Flavinyl Peptides. II. Intramolecular Interactions in Flavinyl Aromatic Amino Acid Peptides*

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ABSTRACT: Close association of flavin coenzymes with aromatic amino acid residues as may occur in flavoproteins was simulated with a model system. L-Tryptophan, L-tyrosine, and L-phenylalanine were attached by peptide linkage to N-3 and N-10 ω-carboxyalkylflavins. The dependence of spectral and fluorescence properties of the flavin upon variation of the chain length separating the two chromophores was investigated. Fluorescence quenching due to collisions in the excited state, but especially that due to ground-state complex formation, increases as the chain length of the 10-substituted flavinyltryptophan and -tyrosine peptides decreases from 5 to 1 methylene groups. Hydrophobic forces are undoubtedly involved in complex formation in aqueous solution, but dispersion forces are also effective, since significant fluorescence quenching occurs in organic solvents. The flavinyltyrosine peptides exhibit intramolecular hydrogen bonding between the tyrosine hydroxyl and the 4-carbonyl group of the flavin in chloroform, but this is not the most important force in maintaining interaction in aqueous solution. Flavinyltryptophan and -tyrosine peptides ($n = 1$) in position 10 of the flavin have fluorescence properties which differ from those of the corresponding peptides substituted in position 3. This indicates that orientation of the complexing moieties influences the extent and type of quenching of the flavin fluorescence. Short-chain flavinyl peptides exhibit a degree of fluorescence quenching of sufficient magnitude to account for similar changes observed upon binding of flavinyl coenzymes to apoenzymes.

Binding of flavin mononucleotide and flavin–adenine dinucleotide to apoenzymes results in alteration of the spectral and fluorescence as well as redox properties of these coenzymes (Beinert, 1960; Massey and Ganther, 1965). The possibility that aromatic amino acid residues in the protein may be responsible for the quenching of the flavin fluorescence was first suggested by Weber in 1950. Associations of flavin coenzymes with tyrosine in flavoproteins have been reported (Strittmatter, 1961; Yagi *et al.*, 1959), and the ability of flavins to form molecular complexes was also demonstrated with phenols and indoles (Tollin, 1968). The tight binding of flavins to apoenzymes could in some cases be partly attributable to a close positioning of the flavin with one or more aromatic amino acid residues. Maintenance of such specific interaction would potentiate molecular complex formation, which results in spectral and fluorescence changes.

Previous investigations on flavin–phenol and flavin–indole complexes have required a large excess of the complexing agent or the crystalline flavin–phenol 1:1 adducts. Lambert *et al.* (1967) used a model system to investigate the intramolecular interactions between flavin–flavin, flavin–purine, flavin–trimethoxybenzene, and flavin–indole pairs. A more specific system has been synthesized (Föry *et al.*, 1968) to investigate the intramolecular interaction of flavin with tyrosine, tryptophan, and phenylalanine. The methyl esters of these amino acids were covalently attached to the flavin nucleus to simulate tight association such as may occur in certain flavoproteins. Hence, any changes in spectral properties from those of the flavin alone can be produced only by a 1:1 association of flavin:amino acid, because this intramolecular interaction can be examined in very dilute solutions as opposed to the known intermolecular cases.

This investigation was undertaken to examine the effects of proximity of the aromatic amino acid to the flavin on the limiting types of fluorescence quenching (*i.e.*, ground-state and excited-state quenching) with the models shown in Chart I.

Experimental Section

Materials. Flavinyltryptophan, -tyrosine, and -phenylalanine methyl ester peptides were synthesized as described earlier (Föry *et al.*, 1968). *O*-Methyl-L-tyrosine was obtained by the method of Izumia and Nagamatsu (1952) and converted to the methyl ester according to Law and du Vigneaud (1960). The *O*-methyltyrosine peptides with one or five methylene functions ($n = 1, 5$) in the flavin side chain were synthesized as described for the corresponding tyrosine peptides. Elemental analyses were performed by Schwarzkopf Microanalytical Laboratory (Woodside, N. Y.) on *O*-methyltyro-

