still an appreciable additional slowing of peptide hydrogen exchange in the native DNA–poly-L-lysine complex. We conclude that in postulated models of the DNA–poly-L-lysine complex, the polypeptide backbone should be positioned to provide at least a partially nonaqueous environment for the peptide hydrogens.

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The Luminescence of Tryptophan and Phenylalanine Derivatives*

Ira Weinryb† and Robert F. Steiner

ABSTRACT: The fluorescence and phosphorescence of a series of tryptophan and phenylalanine derivatives have been examined. For tryptophan compounds, the luminescence is much more sensitive to chemical modification at 25° than at liquid nitrogen temperatures. In particular, the observed phosphorescence lifetimes at 91°K were invariant within experimental uncertainty. The kinetic processes responsible for the variation in yield and lifetime at 25° were found to be first order with respect to the excited state. Proton transfer appears to be a dominant influence in some cases. Luminescence studies of the phenylalanine peptides revealed differences between peptides which persisted at liquid nitrogen temperatures.

During the last few years the luminescence properties of the aromatic amino acids have attracted considerable interest. Numerous investigations have been made of the free amino acids, their derivatives, and related model compounds (Teale and Weber, 1957; White, 1959; Cowgill, 1963a,b, 1967; Nag-Chaudhuri and Augenstein, 1964; Longworth, 1966; Bishai *et al.*, 1967). Synthetic polymers and copolymers containing tryptophan, tyrosine, and phenylalanine have been examined (Fasman *et al.*, 1964; Pesce *et al.*, 1964; Wada and Ueno, 1964; Weber and Rosenheck, 1964; Fasman *et al.*, 1966; Longworth, 1966; Lehrer and Fasman, 1967). The emission properties of these amino acids, when incorporated into proteins have also received considerable attention (Chen *et al.*, 1968, and references therein).

The interpretation of protein luminescence has been hampered by the multiplicity of environments in which the aromatic amino acids may be found, as a consequence of the wide variations in primary, secondary, and tertiary structure for different proteins. The systematic exploration of the behavior of simple peptide

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derivatives of the aromatic amino acids may be expected to be helpful in bridging the considerable gap between the free amino acids and natural proteins. The few existing observations upon such model compounds include the work of Edelhoch *et al.* (1967) upon several tryptophyl peptides and that of Steiner and Kolinski (1968) upon several oligopeptides containing tryptophan and tyrosine.

This paper presents the results of studies upon the fluorescence and phosphorescence of peptide and other derivatives of tryptophan and phenylalanine, with the objective of systematically extending knowledge of the factors governing the luminescence of these residues in a peptide or polypeptide environment.

Experimental Section

Materials

The following compounds were purchased from Sigma Chemical Co., St. Louis, Mo.: L-tryptophan (L-Trp), L-Trp-amide, *N*-acetyl-DL-Trp (Ac-DL-Trp), L-Trp-glycine (L-Trp-Gly), L-Trp-L-phenylalanine (L-Trp-L-Phe), L-Trp-L-tyrosine (L-Trp-L-Tyr), L-Trp-L-Trp, and L-phenylalanine (L-Phe).

N-Carbobenzoxy-L-Trp (Z-L-Trp), Z-L-Trp-amide, Ac-L-Trp-amide, Z-L-Trp-L-Tyr, Z-L-Trp-L-Tyr-amide, Z-L-Trp-L-Tyr methyl ester (Z-L-Trp-L-Tyr-Me), Z-L-Trp-L-Phe, Z-L-Trp-L-Phe-amide, Z-L-Trp-L-Phe-Me, Z-L-Trp-L-alanine (Z-L-Trp-L-Ala), Z-L-Trp-L-leucine (Z-L-Trp-L-Leu), Z-L-Trp-L-serine (Z-L-Trp-L-Ser), Z-L-Trp-Gly, L-Trp-Gly-Gly, Z-L-Trp-Gly-Gly, Z-L-Trp-Gly-Gly-Me, Z-L-Phe-L-Phe, L-methionyl-L-Phe (L-Met-L-Phe), L-histidyl-L-Phe (L-His-L-Phe), L-lysyl-L-Phe (L-Lys-L-Phe), and L-arginyl-L-Phe (L-Arg-L-Phe) were products of Cyclo Chemical Corp., Los Angeles, Calif. All the Cyclo derivatives were of purity Grade I, with the exception of Z-L-Trp, Z-L-Trp-L-Tyr-Me, Z-L-Trp-L-Phe-Me, and Z-L-Trp-Gly-Gly-Me, which were of purity Grade II. Grade I compounds are stated by the supplier to be chromatographically homogeneous in a minimum of two systems (one acidic, one basic), and are subjected to several additional analytical tests (see Cyclo Chemical Corp. catalog for details). Grade II compounds are also of high purity, and where chromatograms show traces of contaminants, such a report accompanies the product. Gly-L-Trp, L-Trp ethyl ester (L-Trp-Et), DL-Ala-DL-Phe, and Gly-DL-Phe were obtained from Nutritional Biochemicals Corp., Cleveland, Ohio.

