Fluorescence Studies with Tyrosyl Peptides*

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ABSTRACT: The influence of carboxyl, amino, and peptide groups on the quantum yield of tyrosyl fluorescence has been evaluated in a series of oligopeptides containing tyrosyl and glycyl residues, and in a group of substituted tyrosine derivatives. In these compounds, tyrosyl fluorescence is quenched by uncharged carboxyl and charged amino groups. Neighboring peptide bonds are an important reason for the low quantum yield of tyrosyl fluorescence in proteins. Their effects are greater when substituted on the carboxyl than on the amino end of tyrosine. Radiationless energy transfer between tyrosyl residues in peptides containing two or more tyrosyl residues has been studied. Energy transfer between two un-ionized tyrosyl residues has been demonstrated by depolarization of fluorescence, and between un-ionized and ionized tyrosyl residues by fluorescence quenching. Ionized tyrosyl residues are more efficient energy acceptors than are un-ionized residues. In peptides containing two tyrosyl residues, the degree of energy transfer can be used as a measure of the intertyrosyl distance. Energy transfer from tyrosine to tryptophan has been demonstrated by a comparison of the fluorescence emission spectra of an homologous series of peptides containing one tyrosyl and one tryptophanyl residue. Radiationless energy transfer may also be responsible for the quenching of tyrosyl fluorescence in proteins.

The quantum yield of tyrosyl fluorescence in native proteins is generally much less than that of simple phenolic compounds (Teale, 1960). Studies with low molecular weight model compounds have shown that tyrosyl fluorescence is quenched by a variety of neighboring functional groups (Cowgill, 1963a) and by peptide (Cowgill, 1963b) and disulfide bonds (Cowgill, 1967). In addition, the quantum yield of tyrosyl emission may be reduced by excitation energy transfer, either to tryptophanyl or to other tyrosyl residues. Cowgill (1966) has suggested that tyrosyl residues which are rendered nonfluorescent by hydrogen bonding may act as energy sinks, thereby quenching the fluorescence of other tyrosyl residues.

The interpretation of protein quantum yields requires an understanding of the various factors which affect tyrosyl and tryptophanyl fluorescence. Several reports have appeared evaluating the influence of carboxyl and amino ionization on copolymers containing tyrosine, glutamic acid, and lysine (Weber and Rosenheck, 1964; Fasman *et al.*, 1964). Recently the effects of carboxyl and amino groups on tryptophanyl emission and the transfer of electronic excitation energy from tryptophan to ionized tyrosyl residues have been measured in small model peptides (Edelhoch *et al.*, 1967). This communication considers the effects of carboxyl, amino, and phenolic ionization and of energy transfer from tyrosyl to tyrosyl and tryptophanyl residues, on tyrosyl fluorescence.

Methods

Fluorescence Spectra. Emission spectra were obtained with a Turner Model 210 recording spectrofluorometer. This instrument, recently described in detail, gives energy-corrected excitation spectra, and presents emission spectra in relative quanta per unit band width (Turner, 1964). The sample compartment was equipped with a water jacket and routinely maintained at 25°.

Fluorescence Quantum Yield. Quantum yields were also determined with the Turner spectrofluorometer. Samples of known optical density were excited at 275 nm unless otherwise stated. Quantum yields, Q, of samples at pH 5.5 were determined by comparing their fluorescence intensities at their emission peaks with that of tyrosine, $Q = (FI/FI_{\mathrm{Tyr}}(OD_{275,\mathrm{Tyr}}/OD_{275})$. Since all of the samples had similar excitation and emission spectra, their relative quantum yields should be precise to within a few per cent. For several compounds, relative quantum yields determined by this method agreed well with those determined from the areas of their total emission spectra relative to that of tyrosine. Quantum yields of samples at pH 1.5 and 8.5 were calculated from the changes in their peak fluorescence intensities relative to pH 5.5. In these calculations, we have ignored the small changes in tyrosyl absorbance due to carboxyl and amino ionizations (Wetlaufer *et al.*, 1958); the error introduced by these omissions is less than 5%.

Fluorimetric Titrations. Samples in 0.1 M KCl–0.02 M lysine were titrated by the addition of small amounts of HCl or KOH. pH was determined on a Radiometer Model 25 pH meter. In general, excitation was at 278 nm, the isosbestic wavelength for tyrosyl phenolic ionization, and emission was measured at 305 nm.

Spectrophotometric Titrations. Phenolic ionization was determined by the increase in optical density at 295 nm, measured in a Beckman Model DU spectrophotometer. The degree of ionization, α, at any pH, was calculated from the spectrophotometric titration curves. The phenolate absorption curves of Tyr, Tyr-Gly, and

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Gly-Tyr agreed very closely with the alkaline portions of their fluorimetric curves. In other words, at any degree of ionization, α, the fluorescence intensity, I_α, was proportional to the fraction of un-ionized phenolic groups $I_\alpha = I_{\max}(1 - \alpha)$. For Tyr-Gly and Gly-Tyr, this agreement indicates that there is little or no overlap between the amino and phenolic ionizations, and that the fluorescence maxima represent the fluorescence in the absence of quenching either by amino or by phenolic ionization.

