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Effect of pH on Fluorescence of Tyrosine, Tryptophan and Related Compounds

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Unlike the absorption spectrum, the fluorescence spectrum of proteins is not the simple sum of the contributions of the aromatic amino acids in neutral water solutions (Teale & Weber, 1956). A study of the behaviour of substituted aromatic amino acids and simple peptides was undertaken in an attempt to elucidate phenomena contributing to the fluorescence of proteins. Ultraviolet excitation was used to reach the lowest excited singlet state of the compounds and interest was concentrated in energy loss by processes competing with fluorescence, i.e. a reduction in the quantum yield of fluorescence. The changes in fluorescence yield with hydrogen-ion concentration were studied.

EXPERIMENTAL

Methods

The fluorescence-excitation spectra were determined by means of the apparatus described by Teale & Weber (1956). Briefly, the principle was to irradiate a cell containing the test solution with monochromatic light and to view the light emitted at right-angles to the incident light by means of a photomultiplier. A filter to block any scattered exciting light was interposed between the cell and the photomultiplier. The photomultiplier was an EMI 6255 or 27M3 Mazda. A Perspex filter absorbing light of wavelengths less than 300 mμ was used to separate exciting light and fluorescence.

Cells of black mat glass with crystalline-quartz windows attached by a non-fluorescent cement were used to reduce to a negligible value cell fluorescence with irradiating light of wavelengths 230–260 mμ (Weber & Teale, 1958).

To eliminate the effect of pH on absorption of the tyrosine derivatives the illuminating wavelength was chosen at the isosbestic point (Fig. 1).

Hydrochloric acid was used to adjust the pH of the aqueous solutions on the acid side of neutrality and sodium hydroxide solution for the alkaline pH values. No buffer solutions were used. The instrument used to measure the pH values was a Cambridge Pye pH meter with a glass and a standard calomel electrode. The pH scale was standardized according to the B.S. specification.

Materials

The methyl esters of tyrosine and tryptophan were prepared by the following modification of the method of Brenner & Huber (1953). A mixture of thionyl chloride, methanol and tyrosine (in the molecular proportions 1:8:1) was allowed to stand for 12 days at room temperature. The methanol was removed under reduced pressure, the residue dissolved in water, and adjusted to pH 6 and extracted with ether. A white crystalline material was obtained (m.p. 134–138° uncorr.), which on a paper chromatogram with butanol–acetic acid–water solvent mixture (4:1:5) had R_F 0·585 compared with 0·3 for tyrosine. In the preparation of the tryptophan methyl ester the thionyl chloride, methanol, tryptophan mixture (in the molecular proportions 1:8:1 as described above) was maintained at 0° for some hours until a heavy salmon-pink precipitate was formed from the dark-red solution. The methanol was removed as described above and an ether extraction performed. Upon crystallization from chloroform white crystals which became yellow on standing were obtained (m.p. 70° uncorr.) *N*-Acetyltyrosine was prepared from L-tyrosine by the method of du Vigneaud & Meyer (1932). *N*-Acetyltryptophan was prepared from DL-tryptophan by the method of du Vigneaud & Sealock (1932). Polytyrosine (average chain length 45) was a gift from Dr E. Katchalski. Glycyltyrosylglycine was a gift from Dr R. Pitt-Rivers. Tyrosine *O*-phosphate was a gift from Dr T. Hofman. The glycyl derivatives were commercially available, glycyltyrosine from Hoffman–La-Roche and Co. and glycyltryptophan from Roche Products Ltd.

RESULTS AND DISCUSSION

Fluorescence-excitation spectra

The fractional absorption spectra at neutrality were found to correspond well with the fluorescence-excitation spectra both at neutrality and in

the region of increased quantum yield, i.e. pH 9·5 for tyrosine derivatives and pH 10·3–11·0 for tryptophan derivatives. The fluorescence-excitation spectrum and the fractional absorption spectrum are defined by Teale & Weber (1956).