Gly-Gly-L-Trp, Gly-Gly-Gly-L-Trp, L-Trp-Gly-L-Trp-Gly, Z-L-Phe-L-Phe, and Z-L-glutamyl-L-Phe (Z-L-Glu-L-Phe) were supplied through the courtesy of Dr. Harold Edelhoch of the National Institutes of Health and were prepared by Dr. M. Wilchek of The Weizmann Institute, Rehovoth, Israel.

The above compounds were used without further purification.

The solvent system used in all cases was a 50% ethylene glycol–50% 0.05 M KH_2PO_4 (v/v) solution, which was titrated to the indicated pH. Because the ethylene glycol present may affect the response of the glass electrode and the activity of hydrogen ion, some care should be exercised in comparing pH values in this solvent system with similar values in a purely aqueous solvent. However, fluorescence titration measurement of the pK_a value for the α-amino group of L-tryptophan in ethylene glycol–phosphate buffer revealed little if any change from the value in water (see Figure 1). Certified Reagent compounds (Fisher Scientific Co., Fair Lawn, N. J.) were employed. Glass-redistilled water was used for the preparation of all solutions.

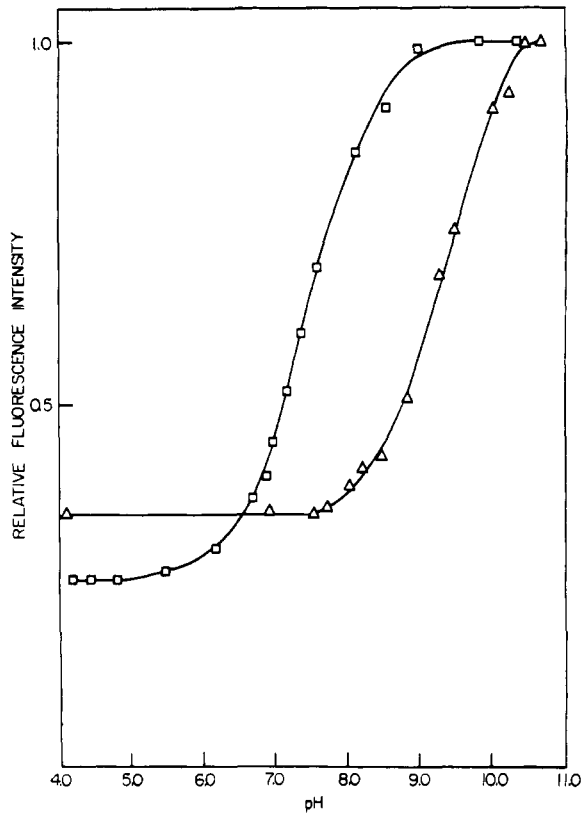

FIGURE 1: Relative intensities of fluorescence at 25° in 50% ethylene glycol–0.05 M PO_4 for two tryptophan derivatives as a function of pH. In each case the intensity relative to that at pH 10.5 is plotted (λ_{ex} = 290 mμ; λ_f = 350 mμ). (△) Trp; (□) Trp-amide.

Methods

Fluorescence Energy Yield Measurements. Fluorescence energy yields were measured at room temperature using an Aminco-Bowman spectrofluorometer equipped with an attachment for spectral compensation, which corrects fluorescence emission spectra for wavelength variations in the detector response. Quartz cells (1 cm) were used. Relative yields at constant absorbed energy were determined by direct comparison with L-Trp or L-Phe for each run according to the method of Parker and Rees (1960); the areas under the emission spectra were cut out and weighed. Base lines were obtained with a solvent blank. Solution absorbances at the excitation wavelength were generally below 0.2; however, all results were corrected for the "inner filter effect," as suggested by Weill and Calvin (1963).