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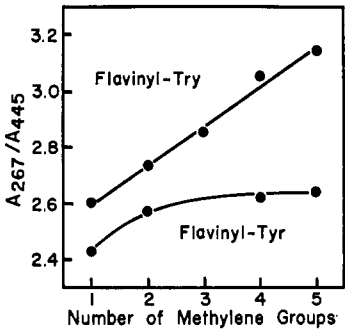


FIGURE 1: Ratio of A_{267}/A_{445} of 10-substituted flavinyltryptophan and -tyrosine peptides as a function of chain length. Flavinyl peptides were 2×10^{-5} M in 0.05 M sodium phosphate (pH 7).

sine peptides. *Anal.* Calcd for $C_{25}H_{25}N_5O_6$ (n = 1): C, 61.1; H, 5.13; N, 14.3. Found: C, 61.1; H, 5.17; N, 14.4. Calcd for $C_{29}H_{33}N_5O_6$ (n = 5): C, 63.6; H, 6.07; N, 12.8. Found: C, 64.0; H, 6.28; N, 12.8. Chloroform, *N,N*-dimethylformamide, and glycerol were Spectroquality from Matheson Coleman and Bell. Ethanol was commercial absolute.

Methods. Absorption spectra were determined with a Cary Model 14 recording spectrophotometer at room temperature. Fluorescence measurements were made with an Aminco-Bowman spectrophotofluorometer equipped with a xenon lamp, photomultiplier tube 1P 28, and slit arrangement 3. Samples were excited at the 450-mμ absorption maximum of flavin and emission was read near 520 mμ. The monochromators were adjusted so as to obtain maximal fluorescence in each solvent. The cell compartment was thermostated with a Haake Model F constant-temperature circulator. The temperature of the cell contents was determined with a Model 10 thermistor temperature indicator from TN-Tronics, New Paltz, N. Y. Fluorescence polarization was measured using a Glan prism accessory. Slit widths were 3 mm at both excitation and emission monochromators. The values of fluorescence polarization of the compounds in glycerol–water mixtures at 20 ± 0.5° were obtained using the formula of Azumi and McGlynn (1962). Solvent fluorescence was negligible except at very high instrument sensitivities necessary to measure the fluorescence of tryptophan peptides (n = 1, 2) where fluorescence background amounted to several per cent. Measurable fluorescence of solvent blanks was subtracted from sample readings before values of polarization were calculated. Glycerol–water mixtures of different viscosities were prepared according to Sheeley (1932). Glycerol concentrations were checked by refractive index measurements with a Bausch & Lomb Type 33-45-56 refractometer.

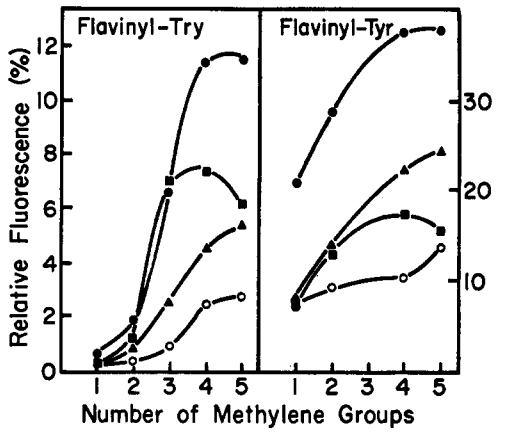
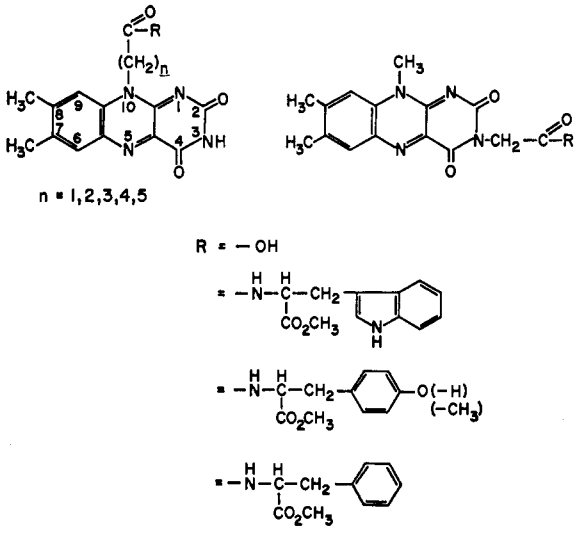


FIGURE 2: Fluorescence of 10-substituted flavinyltryptophan and -tyrosine peptides, expressed as per cent of that of free flavin under the same conditions, as a function of the number of methylene groups in the flavin side chain. Fluorescence measurements were made on 2×10^{-5} M flavin at 25°. Symbols represent: (●—●) *N,N*-dimethylformamide, (■—■) chloroform, (▲—▲) commercial absolute ethanol, and (○—○) 0.05 M sodium phosphate (pH 7).

