Flavinyl Peptides. III. Studies of Intramolecular Interactions in Flavinyl Aromatic Amino Acids by Proton Magnetic Resonance*

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ABSTRACT: Proton magnetic resonance spectroscopy has been applied to an investigation of the solution conformers of flavin aromatic amino acids. The chemical shifts of the protons of ω-carboxyalkylflavins and the corresponding flavinyl peptides, wherein a 7,8-dimethylisoalloxazine portion is conjoined *via* amide linkage with alanine, tryptophan, tyrosine, or phenylalanine or the methyl esters of these amino acids, were measured in D_2O and D_6-dimethyl sulfoxide. In aqueous solution at pH 7 and 37°, the alanine portion of flavinyl peptides with two and five methylene groups in the flavin side chain tends to fold back on the ring system. This also occurs with ω-carboxyalkylflavins where the carboxyl function is appended to chains of sufficient length to allow a similar orientation relative to the benzenoid and heteroaromatic parts of the flavin. The flavinyl peptides of tryptophan, tyrosine, or phenylalanine with two to five methylene groups in the flavin side chain all associate intramolecularly in water in a stacked manner such that the aromatic portions are in a planar orientation. Such conformations are opened at higher temperatures or by dimethyl sulfoxide. In this organic solvent, the flavinyl peptides of the methyl esters of the aromatic amino acids are more unfolded, but the amino acid moiety still somewhat shields the benzenoid portion of the flavin which is more proximal to the side chain.

The possibility that interactions of aromatic amino acid residues with flavin coenzymes cause quenching of the flavin fluorescence in flavoproteins was first emphasized by Weber (1950). Various investigations have shown that indoles and phenols form rather strong molecular complexes with flavins (Tollin, 1968a,b). Yagi *et al.* (1959) concluded, from the inhibitory action of phenol and its derivatives on D-amino acid oxidase, that tyrosyl residues may be involved in binding of flavin. A similar protein–flavin interaction which is completely blocked by iodination of one phenolic group of the apoenzyme was demonstrated by Strittmatter (1961). Although no definitive evidence is available at the present time, indole residues could participate in an association between flavin coenzymes and the tryptophans in certain flavoproteins, *e.g.*, flavodoxin (McCormick, 1969), as well.

A specific model system involving flavinyl aromatic amino acid peptides was used by MacKenzie *et al.* (1969) to investigate the degree and type of molecular interactions of flavins with tyrosine, tryptophan, and phenylalanine as dependent upon the chain length separating the two chromophores. It was shown by this study that hydrogen bonding between the hydroxyl group of the tyrosyl residue and the pyrimidine portion of the flavin and dispersion force-dependent interactions are possible in nonaqueous solvents and, furthermore, that an aqueous solvent is able to promote associations of the aromatic parts of the flavin and amino acid. This fluorescence spectral investigation, however, did not allow any differentiation of possible conformational arrangements of the two aromatic moieties in close proximity.

In recent years, proton magnetic resonance methods have been applied in numerous studies (Kowalsky and Cohn, 1964) to the investigations of biological systems. This technique was specifically employed to study the conformational aspects of inter- and intramolecular interactions of flavin–adenine dinucleotide (Sarma *et al.*, 1968).

In the present report, we have investigated the spatial aspects of the intramolecular interactions of the flavinyl aromatic amino acids, the structures of which are shown in Chart I, in aqueous and nonaqueous media and at different temperatures by means of proton magnetic resonance spectroscopy. The dipolar nature of the flavin, tyrosine, and tryptophan ring systems and the general aromatic character of these chromophores allow that possible hydrophobic forces, hydrogen bonding, and donor–acceptor interactions in connection with the related spatial arrangements between flavin and covalently attached aromatic amino acid be at least quantitatively differentiated by the measurement of chemical shifts of the different protons involved.