TABLE I: The Fluorescence of Tryptophyl Derivatives.[a]

Compound[b]	λ_{max} (mμ)[c]	Rel[d] Energy Yield (Q_r)	Other Values[e]	Fluorescence[f] Lifetime (τ) (nsec)
1. L-Trp	357	1.00		2.9
2. Ac-DL-Trp	361	1.76	1.40	5.2
3. Ac-L-Trp-amide	356	0.87		3.8
4. L-Trp-amide	351	0.59	1.00	1.4
5. L-Trp-Et	349	0.17	0.16	0.5
6. L-Trp-Gly	350	0.65	0.70	1.6
7. L-Trp-Gly-Gly	348	0.48		1.8
8. Gly-L-Trp	356	0.49	0.29	1.4
9. Gly-Gly-L-Trp	362	0.65		2.0
10. Gly-Gly-Gly-L-Trp	360	0.76		2.4
11. Trp-Gly-Trp-Gly	352	0.69		2.0
12. L-Trp-L-Trp	361	0.50	0.45	1.5
13. L-Trp-L-Tyr	351	0.75	0.60	1.9
14. L-Trp-L-Phe	350	0.63		2.0
15. Z-L-Trp	358	1.34		4.3
16. Z-L-Trp-amide	355	1.27		4.0
17. Z-L-Trp-Gly	358	1.30		3.6
18. Z-L-Trp-Gly-Gly	354	1.16		3.3
19. Z-L-Trp-Gly-Gly-Me	354	1.08		3.0
20. Z-L-Trp-L-Tyr	358	1.34		3.8
21. Z-L-Trp-L-Tyr-amide	355	1.09		2.8
22. Z-L-Trp-L-Tyr-Me	356	0.98		2.8
23. Z-L-Trp-L-Phe	357	1.38		4.4
24. Z-L-Trp-L-Phe-amide	365	0.97		3.0
25. Z-L-Trp-L-Phe-Me	356	0.98		3.4
26. Z-L-Trp-L-Ala	356	1.29		4.3
27. Z-L-Trp-L-Leu	354	1.43		4.8
28. Z-L-Trp-L-Ser	353	1.22		4.0

[a] Results obtained at room temperature: 25 ± 2°. [b] Solution pH was 7.5 for N-blocked compounds and 4.7 for compounds with free α-amino groups. [c] Determined from point-by-point photometer galvanometer deflections and not corrected for detector response. Estimated precision: ±2 mμ. [d] Estimated precision: ±7%. [e] From Cowgill (1963a,b). [f] Estimated precision: ±0.3 nsec.

Fluorescence lifetime measurements were performed at room temperature with a TRW nanosecond spectral source system in the laboratory of Dr. Raymond Chen of the National Institutes of Health. The system had been modified to improve the accuracy of measurement at short lifetimes. Details of the operation of the above apparatus have been reported elsewhere (Chen *et al.*, 1967). A 270- or 290-mμ interference (primary) filter and a 9863 secondary filter were employed. Tryptophan derivative concentrations were about 0.3 mg/ml.

Total emission spectra at low temperatures were recorded with an Aminco-Bowman spectrofluorometer adapted for observations at liquid nitrogen temperatures. The instrument and experimental procedure have been described previously (Hoerman and Balekjian, 1966; Steiner and Kolinski, 1968). At equilibrium the temperature of the sample was approximately 91°K, as measured with a Cu-constantan thermocouple. The fluorescence/phosphorescence ratios calculated from photographs of the oscilloscope traces were corrected for the wavelength response of the R136 photomultiplier tube. Compound concentrations for the low-temperature observations were generally 0.3 mg/ml for tryptophan compounds and 2 mg/ml for phenylalanine compounds.

Phosphorescence Lifetime Measurements. Phosphorescence lifetimes were obtained with the low-temperature modified Aminco-Bowman spectrofluorometer, in which the electronic power supply for the photomultiplier had been replaced by a battery power supply. A manually operated rotating shutter which initially admitted the exciting beam was rotated by 90° at zero time, so as to allow passage of the phosphorescent radiation to the detector. Phosphorescence decay curves were observed with an oscilloscope and appeared strictly exponential in all cases, as expected for a single phosphorescent component. Photographs of the oscilloscope traces were used to calculate phosphorescence lifetimes, according to the relationship: $I/I_0 = \exp(-t/\tau)$, where I and I_0 are the intensities in arbitrary

units at times t and zero, respectively, and τ is the phosphorescence lifetime. The lifetimes were computed from the linear slopes of plots of the logarithm of intensity *vs.* time.