Stock solutions of flavins were prepared in dimethylformamide. Dilutions were made into the appropriate solvents just before use. The final dimethylformamide concentration was 0.5–1.0%. Infrared spectra of compounds dissolved in chloroform were obtained with a Perkin-Elmer Model 137B Infracord equipped with 6-mm cells with chloroform as reference.

CHART I



Results

As reported earlier (Föry *et al.*, 1968), the formation of intramolecular complexes of flavin with tryptophan and tyrosine results in a broadening of the visible flavin absorption band to longer wavelengths, without the appearance of distinct new maxima. Flavinyltryptophan peptides show a greater broadening than do flavinyltyrosines, but no measurable shifts in absorbance maxima occur in either series.

In the absence of molecular interaction, the extinctions of absorption spectra of flavin and aromatic amino acid in dilute solution are additive. The ratios of absorbancies at 267 mμ:445 mμ of an equimolar mixture of free flavin and aromatic amino acid methyl ester are 3.1 with tryptophan, and 2.6 with tyrosine. Increasing intramolecular complex formation in the flavinyl aro-

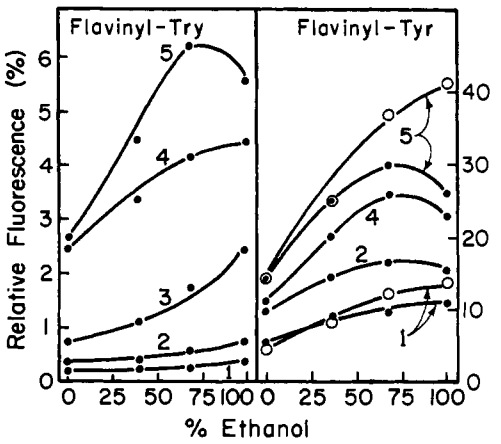


FIGURE 3: Relative fluorescence of 10-substituted flavinyltryptophan and -tyrosine peptides in ethanol–water (w/w) mixtures at 25°. Numbers refer to the methylene groups in the flavin side chain. Open circles represent *O*-methyltyrosine peptides with $n = 1$ and 5. Solutions contained 2×10^{-5} M flavin.

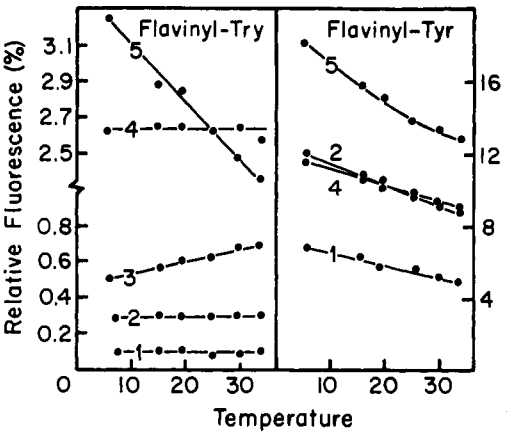


FIGURE 4: Fluorescence of 10-substituted flavinyltryptophan and -tyrosine peptides, expressed as a per cent of that of free flavin, under the same conditions, as a function of temperature. Numbers refer to the methylene groups in the flavin side chain. Flavins were 2×10^{-5} M in 0.05 M sodium phosphate (pH 7).

matic amino acid peptides with decreasing chain length is demonstrated by a decrease in this ratio as shown in Figure 1. This increase in hypochromicity of the 267-mμ band as the number of methylene groups in the flavin side chain is decreased is less for the peptides of tyrosine than of tryptophan.