In tyrosinamide and tyrosine-*N*-methylamide, the amino and phenolic ionization curves overlap slightly, thereby reducing the yield at the maximum, near pH 8.5. It was found that the degree of fluorescence quenching in these two compounds was about 10% less between 0 and 50% ionization than between 50 and 100%. Consequently, the computations of the degree of quenching due to amino ionization have accounted for this quenching effect. In compounds containing more than one phenolic group, all samples gave similar spectrophotometric titration curves, which could be analyzed in terms of a single dissociation constant. In order to calculate energy transfer from un-ionized tyrosyl residues, tyrosyl ionization was assumed to be random. Thus, for a peptide with two tyrosyl residues, at any degree of ionization, α, the fraction which is fully ionized is α^2, totally un-ionized $(1 - \alpha)^2$, and half-ionized $2\alpha(1 - \alpha)$. We assume that the fully ionized peptides are nonfluorescent, and that the fluorescence of the totally un-ionized peptides is independent of pH. The fluorescence of the half-ionized peptides, $I_{1/2}$, can then be calculated from the fluorescence intensity at any α (where I_α is the fraction of the maximum fluorescence at ionization α), $I_{1/2} = (I_\alpha - (1 - \alpha)^2)/2\alpha(1 - \alpha)$. If we assume further that all additional quenching caused by tyrosyl ionization is due to energy transfer, then the percentage of energy transfer is given by $100(1 - I_{1/2})$.

Fluorescence polarization was measured in an Aminco-Bowman spectrofluorometer fitted with Polacoat filters in the excitation beam and Polaroid films in the emission beam, as described by Chen and Bowman (1965). Fluorescence was excited at 290 nm with vertically polarized light, and the emission was analyzed at 310 nm. Polarization, P, is defined as $(I_\mathrm{v} - GI_\mathrm{H})/(I_\mathrm{v} + GI_\mathrm{H})$, where I_v and I_H represent the intensity of vertically and horizontally polarized emission, and G is a grating correction factor defined as $I_\mathrm{v}/I_\mathrm{H}$ with horizontally polarized excitation. The polarization of samples in 99% glycerol was measured as a function of temperature, and an extrapolated polarization, P_e, was obtained from a plot of $1/P$ *vs.* T/η. Interphenol distances in peptides containing two tyrosyl residues were estimated from fluorescence polarization measurements. According to Weber (1966), depolarization of fluorescence due to excitation energy transfer is given by the equation

$$\left(\frac{1}{P} - \frac{1}{3}\right) = \left(\frac{1}{P_0} - \frac{1}{3}\right)\left(1 + \frac{3}{2}\sin^2\theta\bar{n}\right)$$

in which θ is the average change in orientation per transfer, and $\bar{n}$ is the mean number of transfers during the lifetime of the excited state. P_0 is the intrinsic polarization, and P the observed polarization; we have used P_e to indicate the polarization in the absence of Brownian rotation in molecules containing more than one tyrosyl residue. Assuming random orientation of the two phenolic groups and neglecting back-transfers, the above equation becomes

$$\left(\frac{1}{P_\mathrm{e}} - \frac{1}{3}\right) = \left(\frac{1}{P_0} - \frac{1}{3}\right)\left(1 + \frac{2}{3}\left[\frac{R_0}{r}\right]^6\right)$$

in which r is the mean interphenol distance, and R_0 is the critical transfer distance, where the probabilities of spontaneous emission and deactivation by energy transfer are equal. We have previously calculated an R_0 of 8.9 Å for energy transfer between two un-ionized tyrosyl residues (Perlman *et al.*, 1968).

Fluorescence polarization spectra were obtained using an instrument built by Dr. L. Brand, Johns Hopkins University. In these experiments, the fluorescence polarization was measured at 310 nm and 25° of samples dissolved in 99% glycerol and excited with vertically polarized light.

Materials

The glycyl$_{1-3}$-L-tyrosine compounds were synthesized from carbobenzoxyglycine$_{1-3}$ and L-tyrosine benzyl ester by the *N*,*N*′-dicyclohexylcarbodiimide method (Sheehan and Hess, 1955). The free peptides were obtained by catalytic hydrogenation of the protected peptides with palladium on charcoal.

The L-tyrosyl-L-tyrosyl$_{0-4}$-L-tyrosine peptides were synthesized from carbobenzoxy-L-tyrosine and L-tyrosine$_{1-5}$-benzyl ester by the above method. The free peptides were obtained similarly.

The synthesis of L-tyrosyl$_{1-4}$glycine has been described elsewhere (Wilchek *et al.*, 1968).

The preparation and properties of the peptides containing tryptophan and tyrosine are described in Edelhoch *et al.* (1967).

The peptides synthesized had satisfactory elemental analysis and were chromatographically homogeneous in two solvent systems, (*i.e.*, thin-layer chromatography, 1-butanol–acetic acid–water (25:6:25), and 1-butanol–pyridine–acetic acid–water (30:20:6:24)) when treated with ninhydrin or Pauly reagents for free amino and tyrosyl groups, respectively.

N-Acetyl-DL-tyrosine (S788) and L-tyrosine ethyl ester (A5731) were obtained from Mann Research Laboratories and were reported to be homogeneous by paper chromatography. β-Alanyltyrosine was a gift from L. Levenbook (National Institutes of Health, Bethesda, Md).