Syntheses[1]

Carboxyalkylflavins ($VI_{1,2,4,5}$), flavin *p*-nitrophenyl esters ($I_{2,5}$), and flavinylamino acid methyl esters ($II_{1.5}$, $III_{1.5}$, IV_1, V_5, R = R_2) were synthesized by known methods (Föry *et al.*, 1968). Flavinyltryptophans ($II_{2,5}$), -tyrosine (III_5), and -phenylalanine (IV_5, R = R_1) were obtained by aminolysis of the corresponding *p*-nitrophenyl esters ($I_{2,5}$) with L-trypto-

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[1] See Experimental Section for further details.

TABLE I: Effect of Concentration upon the Chemical Shifts of Various Protons of Flavinylalanines and Carboxyalkylflavins.[a]

Compd	Concn (M)		FC_6H	FC_9H	FC_8CH_3	FC_7CH_3
			Flavinylalanines[b]			
V_2	0.12		454.5	447	149.5	139.5
	0.0		461.5	461.5	152	143.5
		Δ[c]	7	14.5	2.5	4
V_5	0.1		444.5	441	147.5	138
	0.0		451.5	451.5	148.5	140
		Δ	7	10.5	1	2
			Carboxyalkylflavins			
VI_1	0.15		450	439	146	139
	0.0		458	443	148.5	143
		Δ	8	4	2.5	4
VI_2	0.12		453.5	447	148	139
	0.0		458	454	150	142
		Δ	4.5	7	2	3
VI_4	0.15		443.5	437.5	147	137
	0.0		451	451	150	142
		Δ	7.5	13.5	3	5
VI_5	0.10		444.5	444.5	148	139
	0.0		458	458	150	142
		Δ	13.5	13.5	2	3

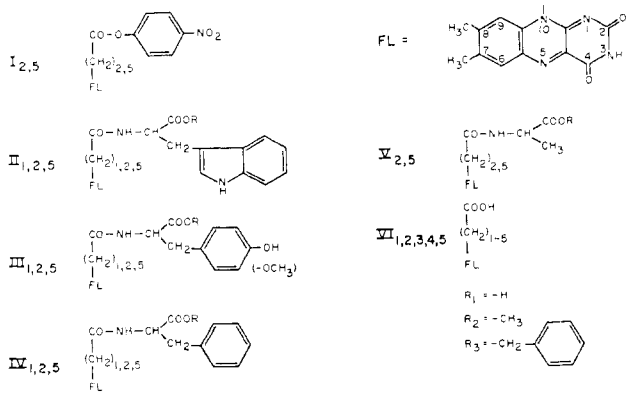
[a] Chemical shifts in cps downfield from internal DSS; values at 0.0 M were obtained from graphical extrapolation; temperature was 37°. [b] R = R_1. [c] Δ values are all positive, indicating upfield shifts with increasing concentration.

phan, L-tyrosine, and L-phenylalanine. The flavinyltyrosine, -phenylalanine, and -alanine benzyl esters (III_2, IV_2, $V_{2,5}$, R = R_3) were obtained similarly by reaction of the corresponding I with the appropriate L-amino acid benzyl ester. Hydrogenolysis of those benzyl esters, followed by air oxidation of the corresponding dihydroflavins, gave the flavinyl-amino acids with R = R_1.

Results and Discussion

I. Interactions between and within Flavin Molecules. The concentration-dependent tendency of flavin molecules to dimerize was shown by Tsibris *et al.* (1965) by means of fluorescence spectroscopy. The proton magnetic resonance study of Sarma *et al.* (1968) helped elucidate the conformational arrangement of the intermolecular interaction between flavins with FAD and FMN molecules. This demonstrated that the mechanism of self-association is that of vertical stacking with the aromatic parts of the flavin face to face, but with the polar ribityl phosphate groups opposing each other to reduce electrostatic and steric repulsion.

CHART I



The mode of the intermolecular flavin interaction in our flavinyl peptide system also had to be investigated, since flavin nucleus and amino acid are separated by nonpolar alkyl side chains of different length with a carboxyl-solubilizing group. Moreover, such a proton magnetic resonance study of the flavinyl peptides ($V_{2,5}$) with a nonaromatic amino acid part is helpful for at least partially distinguishing between the general concentration dependency of flavin self-association and the intramolecular flavin aromatic amino acid interaction. Figure 1A shows proton magnetic resonance spectra of flavinylalanine (V_2) at two different concentrations in the region of the aromatic protons consisting of two distinct resonance peaks which can be assigned to the flavin C_6H and C_9H (Bullock and Jardetzky, 1965). These two peaks appear to fuse at lower concentrations of the flavin peptide.