Tyrosine and derivatives

The pH value at which half of the fluorescence at neutrality is present will be called in the following the pH_f value. For tyrosine methyl ester $pH_f = -0·6$ (Fig. 1). Glycyltyrosine and tyrosine have pH_f values of 3·0 and 2·45. At this concentration the quenching is probably non-collisional and may be envisaged as the transport of H^+ ion by the CO_2^- group from the solution to the neighbourhood of the excited molecule. For collisional quenching one or more collisions must occur during the lifetime of the excited state (Bowen, Barnes & Holliday, 1947; Umberger & la Mer, 1945). From diffusion theory, $Z = 10^{10}.c\tau$, where Z = number of collisions/sec., c = molar concentration of quencher, τ = lifetime of the excited state, with $\tau \simeq 10^{-8}$ sec. For pH_f 3·0, $Z = 0·1$, whereas for pH_f 0, $Z = 100$. The low values (pH_f 0·3 to −0·8) observed for the other derivatives are therefore consistent with the familiar collisional quenching by H^+ ions.

In the alkaline range tyrosine, phenol and tyramine exhibit pH_f 9·7, but other derivatives show a displacement to higher pH values (Fig. 2). The pK values for dissociation of the phenolic hydroxyl group are: phenol 10, tyrosine 10·07 (electrometric titration, Winnek & Schmidt, 1935), glycyltyrosine 10·4 (electrometric titration, Greenstein, 1932), polytyrosine approx. 11·3 (spectrophotometric titration, Katchalski & Sela, 1953). The quenching at alkaline pH follows closely the titration curve of the phenolic hydroxyl, thus showing that the ionized species is non-fluorescent. Where the phenolic group is substituted as in anisole and tyrosine O-phosphate quenching of fluorescence does not occur until pH values greater than 14 are reached.

Crammer & Neuberger (1943) working with ovalbumin and Tanford (1950) with serum albumin have related an increased pK of the phenolic group in proteins with hydrogen bonding. An inhibition of dissociation of the phenolic group in polytyrosine is in keeping with the experimental findings of a high pH of alkaline-quenching of fluorescence.

The alkaline pH_f values in glycyltyrosine, glycyltyrosylglycine and tyrosine methyl ester may correspondingly suggest some inhibition of dissociation of the phenolic group. There may possibly be interaction of the long flexible side chain of the glycyl derivatives with the phenolic group directly and perhaps an indirect effect via the aromatic nucleus where the side chain is short as in the methyl ester.

A feature of the alkaline pH range was the appearance of a maximum in the fluorescence of the glycyl derivatives at pH 9·5 (Fig. 4).

The quantum yields relative to that of tyrosine (Weber & Teale, 1957) were measured with neutral aqueous solutions of a standard optical density at 270 mμ (Table 1). Removal of the side chain, removal of the carboxyl group and N-acetylation leave the quantum yield almost unchanged. Glycylation of the amino group and esterification of the carboxyl group both considerably reduce the quantum yield of fluorescence.

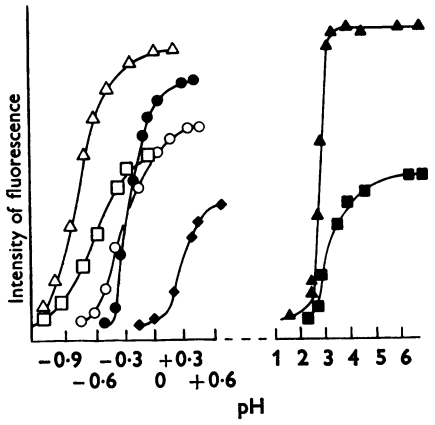

Fig. 1. Acid-quenching of fluorescence of tyrosine derivatives. Wavelength at isosbestic point (given for each compound) was used for excitation. ▲, Tyrosine (268 mμ); ■, glycyltyrosine (268 mμ); ○, glycyltyrosylglycine (268 mμ); ●, phenol (258 mμ); □, tyrosine methyl ester (269 mμ); △, tyramine (265 mμ); ◆, polytyrosine (268 mμ).