Glycyl-L-tyrosylglycine was a gift from H. Cahnmann and T. Shiba (National Institutes of Health, Bethesda, Md.) and was chromatographically pure. L-Tyrosinamide was prepared by Yeda (Rehovot, Israel). *N*-Acetyl-L-tyrosinamide was prepared by acetylation of L-tyrosinamide.

The oxidized derivative of the A chain of bovine insulin was a gift from Dr. John A. Galloway of the Eli-Lilly Co.

TABLE I: The Relative Quantum Yields of Substituted Tyrosine and Glycyltyrosine Peptides.

	Q	
$^{+}NH_3CH(CH_2C_6H_5OH)COO^{-}$	1.00	I
CH_3CONH	0.88	II
$^{+}NH_3CH_2CONH$	0.38	III
NH_2CH_2CONH	0.67	IV
$^{+}NH_3CH_2CH_2CONH$	0.52	V
$^{+}NH_3CH_2CONHCH_2CONH$	0.54	VI
$NH_2CH_2CONHCH_2CONH$	0.54	VII
$^{+}NH_3CH_2CONHCH_2CONHCH_2CONH$	0.58	VIII
$NH_2CH_2CONHCH_2CONHCH_2CONH$	0.58	IX

	Q		
I	1.00	$^{+}NH_3CH(CH_2C_6H_5OH)COO^{-}$	
X	0.15		$COOH$
XI	0.12		$COOCH_2CH_3$
XII	0.25		$CONH_2$
XIII	0.38		$CONHCH_3$
XIV	0.33		$CONHCH_2COO^{-}$
XV	0.22		$CONHCH_2COOH$
XVI	0.22		$CONHCH_2CONHCH_2COO^{-}$
XVII	0.22		$CONHCH_2CONHCH_2COOH$
XVIII	0.45	CH_3CONH	$CONH_2$
XIX	0.22	NH_3CH_2CONH	$CONHCH_2COO^{-}$

Results

The effect of amino ionization on tyrosine emission cannot be evaluated since its ionization curve (pK = 9.28) strongly overlaps that of the phenolic group (pK = 9.63) (Martin *et al.*, 1958). There is no increase in fluorescence in tyrosine, tyrosine ethyl ester, or β-alanyltyrosine prior to phenolic dissociation and quenching. It should be noted that with the loss of phenolic emission at 303 nm, a new emission band develops at longer wavelengths due to the phenolate ion (Cornog and Adams, 1963; Longworth and Rahn, 1967). We are concerned here only with emission of the phenolic group which has its peak near 303 nm.

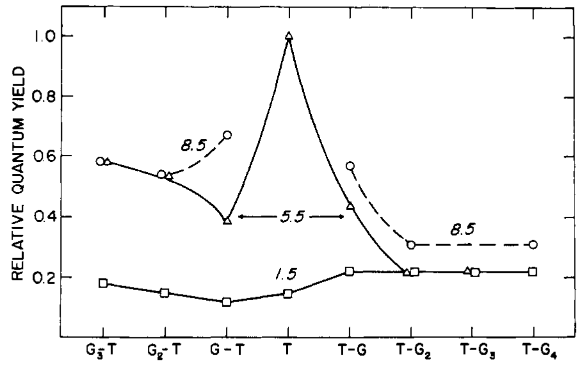

FIGURE 1: Relative quantum yields of tyrosine, Tyr-Gly$_{1-4}$, and Gly$_{1-3}$-Tyr at pH 1.5 (□, NH_3^+, COOH), pH 5.5 (△, NH_3^+, COO^-), and pH 8.5 (○, NH_2, COO^-) in 0.1 M KCl–0.02 M lysine. Quantum yields are all expressed relative to tyrosine at pH 5.5 = 1.

An attempt has been made to evaluate the influence of the peptide bond on the quantum yield of phenolic fluorescence in a series of substituted tyrosine and glycyltyrosine compounds enumerated in Table I. All yields are shown as relative to tyrosine at pH 5.5. If the amino group of tyrosine is blocked by acetylation (II) there is almost no change in quantum yield. Addition of an amino group to the acetyl, forming Gly-Tyr, leads to a marked reduction in yield, the charged form (III) having a lower quantum yield than the uncharged group (IV), as seen in Figures 1 and 2 (Cowgill, 1965). It is clear that during the lifetime of the excited state of phenol, the

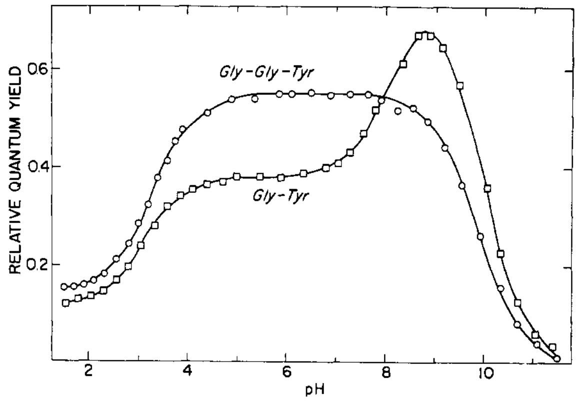

FIGURE 2: Fluorimetric titrations of Gly-Tyr (□) and Gly-Gly-Tyr (○) in 0.1 M KCl–0.02 M lysine. Samples were excited at 278 nm, and their emission was measured at 305 nm. Quantum yields are expressed relative to tyrosine at pH 5.5 = 1.