Data in Table I summarize the effect of concentration on the aromatic proton chemical shift of the flavinylalanines ($V_{2,5}$) and carboxyalkylflavins ($VI_{1,2,4,5}$). Again, it is to be noted that the resonance peaks of the C_6H and C_9H of the flavinyl peptides ($V_{2,5}$) coalesce with decreasing concentration to one peak of twice the intensity, similar to riboflavin, FMN, or FAD. With increasing concentrations, flavin molecules may associate intermolecularly to shield their aryl protons, the chemical shifts of which are more upfield. In addition, there may be an intramolecular interaction between the flavin C_6H, or

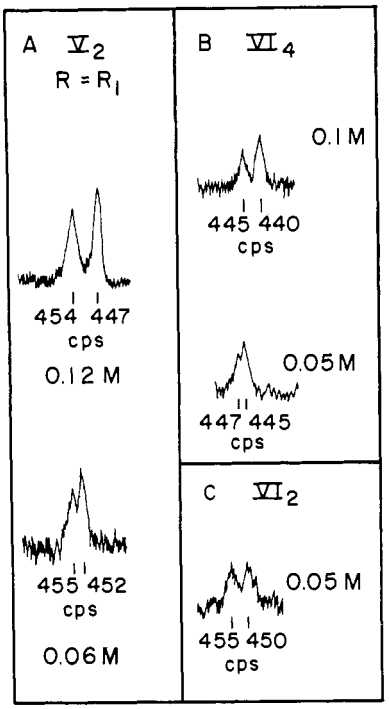


FIGURE 1: Flavin studies. (A) The effect of concentration upon the chemical shift of the aromatic protons of flavinylalanine (V_2, R = R_1), 60-MHz spectra of 0.12 and 0.06 M flavin (pD 7.0); dimethylsilapentanesulfonate was added as internal standard; peaks are (1) flavin C_6H and (2) flavin C_9H. In B and C are shown the effects of length of the carboxyalkyl side chain upon the chemical shift of flavin protons. (B) Carboxybutylflavin (VI_4). (C) Carboxyethylflavin (VI_2).

C_9H and the flavin side chain, both of which could cause the observed chemical shift of either or both of these flavin aromatic protons. To investigate the second possibility, *i.e.*, specific intramolecular interaction between the isoalloxazine nucleus and the side-chain-appended alanyl residue, the proton magnetic resonance spectra of ω-carboxyalkylflavins ($VI_{1,2,4,5}$) with lengthwise similar or shorter chains than that of the flavinylalanine (V_2) were measured. The results obtained are shown in Figure 1B,C and Table I (bottom). The carboxypentylflavin (VI_5) which has a side chain of similar length to that of the flavinylalanine (V_2) tends toward one resonance peak in the region of the flavin aromatic protons, the area of which corresponds to two protons over the concentration range of 0.1–0.05 M. Carboxybutylflavin (VI_4) shows a similar trend, although at higher concentration (0.1 M), the two flavin peaks are well separated (5 cps) as seen in Figure 1B. The peaks coalesce, however, with decreasing concentration through an intermediate state of separation (2 cps at 0.05 M) to one peak, the chemical shift of which is obtained by graphical extrapolation of the measured shifts to zero flavin concentration. The aromatic protons of carboxyethylflavin (VI_2) (Figure 1C), do not show any coalescing tendency over the mentioned concentration range.