Results

Tryptophan Compounds. FLUORESCENCE AT ROOM TEMPERATURE. Since the intensity of fluorescence for tryptophan derivatives is dependent upon the state of ionization of the α-amino and α-carboxyl groups, it is important that different compounds be compared for similar states of ionization. Substitution of the α-carboxyl group may shift the pK of the α-amino to as much as two pH units below that of tryptophan (pK = 9.4), presumably because of removal of the negative charge. This is the case for tryptophanamide (Figure 1).

Derivatives containing a free α-amino group were measured at pH 4.75, which is well below the α-amino pK, but above that of the carboxyl. The results cited thus refer to the state of the molecule in which the α-amino group is protonated (NH_3^+), and the α carboxyl is ionized (COO^-). Compounds in which no free α-amino was present were measured at pH 7.5. For such compounds no significant difference was observed between pH 7.5 and 4.75. The results summarized in Table I therefore refer to comparable states of ionization.

For compounds with blocked vicinal α-carboxyl groups, in which the α-amino group was unblocked, the fluorescence yield and lifetime were invariably greater at pH 7.5, at which the α-amino was partially unprotonated, than at pH 4.75, where it was completely protonated. For example, the relative fluorescence yield and lifetime of Trp-Gly were 1.1 and 4.7 (nsec) at pH 7.5, respectively, and 0.65 and 1.6 at pH 4.75.

Several trends are obvious from an inspection of Table I. (1) Blockage of the α-amino group by acetylation or carbobenzoxylation invariably increases the energy yield and fluorescence lifetime relative to the parent compound, irrespective of the state of substitution of the α-carboxyl group. It is instructive to compare the following pairs: 1, 2; 3, 4; 1, 15; 13, 20; 14, 23; 6, 17; 7, 18; and 4, 16. (2) The formation of an amide or ester derivative of the α-carboxyl group reduces the fluorescence yield and lifetime. This is the case even when the α-carboxyl is separated from the tryptophan by another residue (compare 1, 4; 1, 5; 2, 3; 15, 16; 18, 19; 20, 21; 20, 22; 23, 24; and 23, 25). The effect is in general much less pronounced if the α-amino group is carbobenzoxylated. (3) The formation of a peptide bond at either the α-amino or α-carboxyl end reduces fluorescence yield and lifetime (compare 1, 6; 1, 7; and 1, 8). Again the effect is largely suppressed if the α-amino group is carbobenzoxylated (compare 15, 17, and 18). (4) Progressive removal of the α-amino from the indole nucleus raises the fluorescence yield and lifetime. Both quantities increase in the series $(Gly)_n$-L-Trp as n increases from 1 to 3 (compare 8, 9, and 10).

LOW-TEMPERATURE LUMINESCENCE. At liquid nitrogen temperatures, the relative intensities of fluorescence and phosphorescence for L-Trp were independent of the measured apparent pH value at room temperature for values between pH 2 and 12, in harmony with the findings of Bishai *et al.* (1967). Similarly, the fluorescence/phosphorescence (F/P) ratios were identical at pH 4.75 and 7.5 for L-Trp-amide and L-Trp-Gly. Longworth (1966) has reported that the luminescence of aromatic amino acids is little affected by the state of ionization of their amino and carboxyl groups at 77°K. Accordingly, all determinations were carried out at pH 7.5.

Two conclusions stand out from the results summarized in Table II. The phosphorescence lifetimes of these tryptophan derivatives are little influenced by the nature of the compound; in fact, the lifetime is constant within experimental error and is equal to 6.2 ± 0.3 sec for all the entries in Table II. This is in complete contrast to the fluorescent lifetimes.

Moreover, the F/P ratios are also fairly insensitive

TABLE II: Low-Temperature (91°K) Luminescence Studies on Tryptophyl Peptides and Derivatives.