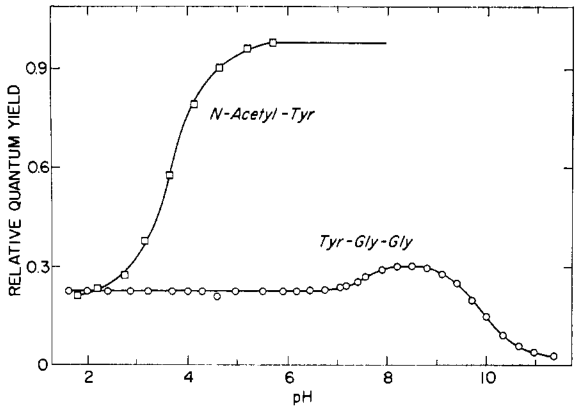

FIGURE 3: Fluorimetric titrations of Tyr-Gly-Gly (○) and N-acetyltyrosine (□) in 0.1 M KCl–0.02 M lysine. Samples were excited at 278 nm, and their emission was measured at 305 nm. Quantum yields are expressed relative to tyrosine at pH 5.5 = 1.

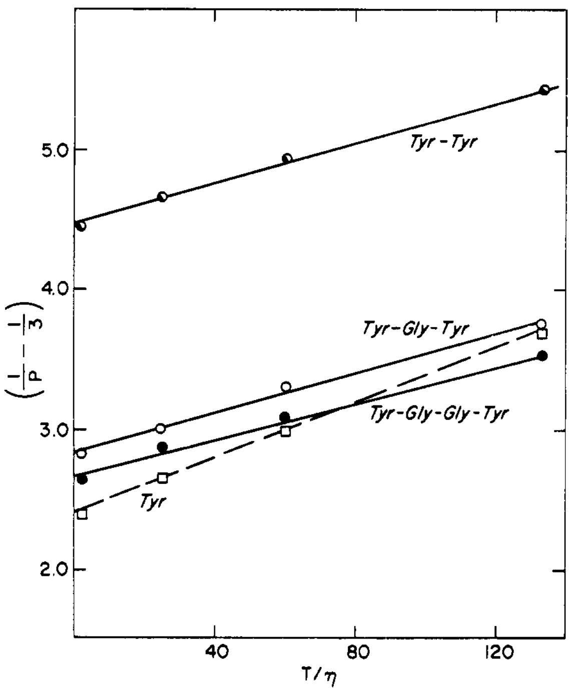

FIGURE 4: Fluorescence polarization of tyrosine and Tyr-Gly_{0-2}-Tyr in 99% glycerol. T/η was varied by varying the temperature and is in degrees Kelvin per poise. Samples were excited at 290 nm with vertically polarized light, and there emission was analyzed at 310 nm.

TABLE II: Effect of Carboxyl Group Ionization on Fluorescence Intensity.

Compound	pK Carboxyl	Percentage Quenching
Phenylalanine	2.3	30
p-Fluorophenylalanine	2.3	43
Tyrosine	2.5	85
m-Tyrosine	2.4	85
o-Tyrosine	2.6	95
3,4-Dihydroxyphenylalanine	2.4	84

amino group of Gly-Tyr significantly reduces the population of excited molecules by radiationless transition to their ground state. Displacement of the charged amino group by one methylene group (β-alanyltyrosine, V) leads to a 50% increase in yield (0.52 *vs.* 0.38). Further displacement by two (VI) or five (VIII) atoms along the backbone only increases the yield slightly (Table I). Moreover in Gly-Gly-Tyr (VI) and Gly-Gly-Gly-Tyr (VIII), the yield is independent of the charged state of amino group (Figures 1 and 2). Apparently, the addition of a series of peptide bonds to the amino end of tyrosine results in about 40% quenching.

Since a plateau appears to be reached in Gly-Gly-Gly-Tyr, we turn our attention to the carboxyl end of tyrosine. There is a profound reduction in quantum yield when the carboxyl group of tyrosine is protonated (X) (White, 1959) or esterified (XI). Very similar results have been reported for γ-(p-hydroxyphenyl)propionic acid and tyrosine methyl ester by Feitelson (1964). (Almost as great a fall occurs with acetyltyrosine (II) as for tyrosine when the carboxylate group is discharged, *i.e.*, 0.98–0.22, Figure 3.) Slightly less, but still strong quenching occurs with the amide group (XII). Substitution of a methyl group on the amide (XIII) increases the yield by 50%. This result illustrates the enormous influence that minor chemical changes can have on yield even when they are far removed from the fluorophore (Cowgill, 1963a). Perhaps of even greater interest is the effect of the discharge of the α-amino group. The yield of XIII doubles (0.38–0.76).