From this set of data, it can be concluded with some certainty that the flavin C_6H experiences a shielding from a carbonyl function of a carboxy or amide group. Another indication that the resonance of the flavin C_6H and not as much C_9H is additionally upfield shifted can be deduced from the

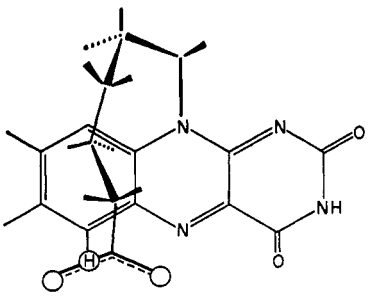


FIGURE 2: Model illustrating proposed arrangement of the carboxypentyl side chain.

fact that a minimal chain length separating the carbonyl function from the isoalloxazine nucleus is prerequisite for this shielding effect. A sizeable upfield shift of the flavin C_9H would be in disagreement with the presented experimental data. This specific orientation of the side chain relative to the ring system of the flavin may be the result of a polar interaction between the charged carboxyl group and the heteroaromatic part of the flavin molecule, and additionally, of a hydrophobic interaction between the nonpolar alkyl side chain and the aromatic moiety of the flavin. The latter explanation is supported by the fact that the carboxypentylflavin exists in a more extended conformation in 50% aqueous dimethyl sulfoxide, shown by the greater separation of two resonance peaks of the flavin C_6H and C_9H in this solvent mixture. A conformation of the carboxyalkyl side chain relative to the flavin compatible with the results is diagrammatically illustrated in Figure 2.

As the present flavins have rather low solubility in D_2O, the chemical-shift differences due to their association tendency, studied over a concentration range of 0.1–0.05 M, are less pronounced than those reported earlier (Sarma *et al.*, 1968) for FMN and FAD at higher concentrations. However, the observed upfield shifts of the aromatic flavin C_6H and C_9H, as a manifestation of the ring-current diamagnetic anisotropy effects of the flavin molecules in stacks, generally follow a comparable trend for flavin–flavin association. Thus, it can be seen from the results in Table I that the chemical shift of the flavin C_9H is more sensitive to concentration changes than that of the C_6H. This strongly indicates a preferred conformation of the flavin molecules in a stack, in which each C_9H is positioned in the shielding sphere of the benzenoid portion of a neighboring flavin molecule.

II. Intramolecular Interactions between Flavin and Aromatic Amino Acid. A. FLAVINYLTRYPTOPHANS. The proton magnetic resonance spectrum of the flavinyltryptophan (II_5) and that of the aromatic region of the short-chain peptide (II_2) are shown in Figure 3. Two experimental assignments (Bullock and Jardetzky, 1965; McCormick, 1967) as well as a theoretical rationalization of the proton magnetic resonance spectrum of flavin (Sarma *et al.*, 1968) have appeared in the literature. These studies assigned the high-field peaks to C_8CH_3 and C_7CH_3 of the isoallozazine ring and the low-field peaks to C_6H and C_9H of the benzene portion of the flavin; resonance of C_9H is more upfield than C_6H, and C_7CH_3 is more upfield than C_8CH_3. The methyl resonance peaks (7 and 8 of Figure 3) can be differentiated on the basis that the C_8CH_3 of the flavinyl peptides shows a rather facile deuterium exchange in

