate, (3) radiationless energy transfer as described by Förster (1959).

A value of 13.3 A has been computed for R_0, the distance where the rate of tryptophan emission is equal to the rate of energy transfer to ionized tyrosine, from the Förster equation, $R_0^6 = (0.538 \times 10^{-33}/n^2)(\tau J(\bar{\nu})/\bar{\nu}_0^2)$, for resonance transfer (Förster, 1959). The refractive index (n) is that of water (1.33); the lifetime (τ) of the excited state of tryptophan is 2×10^{-9} sec; $\bar{\nu}_0$ is the mean of the wavenumbers of absorption and emission of tryptophan (32.2×10^3 cm^{-1}). $J(\bar{\nu})$ is the overlap integral calculated graphically from the absorption spectrum of ionized tyrosine and the fluorescence spectrum of tryptophan, both in water. The constant in the above equation has been corrected according to Latt *et al.* (1965).

The backbone chain length at 50% quenching is near four residues, *i.e.*, Trp-Gly-Gly-Tyr (Figure 7). If the four residues were in their fully extended form the distance between the ends would be 14.46 A. Of course, the orientation of the indole and phenol groups and free rotation in the peptide backbone would affect the distance between donor and acceptor. Nevertheless there appears to be a rough agreement between the latter distance and that computed for R_0, *i.e.*, 13.3 A.

The finding that aqueous solutions of ethyl alcohol

and urea, which might be expected to weaken ring–ring interactions, have no influence on the degree of quenching makes the first possibility unlikely. The other two mechanisms are feasible only if the emission spectrum of the donor overlaps the absorption spectrum of the acceptor. Overlap occurs with the phenolate but not the phenol form of the tyrosyl residue, since ionization produces a shift in absorption maximum from 273.5 to 293 mμ and an increase in molar extinction coefficient from 1400 to 2400 (31).

In the case of "trivial transfer" there should be a change in the emission spectrum of tryptophan. Little or no change was observed. Concentration quenching either of the exciting or emitted light due to higher extinction of the phenolate as opposed to the phenol is unlikely since dilute solutions were used for all the measurements.

The Förster mechanism has already been invoked to explain intramolecular energy transfer of reduced diphosphopyridine nucleotide (Weber, 1958) (from the adenine to the reduced nicotinamide). In this case transfer is from a nonfluorescent donor to a fluorescent acceptor. Direct evidence for the validity of Förster's theory has been obtained using two fluorescent chromophores attached to a steroid backbone (Latt *et al.*, 1965). In this case transfer takes place from a fluorescent donor to a fluorescent acceptor. A similar situation prevails in the intramolecular transfer of energy from indole to naphthalene in dimethylaminonaphthalenesulfonyl-L-tryptophan conjugates (Conrad and Brand, 1965). In the model compounds described in the present report intramolecular transfer would be from a fluorescent donor to a nonfluorescent acceptor. In this type of situation other forms of quenching must be excluded before radiationless energy transfer can be invoked as the mechanism responsible for quenching.

Another functional group in proteins that has profound effects on the fluorescence intensity of tryptophan is the amine group. In tryptophan itself the quantum yield decreases from about 0.6 to 0.2 as the α-amine is converted from the neutral to the protonated form. When the amine is displaced from the indole as in the $(glycyl)_n$-L-tryptophan series of compounds the protonated form of the amine has less of an influence on the fluorescence intensity. In $(Gly)_3$-L-Trp a change of only 10% was observed. Nevertheless it is significant that a tryptophan residue removed by three residues from an N-terminal amine could still have its fluorescence partially quenched by that group.

The ε-amines of lysines may also influence the fluorescence of tryptophan in proteins. The data obtained with the compounds of the type $NH_2(CH_2)_nCO$-Trp are relevant to their influence on the quantum yields. It is of interest to note that *both* the charged and neutral forms of the amine can act as quenchers. The distance of the amine from the indole in the excited state determines which of the two species will quench the most.