The fluorescence of the flavinyl aromatic amino acid peptides, expressed as per cent of that of free flavin, is shown in Figure 2. The fluorescence of flavinyltryptophans is least in aqueous solution and increases through ethanol < chloroform < dimethylformamide. In all solvents there is a decrease in flavin fluorescence with shortening of the chain length that is pronounced with three or fewer methylene groups. However, the emission maxima of the flavinyl peptides were not measurably shifted from that of free flavin in each solvent.

The flavinyltyrosine peptides show severalfold less fluorescence quenching than the corresponding flavinyltryptophans. The decrease in fluorescence of these tyrosine peptides with decrease in chain length is observed in the same series of solvents. Flavinyltyrosines in the dipolar solvent, dimethylformamide, are present to a greater extent in the noncomplexed, fluorescent form than are the corresponding flavinyltryptophans. In addition, the fluorescence of these tyrosine peptides in ethanol is greater than in chloroform, the inverse of which is true for the flavinyltryptophans. The flavinylphenylalanine ($n = 1$) is essentially as fluorescent as the free flavin.

These different effects of solvents on the relative fluorescence of flavinyltyrosine and flavinyltryptophan peptides suggest that different or additional forces are important in maintaining intramolecular interaction in the case of flavinyltyrosines. One possibility seen with space-filling models is intramolecular hydrogen bonding between the tyrosine hydroxyl group and a flavin carbonyl group. To investigate the possibility of hydrogen bonding, fluorescence of the peptides was measured in different mixtures of ethanol–water, as shown in Figure 3. Relative fluorescence of flavinyltryptophan peptides ($n = 1$–4) increases as the percentage of water decreases. This behavior contrasts with that of the flavinyltryptophan ($n = 5$) and flavinyltyrosines ($n = 1$–5) which show an optimal fluorescence in 65% aqueous ethanol solution. That hydrogen bonding is effective in flavinyltyrosines is shown by the loss of the optimum of the fluorescence with the *O*-methyltyrosine peptides where $n = 1$ and 5. Instead, an increase in fluorescence with increasing ethanol concentration occurs which is similar to the behavior of the flavinyltryptophans ($n = 1$–4). Infrared spectroscopy gives further evidence for hydrogen bonding. Flavinyl-*O*-methyltyrosine, but not the flavinyltyrosine ($n = 1$), shows an absorption band in the 4-carbonyl region of the flavin near 1740 cm^{-1}.

Quenching of the flavin fluorescence can occur by interaction between flavin and the aromatic amino acid moiety in the ground state to form a nonexcitable complex, or by molecular collisions involving the singlet excited state of the flavin that result in loss of excitation as kinetic energy. Sufficiently high temperatures can increase the dissociation of the ground-state complex, and thus increase fluorescence. Molecular collisions are favored by increased temperature, and excited-state quenching is thereby increased. In an attempt to distinguish between these two quenching mechanisms, the effect of temperature on the relative fluorescence of the peptides was investigated, as shown in Figure 4. A marked decrease in relative fluorescence with increasing temperature from 5 to 35° is seen with the flavinyltryptophan where $n = 5$. The fluorescence of the peptide where $n = 4$ is little affected over this range of temperature while that of the peptide where $n = 3$ increases with higher temperatures. The inversion of slope in going from five through four to three methylene groups establishes that at least two types of fluorescence quenching occur with the tryptophan peptides. The extensive fluorescence quenching of the flavinyltryptophans where $n = 1$ and 2 remains constant over this temperature range. All flavinyltyrosines show a decrease in fluorescence with increasing temperature. An

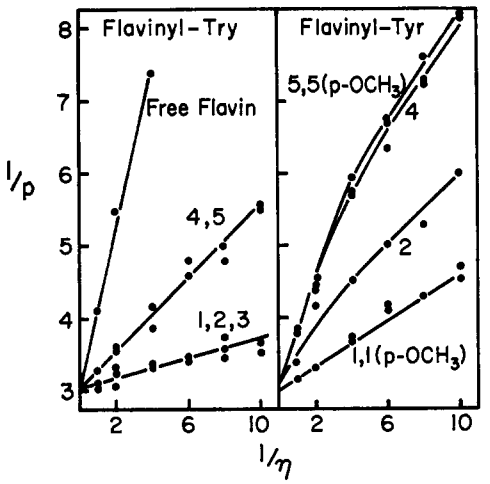


FIGURE 5: Double-reciprocal plot of polarization of fluorescence of 10-substituted flavinyl peptides in aqueous glycerol solutions of varying viscosity. Numbers refer to the methylene groups in the flavin side chain. Solutions contained 2×10^{-5} M flavin.

estimate of the relative contributions of the two mechanisms can be obtained from polarization studies.