Compound[a]	Rel Fluorescence/ Phosphorescence Ratio (F/P)[b]	Phosphorescence Lifetime (sec)[c]
1. L-Trp	1.00	6.3
2. Ac-DL-Trp	1.26	6.0
3. Ac-L-Trp-amide	1.15	6.4
4. L-Trp-amide	0.94	6.1
6. L-Trp-Gly	1.00	6.2
7. L-Trp-Gly-Gly	1.00	6.2
9. Gly-Gly-L-Trp	1.07	6.2
10. Gly-Gly-Gly-L-Trp	1.00	5.9
11. L-Trp-Gly-L-Trp-Gly	0.90	6.2
12. L-Trp-L-Trp	0.99	6.3
13. L-Trp-L-Tyr	0.89	6.5
14. L-Trp-L-Phe	0.97	6.2
15. Z-L-Trp	1.11	6.3
16. Z-L-Trp-amide	1.15	6.3
23. Z-L-Trp-L-Phe	1.00	6.1
24. Z-L-Trp-L-Phe-amide	1.01	6.1
25. Z-L-Trp-L-Phe-Me	1.25	6.1
26. Z-L-Trp-L-Ala	0.93	6.1
27. Z-L-Trp-L-Leu	1.04	6.4
28. Z-L-Trp-L-Ser	1.01	6.3

[a] Solution pH at room temperature = 7.5. [b] Estimated precision = ±10%. [c] Estimated precision = ±0.3 sec.

to the chemical nature of the tryptophan derivative. In some cases, blockage of the α-amino group by acetylation or carbobenzoxylation results in a probably significant, although small, increase in F/P ratio relative to the parent compound (compare 1, 2; 1, 15; 3, 4; and 4, 16). The efficiency of intersystem crossing thus appears to be slightly decreased by this kind of substitution. Note also the significant increase in F/P when Z-L-Trp-L-Phe is O methylated (compare 23 and 25), despite the fact that this substitution decreased the fluorescent yield and lifetime at room temperature.

A comparison of relative fluorescence yields at room temperature and liquid nitrogen temperature is also of particular interest (Table III). The ratio of yields

TABLE III: Temperature Dependence of Fluorescence Yield of Several Tryptophyl Compounds.

Compound	Excitation Wavelength (mμ)	Fluorescence, 298°K/ Fluorescence, 91°K	Rel Fluorescence Energy Yield (298°K)
L-Trp	260	0.42	1.00
L-Trp-Gly	270	0.28	0.65
L-Trp-amide	250	0.24	0.59
L-Trp-Et	260	0.07 (3)	0.17

at 25°C and 91°K (third column of Table III) vary considerably for the four compounds examined. The thermal dependence of fluorescence yield is thus very sensitive to the nature of the tryptophan derivative. In addition the relative yields at 91°K (the ratio of column 4 to column 3) are constant within experimental error, despite a fivefold variation in yield at 25°.

Phenylalanine Compounds. FLUORESCENCE AT ROOM TEMPERATURE. Both the quantum yield and the molar absorbancy of phenylalanine are much lower than those of tryptophan. At accessible concentrations the intensities of fluorescence were too low to permit reliable measurements of excited lifetime. However, measurements of relative fluorescence yield could be made with reasonable precision and are cited in Table IV for a series of phenylalanine peptides in 50% ethylene glycol at pH 4.7.

Inspection of Table IV shows that, in contrast to the tryptophan case, formation of a peptide bond does not invariably reduce the fluorescence yield. The yields for Gly-Phe and Ala-Phe are larger than that of Phe. The proximity of a basic residue at the N-terminal end of a dipeptide has a significant quenching effect (compare Lys-Phe, His-Phe, and Arg-Phe with Gly-Phe).

The most dramatic effect is observed in the case of Met-Phe, whose yield is less than half that of Phe.

TABLE IV: Fluorescence Studies on Phenylalanyl Peptides.[a]

Peptide[b]	λ_{max} Fluorescence,[c] (mμ)	Rel Energy Yield[d]
Gly-DL-Phe	287	1.15
DL-Ala-DL-Phe	285	1.10
L-Phe	287	1.00
L-Lys-L-Phe	287	0.98
L-His-L-Phe	288	0.82
L-Arg-L-Phe	286	0.77
Z-L-Phe-L-Phe	288	0.69
Z-L-Glu-L-Phe	287	0.52
L-Met-L-Phe	287	0.44

[a] Studies at room temperature = 25 ± 2°. [b] Solution pH 4.7. [c] Determined from galvanometer deflection. Estimated precision = ±2 mμ. [d] Estimated precision = ±7%.

In view of the results of Cowgill (1967), it is possible that the presence of a sulfur atom is the dominant influence.

Both of the carbobenzoxylated dipeptides examined have reduced fluorescence yields, as compared with phenylalanine itself. However, a direct comparison is difficult, since the primary absorption band of the carbobenzoxy group overlaps that of phenylalanine itself.