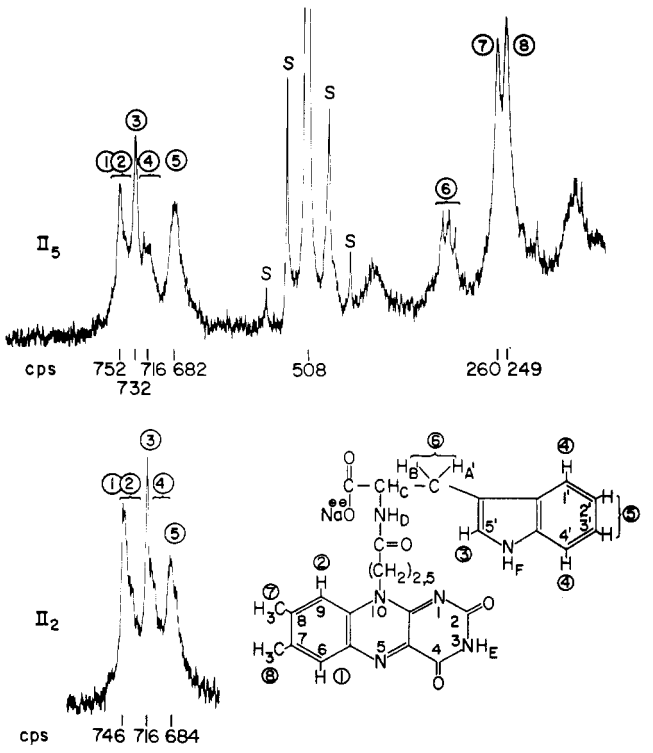


FIGURE 3: 100-MHz proton spectra of 0.1 M flavintryptophans ($II_{2,5}$), pD 7.0, 37°; tetramethylsilane sealed in a capillary was used to provide the field-frequency lock signal. Spinning side bonds are indicated by S. The strong line at 508 cps arises from HDO.

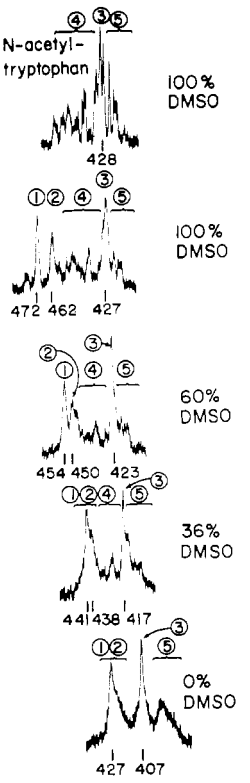


FIGURE 4: 60-MHz proton spectra of 0.1 M flavinyltryptophan (II_2) in dimethyl sulfoxide–water (v/v) mixtures; 37°; dimethylsilapentanesulfonate was added as internal standard; for peak assignments, see Figure 3.

D_2O at elevated temperatures as was reported by Bullock and Jardetzky (1965) for the case of FMN. In addition, the appearance of the flavin C_8CH_3 peak at a lower field than that of C_7CH_3 can be explained by the fact that the flavin 8-methyl protons exhibit acidic character, the magnitude of which is sufficient for the flavin to undergo aldol condensation with *p*-chlorobenzaldehyde or dimerization to yield 8,8′-biflavin (Hemmerich *et al.*, 1959). Some difficulty was experienced in the analysis of the aromatic region (760–660 cps) of the 100-MHz spectrum in Figure 3, since peak overlaps occur due to the intramolecular interaction-dependent upfield shift. Lacking specifically deuterated analogs, the assignments of the resonance peaks of this complex spectral pattern had to be done in a somewhat indirect manner.

Proton magnetic resonance studies of dipolar or aromatic solutes in aromatic or dipolar solvents have shown that there are attraction forces involved between solute and solvent (Schneider, 1962; Diehl, 1964). The solvent dipole tends to lie in a plane parallel to the molecular plane of the polarizable aromatic solute (Kuntz and Johnston, 1967). It was found that the ω-carboxypentylflavin in dimethyl sulfoxide is able to unfold by forming such an anisotropic solute–solvent complex which competes successfully with the interaction between the flavin ring and the alkyl side chain. Flavinyltryptophans ($II_{2,5}$) show a similar behavior in this dipolar solvent, and in contrast to the pronounced shifts in aqueous solution, the proton resonance of the mentioned peptides and the flavins in general are essentially independent of concentration in this solvent.

The pmr spectra of the flavinyl peptide (II_2) were measured in different dimethyl sufoxide–water mixtures and are shown in Figure 4. Peak assignments for the tryptophan spectrum have been previously reported (McDonald and Phillips, 1967; Gerig, 1968). It can be seen from Figure 4 (top) that the proton magnetic resonance spectrum of the flavinyl peptide exhibits some line broadening compared with that of *N*-acetyltryptophan. This slightly increased line width may be attributable to a diminished rate of molecular motion due to some dispersion force-dependent interaction between tryptophan and flavin. Judged, however, by the decreased fluorescence quenching (MacKenzie *et al.*, 1969) and this small line broadening in dimethyl sulfoxide compared with these properties in water, one can assume that the flavinyltryptophan exists in dimethyl sulfoxide in a rather extended conformation. Thus, the peak assignments of the protons or proton groups of the flavin and tryptophan aromatic regions in aqueous medium can be achieved by observing the different coalescing tendencies of the aromatic flavin and tryptophan protons, shown in Figure 4, in going from an extended conformation in 100% dimethyl sulfoxide to a folded conformation in 100% water. The analysis of the aromatic region of the long-chain flavinyltryptophan (II_5) has been similarly made.