Addition of a carboxyl group to XIII, forming Tyr-Gly (XIV), has only a small effect on the yield (Table I). Discharge of the carboxylate group of XIV quenches its fluorescence by 33%. The effect of carboxyl ionization disappears completely when a second glycyl residue is added, thereby extending the chain by three atoms (XVI) (Figures 1 and 3). No further changes are observed in either Tyr-Gly_3 or Tyr-Gly_4 (Figure 1).

It appears that the effects of charge and peptide groups on the yield of tyrosine are largely eliminated after two glycyl residues have been added to both termini of tyrosine. The peptide group has little influence on the amino end. The major quenching on this side is due to the amino group. On the other hand, the peptide group contributes most of the quenching on the carboxyl end. The carboxyl group, however, has a very important influence when it is in the α position (Table I).

In XVIII and XIX, both ends of tyrosine are blocked by groups which have been independently evaluated. The yield in both compounds is greater than one would predict if both groups acted independently. The quenching processes must therefore be at least in part competitive.

The profound quenching of tyrosine fluorescence by the discharged carboxyl group prompted an evaluation of its effect on tyrosine derivatives where the fluorophore itself was modified. The two tyrosine analogs, as well

TABLE III: Limiting Polarization Values and Interphenol Distances in the Oligopeptide Series Tyr-Gly$_{0-2}$-Tyr.

Compound	P_e	r (Å)
Acetyltyrosinamide	0.36	
Tyrosine	0.36	
Tyr-Tyr	0.21	8.6
Tyr-Gly-Tyr	0.31	11.0
Tyr-Gly-Gly-Tyr	0.34	13.1

as 3,4-dihydroxyphenylalanine, show quenching either as large as or greater than observed in tyrosine (Table II). The emission of phenylalanine is much less affected than is that of tyrosine by protonation of its carboxylate group (Feitelson, 1964). Similar quenching was observed with *p*-fluorophenylalanine as in phenylalanine. The effect of carboxyl discharge is even smaller in tryptophan (22%) (Edelhoch *et al.*, 1967). If proton transfer from uncharged carboxyl is an important mechanism of quenching, then the phenol chromophore is the best and indole the poorest acceptor.

Excitation Energy Transfer. A. TYROSYL-TYROSYL. Radiationless energy transfer between tyrosyl residues in a peptide can be shown by fluorescence polarization measurements. Weber has demonstrated energy transfer between phenol molecules in propylene glycol films at −70° (1960a), and between tyrosyl residues in insulin, zein, and ribonuclease (1960a) by this technique. Energy transfer in a series of peptides containing two terminal tyrosyl residues, Tyr-(Gly)$_{0-2}$-Tyr, has been evaluated from their fluorescence polarization in 99% (v/v) glycerol–water solutions (Figure 4). A linear dependence of the emission anisotropy, $(1/P - 1/3)$, upon T/η was observed for all compounds, as well as for the models, tyrosine and acetyltyrosinamide (not shown), which contain only a single phenolic chromophore. Due to the low efficiency of phenol–phenol transfer, intermolecular energy transfer is very improbable in the dilute solutions employed.

Polarization values in the absence of rotational depolarization were obtained by extrapolation of the emission anisotropy to infinite viscosity ($T/\eta = 0$) and are presented in Table III). These extrapolated polarizations, P_e, are the intrinsic polarizations, P_0, for tyrosine and acetyltyrosinamide. These values, 0.36, are in good agreement with the value of 0.21 for tyrosine and cresol obtained by Weber (1960a) with unpolarized excitation ($P_p = 2P_n/(1 + P_n)$, where P_p and P_n are the polarizations obtained using polarized and unpolarized excitation, respectively). The P_e for Tyr-Tyr, 0.21, is appreciably smaller than that of tyrosine, indicating a significant amount of energy transfer between the two tyrosyl residues. As the distance between phenolic groups is increased by the interposition of glycyl residues, the efficiency of energy transfer decreases, and P_e approaches P_0 (Table III). Table III also presents the mean interphenolic distances computed as described in the Methods section. The increase of 2.0–2.5 Å/glycyl residue is reasonable, and suggests that the assumption of random orientation of the two chromophores used in the Forster equation is applicable to these molecules. The significance of the absolute values of the interphenolic distances is uncertain, owing to the numerous assumptions made in the application of the Forster and Weber equations. Further, since the rate of energy transfer varies inversely with the sixth power of the distance between donor and acceptor, the calculated mean distances are strongly weighted in favor of the shortest distances.

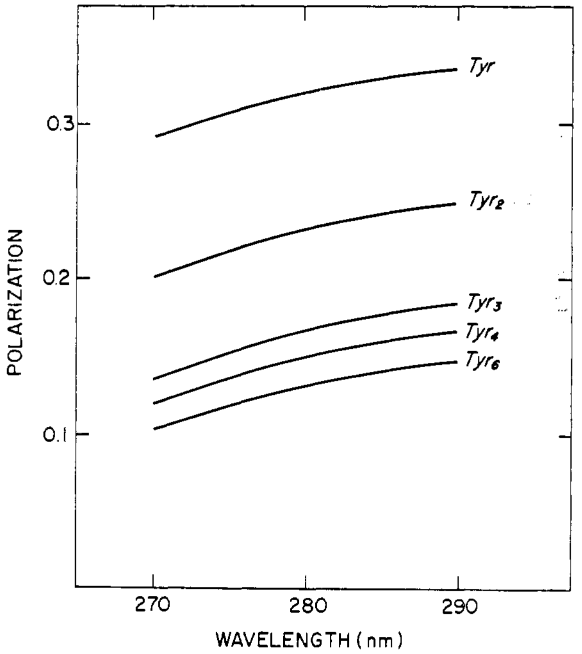

FIGURE 5: Excitation polarization spectra of Tyr$_{1-4,6}$ in 99% glycerol at 25°. Samples were excited with vertically polarized light, and their emission was analyzed at 310 nm.