LOW-TEMPERATURE LUMINESCENCE. Again in contrast to the tryptophan series, the F/P ratios for phenylalanine peptides at liquid nitrogen temperatures show significant variations according to their chemical nature (Table V). Several of the basic peptide deriva-

TABLE V: Low-Temperature (91°K) Luminescence Studies on Phenylalanyl Dipeptides.

Peptide[a]	Rel Fluorescence Phosphorescence Ratio (F/P)[b]	Phosphorescence Lifetime (sec)[c]
L-Phe	1.00	5.6
DL-Ala-DL-Phe	0.98	5.1
Z-L-Phe-L-Phe		3.6
L-Met-L-Phe	0.91	5.6
L-Arg-L-Phe	0.85	5.6
Gly-DL-Phe	1.02	5.8
L-His-L-Phe	0.83	5.4
Z-L-Glu-L-Phe		4.0
L-Lys-L-Phe	0.80	5.7

[a] Solution pH at room temperature = 7.5. [b] Estimated precision = ±10%. [c] Estimated precision = ±0.3 sec.

tives have somewhat reduced values of F/P, compared with phenylalanine itself. This is the case for His-Phe, Arg-Phe, and Lys-Phe. The value for Met-Phe is similar to that for phenylalanine itself.

The phosphorescence lifetimes are also very similar for all the phenylalanine dipeptides, with two exceptions. The two exceptions are the carbobenzoxylated dipeptides, which have reduced lifetimes.

Discussion

Tryptophan Compounds. A consistent feature of the results shown in Table I is the approximate constancy of the ratio of relative energy yield to fluorescent lifetime. The ratios do not differ from that for tryptophan itself (0.34 ± 17%) to an extent in excess of experimental uncertainty, with the possible exception of acetyl tryptophanamide, whose deviation is not greatly outside of error. This constancy of ratio is observed despite variations as much as tenfold in quantum yield and lifetime.

If all quenching processes are first order with respect to the concentration of the lowest excited state, then Q, the quantum yield, is given by

$$Q = k_f/(k_f + k_c + k_x) \quad (1)$$

where k_f, k_c, and k_x are the rate constants for fluorescence emission, radiationless deactivation, and intersystem crossing, respectively. The excited lifetime, τ, is given by

$$\tau = 1/(k_f + k_c + k_x) \quad (2)$$

For two different fluorescent molecules

$$\frac{Q_1/\tau_1}{Q_2/\tau_2} = k_{f1}/k_{f2} \quad (3)$$

If Q/τ is constant ($(Q_1/\tau_1)/(Q_2\,\tau_2) = 1$), the implication is that all quenching processes are first order and that k_f is the same for all species. In the present case it follows that: (1) The characteristics of the lowest excited state of indole are probably similar for all members of this series of compounds, in view of the constancy of k_f. The magnitude of k_f is a measure of the probability of an electronic transition between the lowest excited state and the ground state, which is in turn dependent upon the oscillator strength of the primary absorption band. (2) The factors responsible for the variations in quantum yield probably involve only quenching processes which are first order with respect to the excited state. In particular, interactions of the ground state (which would alter Q, but not τ) are probably not a dominant influence. Quenching processes of interest, which may occur with constant probability during the lifetime of the excited state, include intersystem crossing to the triplet state and interaction of the excited state with solvent or with an atom or group transferred from another part of the same molecule.

The efficiency of intersystem crossing is largely dependent upon the energy gap between the lowest excited singlet and triplet states and upon the extent of singlet–triplet mixing in the latter. While no direct measurements of intersystem crossing are available for these compounds at 25°, the rates of intersystem crossing at 91 °K do not vary greatly.

Walker *et al.* (1967) have stressed interactions of the excited state with solvent ("exciplex" formation) in explaining the effects of solvent upon the quantum yield of substituted indoles. There is an implication that this may compete to an important extent with direct emission by the initial excited state.

A second relevant factor is the possibility of intramolecular transfer of a group to an excited indole from the balance of the molecule. A plausible, although conjectural, mechanism of this kind is the transfer of a proton to the excited indole ring. The quenching effect of the protonated α-amino (NH_3^+) group of tryptophan has been explained on this basis by White (1959) and by Weber (1961). A formal possibility for the proton acceptor would be the imino (NH) group of indole. Objections to this model have been raised on the grounds that the imino nitrogen of excited indole is too electron deficient to be a good proton acceptor (Walker *et al.*, 1967). Indeed, the mechanism proposed for fluorescence quenching of indole and tryptophan in alkaline solution involves *abstraction* of a hydrogen ion from the indole ring imino nitrogen by a hydroxyl ion (Weber, 1961; White, 1959). Hence proton transfer may be to another part of the indole ring, if it is indeed a factor.