The effect of viscosity on polarization of fluorescence was described by Perrin (1926)

$$\frac{1}{P} = \frac{1}{P_0} + \left(\frac{1}{P_0} - \frac{1}{3}\right)\frac{RT}{\eta V}\tau_0 \qquad (1)$$

where P is the polarization of the fluorescence of a substance with a mean lifetime in the excited state, τ_0, and molar volume, V, in a medium of viscosity, η. R is the gas constant, T the absolute temperature, and P_0 the fundamental polarization. Figure 5 is a double-reciprocal plot showing the effect of viscosity, η, on the polarization, P, of the flavin fluorescence, where the slope, S, of the lines is given by

$$S = \left(\frac{1}{P_0} - \frac{1}{3}\right)\frac{RT}{V}\tau_0 \qquad (2)$$

It is seen that the ratio of the slopes of two lines is dependent upon both the lifetime of the excited state and the molecular volume (Weber, 1950), so that

$$\frac{S_1}{S_2} = \frac{V_2\tau_1}{V_1\tau_2} \qquad (3)$$

Weber (1966) showed that the polarization of flavin fluorescence is roughly proportional to molecular weight. Assuming a ratio of molecular volume of free flavin to molecular volume of flavinyl peptide of two-thirds, which very closely approximates the ratio of molecular weights, it can be seen that a decrease in the lifetime of the excited state occurs with both tryptophan and tyrosine peptides. In addition, the tyrosine peptides where $n = 2$, 3, and 5 exhibit a biphasic curve. The polarization of flavinyltyrosine ($n = 5$) in solutions of high viscosity is that expected from the estimated $^8/_2$-fold change in molecular volumes. The *O*-methyltyrosine peptides ($n = 1$, 5) show the same polarization as the corresponding tyrosine peptides. The intercept value for riboflavin under these conditions (uncorrected for 0.5% dimethylformamide) was 2.5.

TABLE I: Estimate of Fluorescence Quenching Due to Ground-State Complex Formation.

Peptide	n	τ_0/τ	I_0/I	Quenching Due to Ground-State Complex (%)[a]
	10 Position			
Flavin–tryptophan	5	3	15	86
	4	3	23	91
	3	10	86	89
	2	10	212	96
	1	10	500	98
Flavin–tyrosine	5	2	3	50
	5-(*p*-OCH_3)	2	2.9	48
	4	2	3.3	57
	2	3	5.5	56
	1	4	11.7	73
	1-(*p*-OCH_3)	4	9.9	66
	3 Position			
Flavin–tryptophan	1	6	20	74
Flavin–tyrosine	1	1.8	1.8	0

[a] Calculated from $[(I_0/I - 1) - (\tau_0/\tau - 1)]/(I_0/I - 1)$ assuming that $I_0/I = \tau_0/\tau$ for collisional quenching, and where I_0 is the fluorescence in the absence of quenching, I with quenching; τ_0 is the lifetime of the excited state in the absence of quenching, τ with quenching.

From the slopes of the curves at low viscosity ($1/\eta > 4$), and assuming a ratio of molar volumes of two-thirds, the ratios of lifetimes of the excited state, τ_0/τ, were calculated according to eq 3. Also, the ratios of fluorescence of free flavin to flavin peptides, I_0/I, were measured in dilute glycerol solution ($1/\eta = 10$). The symbols I_0 and τ_0 represent fluorescence and mean lifetime of the excited state of free flavin, while I and τ are the corresponding symbols for the flavinyl peptides. These data are shown in Table I. From the relationship $I_0/I = \tau_0/\tau$ for entirely excited-state quenching, an estimate was made of fluorescence quenching due to ground-state complex formation. There is an increase in fluorescence quenching due to ground-state complex formation with both tyrosine and tryptophan peptides as the side chain is shortened. Ground-state

complex formation occurs to a greater extent with tryptophan than with tyrosine peptides. These values can be considered to be only semiquantitative due to the limited accuracy of polarization measurements on the Aminco-Bowman spectrophotofluorometer.