Excitation polarization spectra were measured on the series of tyrosyl oligopeptides, Tyr$_{1,2,3,4,6}$, in 99% glycerol at 25° and are presented in Figure 5. The spectrum of tyrosine from 270 to 290 nm is similar to that reported by Weber (1960a). The shape of the spectra for the peptides remains the same as the values fall with increasing size. The decrease in polarization with increasing chain length clearly indicates energy transfer between residues. Because of the multiplicity of energy donors and acceptors, it is difficult to interpret the data quantitatively. However, the absolute polarizations seem to be approaching a limit with increasing tyrosyl residues indicating that energy transfer occurs primarily between close neighbors, and that there is probably only one or at most a few transfers before emission (Figure 6). The quantum yield decreased rapidly in the smaller peptides and appears to be reaching a limit in the hexapeptide (Figure 6).

B. TYROSYL-IONIZED TYROSYL. The transfer of electronic excitation energy from un-ionized to ionized tyrosine is more efficient ($R_0 = 15.2$ Å) than between two un-ionized tyrosyl residues since there is greater overlap between the tyrosine emission spectrum and the absorption spectrum of ionized tyrosine (Perlman *et al.*, 1968).

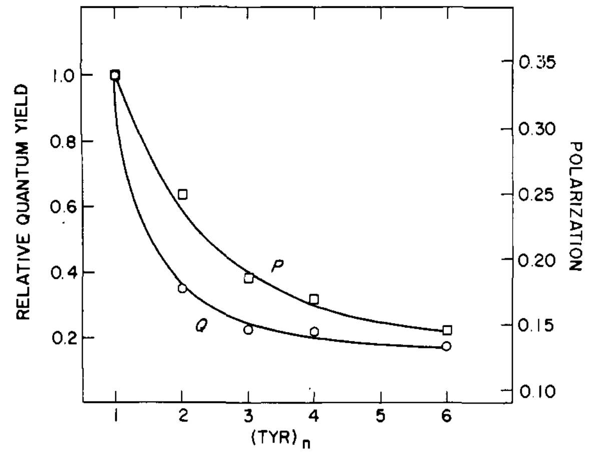

FIGURE 6: Relative quantum yields (○) and fluorescence polarizations (□) of $\text{Tyr}_{1-4,6}$. Quantum yields were measured in 0.005 M sodium acetate (pH 5.5). Fluorescence polarizations at 290-nm excitation were taken from Figure 5.

The efficiency of energy transfer to ionized tyrosyl residues in peptides containing two or more tyrosyl residues can be evaluated by comparing their fluorimetric and spectrophotometric titration curves. The degree of ionization of the phenolic groups is given directly by the spectrophotometric titration curve. In our analysis of energy transfer in these peptides, we have assumed that all tyrosyl residues have the same pK values and consequently that tyrosyl ionization was random.

In the three peptides Tyr-$(\text{Gly})_{0-2}$-Tyr, the residual fluorescence intensity at any degree of ionization, I_α, was equal to the fraction of totally un-ionized peptides, $(1 - \alpha)^2$. Thus, there was no detectable fluorescence due to the half-ionized peptides, and energy transfer from phenolic to phenolate groups in these peptides was complete. From the calculated value of R_0 and the previously determined interphenolic distances, only about 70% energy transfer would be expected in Tyr-Gly-Gly-Tyr (transfer efficiency = $1/(r/R_0)^6 + 1$, where r is the mean distance between donor and acceptor, and R_0 is the critical transfer distance). This discrepancy reflects either the approximations and simplifying assumptions used in the calculations or a structural difference between the un-ionized and ionized forms in which the phenolic groups are closer in the latter than in the former species. In any case, there is qualitative agreement between theory and experiment, in that ionized tyrosyl residues are more efficient energy acceptors than the un-ionized residues.

The A chain of insulin contains two tyrosyl residues separated by four other amino acids (-Tyr-Gln-Leu-Glu-Asn-Tyr-). The fluorimetric and spectrophotometric titrations of this peptide are compared in Figure 7, where the fluorescence intensity is plotted as a function of the degree of ionization. The experimental data can be approximated by a theoretical line corresponding to 70% energy transfer. Figure 7 also shows the limits to be expected in the cases of 0 and 100% energy transfer. Obviously, the residual fluorescence intensity is not a very sensitive function of the efficiency of energy transfer. Using the value of 70% quenching, the mean interphenol distance in this peptide can be estimated as 13.2 Å. While this value is reasonable, it must be taken only as an approximation.