To investigate the conformational arrangements upon intramolecular interaction between the flavin and tryptophan and to distinguish between this intramolecular complex formation and the intermolecular association of flavins mentioned previously, the chemical-shift values of the flavin protons of the flavinyltryptophan have to be subtracted from those of flavinylalanine and *N*-acetyltryptophan. The chemical shift

TABLE II: Upfield Shift (Δ)[a] of the Flavinyl Aromatic Amino Acid Protons Relative to Those of Flavinylalanine and Corresponding *N*-Acetyl Aromatic Amino Acids as a Manifestation of Intramolecular Flavin–Amino Acid Interaction.[b]

Compound		Flavin Protons[c]					Flavin Amino Acid Protons	
						Flavinyltryptophans[c]		
		FC_6H	FC_9H	FC_8CH_3	FC_7CH_3		FL-Trp-$C_{5'}H$	FL-Trp-$C_{2',3'}H$[d]
II_2	Δ ($V_2 - II_2$)	39	31	14	10	Δ (*N*-Ac-Trp-II_2)	28	35
II_5	Δ ($V_5 - II_5$)	18	11	4	1	Δ (*N*-Ac-Trp-II_5)	14	26
						Flavinyltyrosines[e]		
							FL-Tyr-H_A	FL-Tyr-H_B
III_2	Δ ($V_2 - III_2$)	17	12	6	6	Δ (*N*-Ac-Tyr-III_2)	20	20
III_5	Δ ($V_5 - III_5$)	8	10	1	1	Δ (*N*-Ac-Tyr-III_5)	9	13
						Flavinylphenylalanines[f]		
							FL-Phe-C_6H_5-H	
IV_2	Δ ($V_2 - IV_2$)	13	10	3	3	Δ (*N*-Ac-Phe-IV_2)	16	
IV_5	Δ ($V_5 - VI_5$)	9	9	1	1	Δ (*N*-Ac-Phe-IV_5)	10	

[a] Chemical-shift differences (Δ) are positive indicating upfield shift of flavinyl (FL) aromatic amino acid protons relative to those of the corresponding flavinylalanine and *N*-acetyl aromatic amino acid. [b] Chemical-shift values in cps downfield from internal dimethylsilapentanesulfonate, used in Table I, were obtained by extrapolation to zero flavin concentration. [c,f] [e] For peak assignments, see Figures 3, 7A,B, respectively. [d] Center of poorly resolved multiplet.

of the latter compound is concentration independent over the concentration range applied in this investigation. Data in Table II summarize the effect of intramolecular interaction between flavin and tryptophan dependent upon the chain length separating the two chromophores on the chemical shift of flavinyltryptophans ($II_{2,5}$). It was shown by MacKenzie *et al.* (1969) that the quenching of flavin fluorescence due to ground-state complex formation increases as the chain length of the flavinyltryptophans is shortened from 5 to 1 methylene group. It can be seen from Table II that the chemical shifts of the aromatic flavin and tryptophan protons follow a similar trend in experiencing a more pronounced upfield shift with the short-chain peptide (II_2) than do those with the long-chain flavinyltryptophan (II_5). In addition, it was concluded from spectral investigations in various solvents, that hydrophobic forces are involved to some extent in the complex formation between flavin and aromatic amino acid in aqueous media. The results in Figure 4 and Table II support this conclusion by showing a specific involvement of the aromatic moieties during complex formation in going from nonaqueous to aqueous solvents.