At liquid nitrogen temperatures proton transfer would be blocked. This is consistent with the observation that protonation of the α-amino group drastically reduces the quantum yield of tryptophan at 25°, but does not affect it at 77 °K (Bishai *et al.*, 1967).

It is clear from the results cited earlier that the fluorescent yield and lifetime of tryptophan at 25° are very sensitive to modification of the α-amino or α-carboxyl group (Table I). Formation of a peptide bond at either position or conversion of the α-carboxyl into an ester or amide drastically reduces the yield and lifetime, while acetylation of the α-amino group increases both. Since the differences in yield largely disappear for the rigid glass at liquid nitrogen temperatures (Table III), it is likely that the controlling factor in these cases is a temperature- and viscosity-dependent process. Quenching by intramolecular proton transfer falls into this category. "Exciplex" formation (Walker *et al.*, 1967) may also be blocked at liquid nitrogen temperatures, since this process would be dependent upon contact between a tryptophan and solvent molecule and upon their mutual orientation.

Any model accounting for the variation in luminescent properties among these tryptophan derivatives must explain the wide variations in quantum yield and lifetime at 25°, as contrasted to their relative constancy at 91 °K, the only minor variation in λ_{max} at 25°, and the shift in λ_{max} from 350–360 mμ at 25° to 320 mμ at 91 °K. The third of these could be explained on the exciplex model, as well as by the theory of Lip-

pert (1957). The second may be difficult to interpret in terms of the exciplex theory, which would predict shifts in λ_{max} which are correlated with the variations in energy yield. The bearing of the exciplex model on the first observation must be left open at present, pending more definitive information as to the details of the mechanism.

Although both proton transfer and exciplex formation may figure in the variation of quantum yield and lifetime at 25°, the effects of acetylation and carbobenzoxylation of the α-amino group in raising the quantum yield and lifetime may be interpreted in terms of removal of the permanent charge from the α-amino group and consequent blockage of proton transfer. A CH_3CONH or a $C_6H_5CH_2OCONH$ group should be a less efficient proton donor than an NH_3^+ group. The tentative nature of this model should, however, be stressed.

The effects of ester or amide formation by the α-carboxyl may possibly be partially attributed to an enhancement of proton transfer from the α-amino group, as a consequence of abolition of the negative electrostatic potential of the ionized α-carboxyl. This should increase the mobility of a proton from the NH_3^+ group. Transfer of a proton from the NH_3^+ to the excited indole must be done against a considerable retarding potential for Trp, but not for Trp-Et or Trp-amide. The magnitude of the electrostatic effect is demonstrated by the major drop in pK of the α-amino group upon esterification, from 9.4 to 7.3.

Nevertheless, this is certainly not the complete explanation in the case of Trp-Et. The fluorescence yield is less than about one-third of that of Trp-amide, although the pK of the α-amino group is only slightly lower (7.3 as compared with 7.45). Also, esterification of acetyl tryptophan (Cowgill, 1963a) reduces its quantum yield. It may be necessary to invoke a more direct influence of the ester group itself, possibly affecting the interaction of excited indole with solvent at 25°.

The detailed mechanism of quenching at 25° induced by formation of an amide or peptide bond presents difficulties and a final choice of mechanism must probably be deferred. Both groups have the potentiality of serving as a proton donor. The resonance pattern in both cases is such as to leave a preponderance of positive charge upon the amide or peptide nitrogen. The latter might therefore tend to expel a proton, which is acquired by an excited indole.

However, the time constant for deuterium exchange between a peptide group and water is of the order of seconds, which is much too slow for this kind of mechanism. If proton transfer is an important factor, it would be necessary that transfer to an excited indole occur at a much higher rate than to solvent; *i.e.*, excited indole is a much better proton acceptor than solvent or indole in the ground state.

Cowgill (1963a) has explained quenching by ester or peptide groups in terms of electron transfer from indole. While a mechanism of this kind would not necessarily predict the disappearance of quantum yield differences at liquid nitrogen temperatures, it may be a contributing factor at 25°, possibly by influencing the interaction of the excited state with solvent.