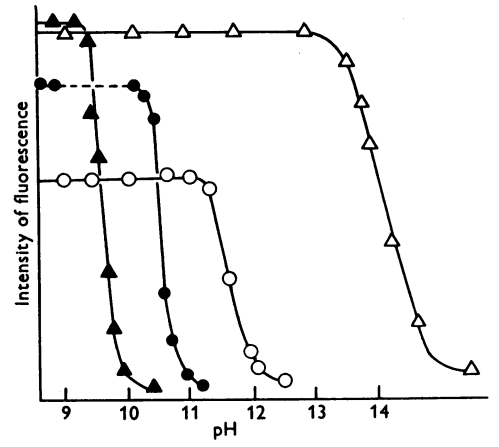

Fig. 2. Alkaline-quenching of fluorescence of tyrosine derivatives. Wavelength at isosbestic point was used for excitation. ▲, Tyrosine, phenol, tyramine; ●, glycyltyrosine, glycyltyrosylglycine, tyrosine methyl ester; ○, polytyrosine; △, tyrosine O-phosphate, anisole.

Table 1. *Quantum yield of fluorescence of tyrosine and tryptophan derivatives in neutral aqueous solution*

Compound	Quantum yield (exciting wavelength 270 mμ)
Tyrosine	0·21
Phenol	0·20
Tyramine	0·21
N-Acetyltyrosine	0·21
Glycyltyrosine	0·079
Tyrosine methyl ester	0·046
Glycyltyrosylglycine	0·05
Polytyrosine	0·072
Anisole	0·166
Tyrosine *O*-phosphate	0·18

Compound	Quantum yield (exciting wavelength 280 mμ)
Tryptophan	0·20
Indole	0·40
Tryptamine	0·40
N-Acetyltryptophan	0·25
Glycyltryptophan	0·055
Tryptophan methyl ester	0·06

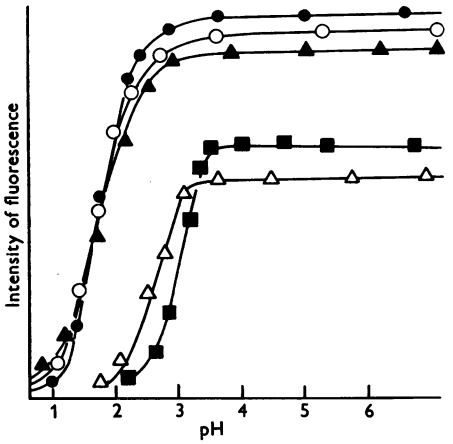

Fig. 3. Acid-quenching of fluorescence of tryptophan derivatives. Wavelength of excitation 280 mμ. ●, Indole; ○, tryptamine; ▲, tryptophan methyl ester; △, tryptophan; ■, *N*-acetyltryptophan.

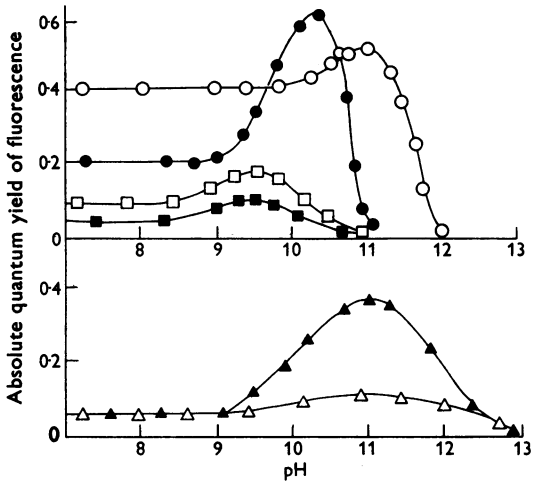

Fig. 4. Occurrence of maxima in alkaline-quenching curves of tryptophan and three derivatives, and two tyrosine derivatives. ●, Tryptophan; ○, tryptamine; ▲, tryptophan methyl ester; △, glycyltryptophan. □, glycyltyrosine; ■, glycyltyrosylglycine.

Tryptophan and derivatives

A comparable set of data was collected for the tryptophan derivatives. Acetylation of the amino group (Fig. 3) left the acid pH_f value at 2·8, which was close to that of tryptophan (pH_f 2·4). As with the tyrosine derivatives, removal or esterification of the carboxyl group displaced the pH of acid-quenching to lower values. Indole is not intermediate as was phenol and the shift in pH values is only −1 pH unit whereas in phenol the displacement was −3 pH units.