Discussion

The apparent absence of new absorption bands minimizes any contribution from classically defined charge-transfer interaction (Mulliken and Person, 1962) even with the most complexed flavinyl peptides. However, the intramolecular interaction in these flavin peptides results in a significant loss in flavin fluorescence.

In aqueous solution, hydrophobic forces are undoubtedly involved to some extent in complex formation with the flavinyltyrosine and -tryptophan peptides. But the phenylalanine peptide ($n = 1$), which lacks significant polarity in the aromatic ring of the amino acid, does not show fluorescence quenching in aqueous media, although conditions for hydrophobic interactions are satisfied. Extensive fluorescence quenching of flavinyltryptophans and -tyrosines occurs in nonaqueous solvents which indicates that dispersion forces must account for some of the interactions. In addition to forces dependent upon the polarity and polarizability of the π systems, hydrogen bonding between the hydroxyl group of tyrosine and the 4-carbonyl of the flavin can be effective in nonaqueous solvents such as chloroform. However, the same degree of fluorescence quenching of tyrosine and *O*-methyltyrosine peptides in water allows the assumption that hydrogen bonding is not the important force in complex formation of the flavinyl peptides in aqueous solution. The extent of complex formation and the relative contribution of the various forces involved are shown to vary with solvent. Consequently, the orientation of the overlapping of the flavin and amino acid may also be affected by solvents.

Hydrogen bonding in chloroform indicates that in this solvent, intramolecular complex formation involves, in part, association of the tyrosine residue with the pyrimidine ring of the flavin. Such interaction may also occur in nonaqueous solution in the absence of hydrogen bonding, due to the dipolar nature of the tyrosine and tryptophan ring systems and the pyrimidine portion of the flavin. The degree of fluorescence quenching of the short peptides in nonaqueous media supports the existence of this dipole–dipole type of interaction. Space filling models show that the aromatic amino acid can overlap with the aromatic ring, the pyrimidine portion, or an intermediate position of the flavin. In aqueous solution, hydrophobic interaction could result in an increase in complex formation which involves greater overlap of the benzene portions of the amino acid and flavin.

A large difference exists in the extent of fluorescence quenching as well as in the contribution from ground-state complex formation between the 10-substituted flavinyltryptophan and -tyrosine peptides ($n = 1$) and the corresponding peptides substituted in position 3 of the flavin. The amino acids are separated from the flavin by the same chain length, so that different orientations of overlap must be responsible for the different fluorescence quenching properties. Any qualitative or quantitative differentiations of orientations of complex formation by fluorescence spectroscopy cannot be decisive, but nuclear magnetic shielding measured by nuclear magnetic resonance is a sensitive probe of molecular interaction. Different vertical stackings may be distinguished by their nuclear magnetic resonance spectra, and thus this technique will be employed to examine orientations of the complexes.

Fluorescence quenching of both the flavinyltyrosines and flavinyltryptophans is inversely related to the chain length separating the two chromophores. Thus, maintenance of close interaction is prerequisite for maximal fluorescence quenching, which is shown to be essentially complete with the tryptophan peptides ($n = 1$) in aqueous solution. Quenching by ground-state complex formation increases more than that due to interaction in the excited state as the amino acid is brought closer to the flavin. Both types of fluorescence quenching were observed with all peptides except for the N-3-tyrosine where interaction between tyrosine and flavin only in the excited state leads to fluorescence quenching.

Most of the flavinyl peptides show a linear dependence of $1/p$ on $1/\eta$. In this respect, the polarization of some tyrosine peptides was anomalous. The biphasic polarization curves obtained for these latter peptides are not due to hydrogen bonding between the tyrosine hydroxyl group and the glycerol solvent, because the *O*-methyltyrosine peptide ($n = 5$) exhibits the same biphasic curve. Kinetic factors can be considered as a possible explanation for this phenomenon. The longer chain tyrosine peptides, which have less fluorescence quenching due to ground-state complex formation, can be considered to exist in solution in a relatively open or extended conformation. Highly viscous solvents impede interaction between the separated flavin and amino acid moieties during the lifetime of the excited state of the flavin. A sufficient decrease in viscosity permits an increase in the number of collisions which lead to fluorescence quenching in the excited state so that the slope of the curve changes. Because the short-chain tyrosine peptide shows considerably more quenching due to ground-state and excited-state interactions than the corresponding longer chain peptides, the viscosity effect in the former, more closed, form is not great enough to significantly decrease collisions during the lifetime of the excited state of the flavin.