The gradual rise in fluorescence yield for the series $(Gly)_xTrp$ as x increases from 1 to 3 presumably indicates that the reduction in efficiency of proton transfer from the α-amino group with increasing separation of the α-amino and indole groups more than compensates for the insertion of additional peptide bonds. In contrast, the series $Trp(Gly)_x$ shows only a progressive reduction in yield.

When the α-amino group is converted into its carbobenzoxy derivative, the influence of amide or peptide formation by the α-carboxyl is much reduced. Thus, comparing pairs 1, 4 and 15, 16; 1, 6 and 15, 17; and 1, 7 and 15, 18 (Table I), it is seen that in each case the relative reduction in yield and lifetime upon peptide or amide formation is much less for the Z-Trp than for the Trp series.

The phosphorescence lifetime is remarkably constant throughout the tryptophan series. The duration of the triplet state at 91°K is not influenced by modification of the α-amino or α-carboxyl groups, or by the nature of the nearest neighbor amino acid substituent. Apparently, none of these factors affect the processes competing with direct emission by the excited triplet state.

The invariance of phosphorescence lifetime for the tryptophan derivatives examined is especially noteworthy in view of the behavior of tryptophan in native proteins, where significant variations in lifetime have been encountered (Nag-Chaudhuri and Augenstein, 1964; Longworth, 1966). It follows that differences in secondary and tertiary structure may exert an important influence upon the phosphorescence lifetime.

The efficiency of intersystem crossing, as reflected by the F/P ratio, is also fairly constant. Acetylation of the α-amino group or carbobenzoxylation appears to reduce intersystem crossing significantly. Again it is of interest that much wider variations in F/P have been observed in proteins than for the simple peptides studied here (Nag-Chaudhuri and Augenstein, 1964; Longworth, 1966).

Phenylalanine Derivatives. The phenylalanine peptides present a considerable contrast to the tryptophan derivatives in several respects. In this case quenching of fluorescence by transfer of a proton to the excited phenyl ring is unlikely, in accord with the invariance of fluorescence yield to the state of ionization of the α-amino group (Bishai *et al.*, 1967).

No systematic effect of peptide-bond formation upon fluorescence yield at 25° was observed, both quenching and exaltation being found in individual cases. Here the nature of the substituent side chain appears to be the dominant influence. Saturated aliphatic residues produce a significant enhancement, while basic substituents result in quenching. In the latter case part of the effect may arise from an increase in intersystem crossing, as reflected by a fall in F/P at 91°K. However, the correlation is not quantitative.

Since Bishai *et al.* (1967) have shown that the sum of the fluorescence and phosphorescence yields is unity for phenylalanine itself at liquid nitrogen temperatures, any fall in F/P for the derivatives must be

reflected by a decrease in fluorescence yield under these conditions. If the fluorescence yield at 91°K, in contrast to the tryptophan case, is not invariant, it is not likely that any form of diffusion-controlled process is the dominant factor at 25°.

Again in contrast to the tryptophan case, the efficiency of intersystem crossing shows significant variations among the phenylalanyl peptides. The basic peptides Arg-Phe, Lys-Phe, and His-Phe appear to have somewhat reduced values of F/P at 91°K, although the differences are not profound. With the exception of Lys-Phe, the change is in the direction expected from the relative fluorescence yield.

However, the phosphorescence lifetimes are constant for the above series of phenylalanine dipeptides, indicating that the processes competitive with respect to direct emission by the triplet state do not vary greatly for this group of derivatives. The considerably lower lifetimes observed for Z-Phe-Phe and Z-Glu-L-Phe may reflect a direct influence of the carbobenzoxy group.

In the case of Met-Phe, the fluorescent yield at 25° is reduced sharply and the F/P ratio at 91°K is lowered slightly. Qualitatively both effects can probably be attributed to the influence of the sulfur atom. Sulfur is the atom of highest atomic number occurring in the noniodinated amino acids. Its effect may be an example of the well-known *intramolecular heavy-atom effect* (Hercules, 1966), which has been observed for aromatic hydrocarbons, for which the introduction of a heavy nucleus tends to increase intersystem crossing through alteration of spin-orbit coupling. The decrease in fluorescence yield at 25° is much too large to be accounted for by the drop in F/P observed at 91°K. However, the mutual orientations of the Met and Phe residues may be different for the two sets of conditions.

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