The tryptophan derivatives examined showed alkaline pH_f values ranging from pH_f 10·9 in tryptophan to pH_f 12·75 in tryptophan methyl ester. The compounds exhibiting this displacement include tryptamine and indole, in contrast with the similarity of alkaline pH_f shown by tyrosine, tyramine and phenol.

Four of the tryptophan derivatives exhibited a fluorescence maximum (Fig. 4). These and the two tyrosine derivatives which show this peak in the alkaline-quenching curve have a free amino group, and the region of rise in fluorescence corresponds with titration of the amino group. The maximum is therefore due to the combination of two factors: (i) titration of the amino group giving an uncharged species with a higher fluorescent yield and (ii) either quenching by hydroxyl ions or ionization of the phenolic hydroxyl group. Because of variations in either or both of these factors the height and position of the maxima may be expected to show considerable variation.

The quantum yields of fluorescence of the tryptophan derivatives in neutral aqueous solution with exciting light of 280 mμ are given in Table 1. Where no side chain is present, as in indole, and where the side chain is small and lacking a carboxyl group, as in tryptamine, the quantum yield of fluorescence is greater than with tryptophan. *N*-Acetyltryptophan gives a quantum yield between that of indole and of tryptophan. However, glycyltryptophan and the tryptophan ester show a drastic reduction in quantum yield as with the corresponding tyrosine derivatives.

The alkaline-quenching has been found to be a particular case of the quenching of fluorescence by transfer of a H^+ ion from a nitrogen atom belonging to an excited molecule to the OH^- ion (G. Weber & A. White, unpublished work). Such cases were first observed by Boaz & Rollefson (1950). The necessity of a mobile hydrogen atom in NH for this reaction to occur has been checked by observing the lack of quenching of fluorescence of *N*-methyl indole by OH^- ions in 5N-sodium hydroxide. The reactions may be represented as:

$$R{\cdot}NH + h\nu \rightarrow R^*{\cdot}NH \text{ (excited singlet state)}$$

$$R^*{\cdot}NH + {}^-OH \rightarrow R^*{\cdot}N^- + H{\cdot}OH$$

$$R^*{\cdot}N^- \rightarrow R{\cdot}N^- \text{ (radiationless)}$$

$$RN^- + H{\cdot}OH \rightarrow R{\cdot}NH + {}^-OH$$

The reaction $R^*{\cdot}N^- \rightarrow R{\cdot}N^-$ is either radiationless (α-naphthylamine) or radiative with low efficiency (β-naphthylamine) (Boaz & Rollefson, 1950). In indole derivatives in N-sodium hydroxide no detectable fluorescence has been found to be emitted at any wavelengths.

SUMMARY

1. The influence of pH on the fluorescence of tyrosine, tryptophan and related compounds was investigated.

2. The pH of acidic half-quenching of fluorescence (pH_F) of tyrosine and tryptophan and their derivatives with an intact carboxyl group corresponds approximately to the pK of the carboxyl and falls to a much lower value in those derivatives where the carboxyl group is substituted or replaced.

3. Tyramine, tyrosine and phenol are converted into non-fluorescent species on dissociation of the phenolic hydroxyl group, the quenching of the fluorescence appearing as a sensitive test of this dissociation as shown by the effect of substituents, hydrogen-bonding and chemical substitution of the hydrogen.

4. The existence of a peak in the alkaline pH range is described for two tyrosine and four tryptophan derivatives. A free amino group is apparently a necessary but not a sufficient condition for this maximum to be present.

5. The relative quantum yield in neutral aqueous solution and at a chosen wavelength was measured for the compounds mentioned.

6. The fluorescence-excitation spectra of the majority of the derivatives were determined in neutral and alkaline solutions and found to correspond with the fractional absorption spectra at neutrality.

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The Nature of the Extra Protein Fraction from Myofibrils of Striated Muscle

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When isolated myofibrils are extracted with solutions considered to be selective for myosin most of the material responsible for the difference in refractive index between the A and I bands (A substance) is removed, Under these conditions Szent-Györgyi, Mazia & Szent-Györgyi (1955) have claimed that, in addition to the myosin, protein material amounting to approximately 20 % of the total myofibrillar nitrogen passes into solution. Evidence has been presented by de Villafranca (1956) that this additional material, the so-called

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