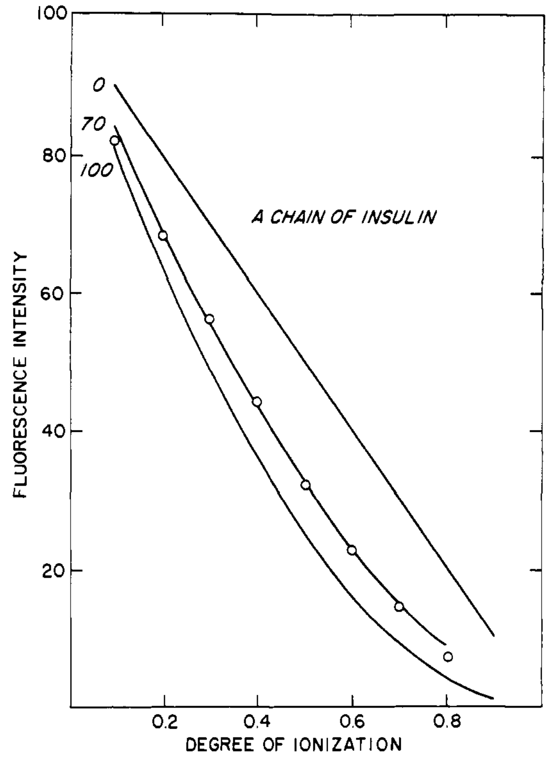

FIGURE 7: Fluorimetric *vs.* spectrophotometric titrations of the oxidized insulin A chain. Fluorescence intensity (maximum 100) is plotted against α, the fraction/of tyrosyl residues ionized, determined from the spectrophotometric titration. The points are the experimental values; the curves are the theoretical curves corresponding to 0, 70, and 100% energy transfer between tyrosyl residues in peptide containing one ionized and one un-ionized tyrosyl residue.

Energy transfer to ionized tyrosyl residues was also studied in a series of oligotyrosine peptides, $(\text{Tyr})_{1-6}$. Figure 8 shows the fluorimetric titrations of several of these peptides. Clearly, as the chain length increases, the titration curve is shifted to lower pH values, and the pH dependence becomes greater, indicating that fewer ionized tyrosyl residues are required to quench fluorescence. The pK's of the tyrosyl residues in all of these peptides were very similar. Analysis of this data suggests that ionization of one tyrosyl residue in any peptide, including the hexamer, is sufficient to quench the fluorescence of all other residues. Knopp and Longworth (1968) also found 100% energy transfer between un-ionized and ionized tyrosyl residues in Tyr-Tyr and in Tyr-Tyr-Tyr. These authors used a complex solvent, containing water, dimethyl sulfoxide, and ethylene glycol. In this solvent, they noted a small electrostatic interaction between the tyrosyl residues in their peptides, so that ionization of these tyrosyl residues was not random. In aqueous solution, however, we find that the tyrosyl ionization is apparently random, and therefore that the electrostatic interaction between tyrosyl residues is insignificant. Previously, Longworth and Rahn (1967) estimated that 1 ionized tyrosyl residue in polytyrosine can quench 50 un-ionized residues.

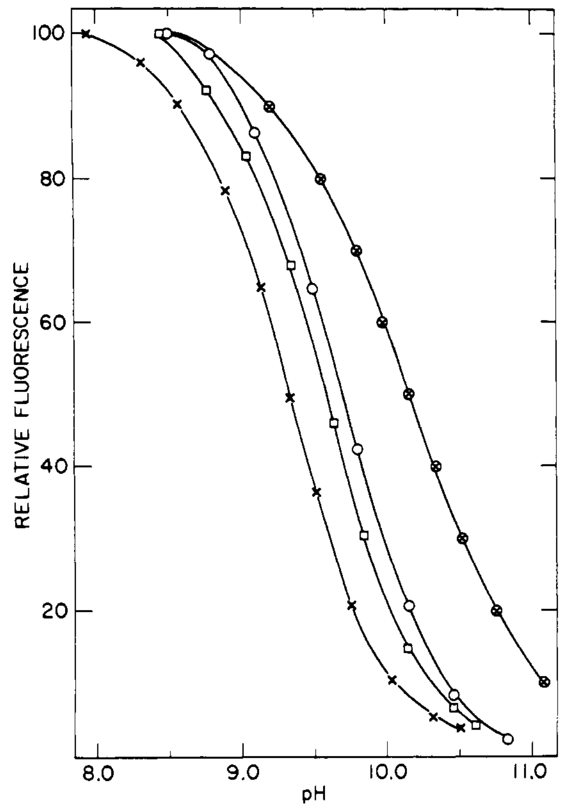

FIGURE 8: Fluorimetric titrations of Tyr$_{1-3,6}$ (⊗, O, □, and ×, respectively) in 0.1 M KCl–0.02 M lysine. Samples were excited at 278 nm, and their emission was measured at 305 nm. Here, the maximal fluorescence intensity for each sample was set to 100. All samples had similar spectrophotometric titration curves, with 50% ionization at about pH 10.