The changes in fluorescence which occur in these flavinyl peptides, due to intramolecular interactions, are of sufficient magnitude to account for similar effects upon binding of flavin coenzymes to apoenzymes. Further studies will be carried out to determine whether the fluorescence changes observed with these peptides are also accompanied by significant changes in flavin redox potential.

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The Oxidative Cleavage of Tyrosyl–Peptide Bonds during Iodination*

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ABSTRACT: In addition to the usual diiodination of the phenolic ring, *N*-iodosuccinimide is capable of effecting an oxidative transformation of tyrosine models and derivatives by which dienone lactones are formed. Thus, diiodophloretic acid, *N*-carbobenzyloxy-3,5-diiodotyrosine, and *N*-benzoyl-3,5-diiodotyrosine are converted into their respective dienone lactones (**2a–2c**) in yields of 57–95% in acetate buffer, pH 4.5. Similarly, *N*-iodosuccinimide effects the oxidative cleavage of the peptide bond in diiodophloretylglycine and in *N*-carbobenzyloxytyrosylglycine. Because the oxidation potential of iodine is considerably lower than that of *N*-iodosuccinimide, only ring halogenation is observed with the former reagent.

The reactivity of *N*-iodosuccinimide is decreased markedly in the presence of acetonitrile. The possibility of peptide-bond cleavage in the course of protein iodination is considered.

Previous studies in this laboratory (Wilson and Cohen, 1963) have demonstrated the facile cleavage of tyrosyl–peptide bonds in simple peptides and in proteins as a result of the oxidative action of bromine or of *N*-bromosuccinimide. Although positive iodine reagents are generally weaker oxidants than their bromine counterparts, it is entirely reasonable to expect that iodine oxidants of sufficient potential would effect analogous cleavage reactions. The possibility is particularly significant in view of the extensive use of iodination as a technique in protein modification.[1] Such cleavage has, indeed, been found to occur and, in this report, the oxidative fission of simple tyrosyl dipeptides by *N*-iodosuccinimide is described.

Experimental Section

Iodination of Phloretic Acid with N-Iodosuccinimide. To a stirred solution of 166 mg (1 mmole) of phloretic acid (3-*p*-hydroxyphenylpropionic acid, Aldrich Chemical Co.) in 10 ml of acetonitrile and 40 ml of acetate buffer (0.2 M, pH 4.5) was added 900 mg (4 mmoles) of *N*-iodosuccinimide in one portion. After 20-min stirring of the reaction mixture in the dark, the crystalline precipitate was collected, washed with 40% acetone–water and with carbon tetrachloride, and dried, 295 mg, 71%. The dienone lactone (**2a**) was recrystallized from acetonitrile–water: mp 223–226°,[2] ultraviolet maximum (ethanol) 295 mμ (ε 3120) and 265 mμ (ε 3560), and infrared spectrum (KBr) 1780 (lactone C=O) and 1670 cm^{-1} (dienone C=O).

Anal. Calcd for $C_9H_6I_2O_3$: C, 25.99; H, 1.45; I, 61.02. Found: C, 26.24; H, 1.94; I, 61.18.

Iodination of phloretic acid with excess iodine gave 3,5-diiodophloretic acid (**1a**) (Barnes *et al.*, 1950): (a) iodine–potassium iodide, Tris buffer, pH 8.9, 45% yield; (b) iodine–chloroform, phosphate buffer, pH 6.8, 75%; (c) iodine–acetonitrile, acetate buffer, pH 5.4, 41%. In no case could **2a** be detected in these iodinations, either by thin-layer chromatography or following work-up.

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[1] For a recent review, see Cohen (1968).

[2] Matsuura *et al.* (1967) report mp 225–26°.