The reversal in the influence of amine ionization with size of the methylene chain in the model compounds strongly suggests that a modification in structure in the excited state occurs, bringing the uncharged amine closer to the indole ring in the longer chain compounds. The quenching by charged amine groups has been explained by Weber as a donation of a proton to the excited state of the indole (Weber, 1961). In the $NH_2(CH_2)_nCO$-Trp series of compounds described here the quenching by the charged amine falls off with the distance of the amine from the indole.

The quenching by uncharged amine may be explained by a proton transfer of the indole hydrogen to the amine during the lifetime of the excited state. This is analogous to the mechanism proposed for base quenching of tryptophan (Weber, 1961). This explanation would be in keeping with the fact that NH_2 has little or no effect on the absorption spectrum of indole but has a large effect on the fluorescence intensity. The quenching by the neutral form of the amine as the number of methylene groups in this series reaches or exceeds 4 can be explained by the assumption that an intramolecular cyclization occurs bringing the amine closer to the indole. The number of atoms in the ring should, therefore, exceed 8 excluding the indole nucleus. Increasing number of methylene groups results in decreasing water solubility. In order to diminish the number of contacts of this chain with water molecules, the nonpolar surfaces might tend to interact and form hydrophobic bonds. Bonds of this type between the methylene groups and the indole ring in the excited state might stabilize the cyclic structure responsible for the increased quenching by neutral amine as the methylene chain is extended from 1 to 4.

The reduction of amine quenching by ethanol might be explained by the breaking of hydrophobic bonds and consequent loss of the cyclic structure required to bring the amine into proximity of the indole. It must be noted, however, that urea and guanidine, which are also thought to affect hydrophobic bonds, have only a small influence on the degree of quenching as compared to ethanol.

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Fluorescent Probes for Conformational States of Proteins. II. The Binding of 2-*p*-Toluidinylnaphthalene-6-sulfonate to α-Chymotrypsin*

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ABSTRACT: 2-*p*-Toluidinylnaphthalene-6-sulfonate (TNS) fluoresces strongly when bound to hydrophobic regions of proteins, although alone it is virtually nonfluorescent in aqueous solution. TNS was used as a hydrophobic probe to study the conformation of chymotrypsin in the presence and absence of competitive inhibitors. Chymotrypsin and chymotrypsinogen each bind TNS with similar dissociation constants (2–5 × 10^{-4} M). The binding of substrate analogs to chymotrypsin inhibits the fluorescence of the TNS–chymotrypsin system without altering the apparent dissociation constant of the TNS–chymotrypsin complex. Similarly, the binding of TNS noncompetitively inhibits the hydrolysis of acetyl-L-tyrosine ethyl ester by chymotrypsin. These findings indicate that there is a hydrophobic binding site in chymotrypsin which is not part of the active site of the enzyme. Studies of the binding of TNS by chymotrypsin as a function of pH showed a peak of fluorescence intensity at pH 7.8. If chymotrypsin is replaced by either of the enzymatically inactive species, chymotrypsinogen or phenylmethanesulfonylchymotrypsin, this peak is no longer seen. This suggests that the binding of substrate analogs or protons to chymotrypsin may alter the enzymatic conformation.

Certain derivatives of aminonaphthalenesulfonic acids do not fluoresce in water, although they have relatively high quantum yields of visible fluorescence when dissolved in organic solvents or aqueous solutions of various proteins (Weber and Laurence, 1954). The mechanism of fluorescence of one of these compounds (TNS)[1] is described in the first communication of this series (McClure and Edelman, 1966). TNS fluorescence was enhanced by solvents of low dielectric constant or, to a lesser extent, by solvents of high viscosity.

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[1] Abbreviations used: TNS, 2-*p*-toluidinylnaphthalene-6-sulfonate; ANS, anilinonaphthalenesulfonate; ATEE, acetyl-L-tyrosine ethyl ester.