C. TYROSYL-TRYPTOPHANYL. Energy transfer from indole to un-ionized phenol does not occur, since there is negligible overlap between the indole emission spectrum and the phenol absorption spectrum. However, there is spectral overlap and the possibility of energy transfer in the reverse direction. Weber has demonstrated energy transfer from phenol to indole in propylene glycol at −70° (1960a). In order to enhance the possibility of observing the transfer of excitation energy from tyrosine to tryptophan in peptides, we have used 233-nm excitation where the ratio of molar extinction coefficients of acetyltyrosinamide to acetyltryptophanamide is maximal (Edelhoch *et al.*, 1968). The emission spectra of the series of compounds Trp-(Gly)$_{0,2,4}$-Tyr are presented in Figure 9. These spectra have been normalized in the region above 350 nm, where only tryptophanyl fluorescence occurs. A short-wavelength shoulder, representing tyrosyl emission, is clearly evident in tetra- and hexapeptides. Its absence in Trp-Tyr must be due to energy transfer from tyrosine to tryptophan in this peptide. Since the R_0 calculated for this process is 14.7 Å (Perlman *et al.*, 1968), it is not surprising that energy transfer also occurs in the tetrapeptide.

Discussion

The complexity of the effects of neighboring charged groups on tyrosyl fluorescence becomes apparent when we compare the model glycyl-tyrosyl peptides studied here with large copolypeptides of glutamic acid or lysine containing a few per cent tyrosyl residues. In the latter polypeptides, tyrosyl fluorescence is strongly quenched by uncharged amino and by charged carboxyl groups. Quenching of tyrosyl fluorescence by these two functional groups is consistent with the quenching of phenol fluorescence by carboxylates and amines. Weber and Rosenheck (1964) have interpreted these effects as due to proton transfer from tyrosine in the excited state to an acceptor, followed by a radiationless transition to the ground state. Stryer (1966) has demonstrated an enhancement of tyrosine quantum yield in D_2O, indicating that excited-state proton-transfer reactions are important in the quenching of tyrosine fluorescence.

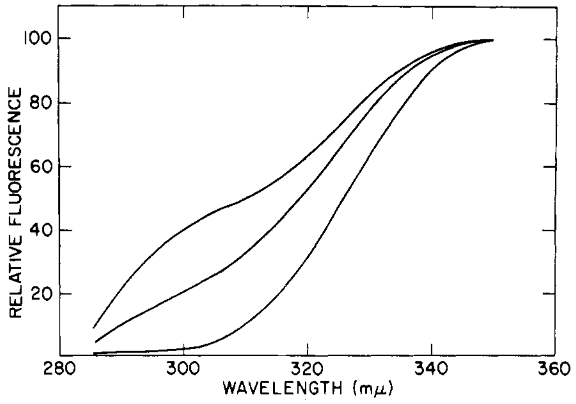

FIGURE 9: Fluorescence emission spectra of Trp-Tyr (lower curve), Trp-Gly$_2$-Tyr (middle), and Trp-Gly$_4$-Tyr (upper), in 0.005 M sodium acetate (pH 5.5). Samples were excited at 233 nm. The emission spectra have all been normalized in the region above 350 nm.

In contrast to the behavior of these copolypeptides, in the present series of compounds tyrosyl fluorescence is quenched by charged amino and uncharged carboxyl groups. Phenol fluorescence is quenched by H^+ ions with a K of 0.28 l./mole (White, 1959). It seems plausible that the quenching of tyrosine fluorescence by charged amino groups is due to proton transfer from the amino group to the excited phenol. Of course, the degree of quenching of tyrosine fluorescence by charged amino groups will depend upon other factors, such as the distance between the amino group and the phenolic ring, the lifetime of the excited state, and probably on the pK of the amino group.

Quenching of tyrosine fluorescence by uncharged carboxyl groups may also involve proton transfer to the excited phenol, as suggested by Weber and Rosenheck (1964). The latter authors also suggest that a dark complex is probably formed between the aromatic ring and the carboxyl group.

Quenching of tyrosyl emission by peptide bonds is probably an important reason for their very low quantum yields in proteins. In the diketopiperazine of glycine and tyrosine, the tyrosyl group forms a complex with the dipeptide ring which strongly quenches its fluorescence (H. Edelhoch, M. Wilchek, and R. Bernstein, unpublished). In addition, however, tyrosine fluorescence in proteins may be quenched by other noncovalent interactions and by energy transfer. Since energy transfer from tyrosine to tryptophan ($R_0 = 15$ Å) is more efficient than transfer from tyrosine to tyrosine ($R_0 = 9$ Å), tryptophan is probably the more important energy acceptor in proteins. Comparing the tetrapeptides, Trp-Gly-Gly-Tyr and Tyr-Gly-Gly-Tyr, we have observed significant energy transfer in the former (by decrease in tyrosyl emission) and negligible energy transfer in the latter (by depolarization of fluorescence). Because these critical transfer distances are relatively small, energy-transfer processes are relatively unimportant in the unfolded state, compared with their importance in the native globular conformation. Quenching of tyrosyl fluorescence by energy transfer in an unfolded polypeptide, however, will occur if the donor and acceptor are close together along the chain. Since these energy-transfer processes are so sensitive to interchromophore distances and angles, they provide a convenient way to monitor changes in protein structures.

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