The flavin C_6H of the flavinyltryptophan, II_2, is more shielded than that of the corresponding long-chain analog, II_5 (39 *vs.* 18 cps), and within the flavin molecule the C_6H experiences a slightly greater shielding effect than the C_9H (39 *vs.* 31 cps in II_2 and 18 *vs.* 11 cps in II_5). The upfield shifts of the well-resolved vinyl proton of the tryptophan moiety of both short- and long-chain flavinyltryptophans are 28 and 14 cps, respectively. A pronounced line broadening occurs, especially for the $C_{1'}$,$_{4'}$ and $C_{2'}$,$_{3'}$ proton groups, as expected for formation of molecular aggregates. If one assumes that a given spatial arrangement is rapidly breaking up and reforming, only the average magnetic environment contributes to the observed shifts, whereby individual resonance peaks are averaged into single resonance peaks of enhanced line width. Because of this line broadening, it is impossible with the present experimental conditions to measure the upfield shift and to resolve the pattern of the mentioned tryptophan protons into individual resonance peaks. In a semiquantitative manner, however, it can be seen that the $C_{2'}$,$_{3'}$ protons of the tryptophan peptides ($II_{2,5}$) appearing as a poorly resolved multiplet, experience an upfield shift (35 cps in II_2 and 26 cps in II_5) of a magnitude comparable with the vinyl proton of tryptophan. Since the line widths of the flavinyl aromatic amino acid protons generally do not decrease with decreasing flavin concentration, intermolecular interaction including intercalation of two or more flavinyl peptides as a possible form of flavinyl amino acid interaction is not of great importance.

Taking into account the upfield shifts of flavin C_6H, C_9H, C_7CH_3, and C_8CH_3, together with those of the tryptophan protons, $C_{5'}H$ and $C_{2'}$,$_{3'}H$ (multiplet), an average spatial arrangement of the flavinyltryptophans (II_2 or II_5) can be reasoned as depicted in Figure 5. This illustration of the stacked conformations is based on a molecular model of the flavinyltryptophan (II_2) built from space-filling models. The actual

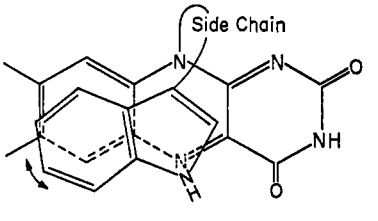


FIGURE 5: Model illustrating proposed arrangement for flavinyltryptophans ($II_{2,5}$) in a folded conformation.

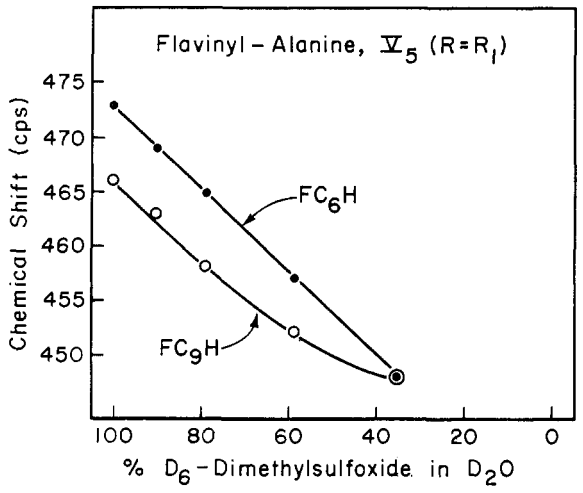


FIGURE 6: Chemical shifts of flavin C_6H and C_9H in flavinylalanine (V_5) dependent upon side-chain conformation in dimethyl sulfoxide–water (v/v) mixtures.

average conformation may be somewhat different from that shown in Figure 5.

B. FLAVINYLTYROSINES AND -PHENYLALANINES. It was demonstrated in the previous section (IIA) that intramolecular interaction between flavin and aromatic amino acid can be monitored by the upfield shift of the protons of the aromatic moieties compared with those of the corresponding flavinylalanine and *N*-acetylamino acid. In one respect, the flavinylalanine has optimal reference characteristics, because of the identical arrangements of side chain, amide function, and charged carboxyl group. On the other hand, as the resonance peaks of C_6H and C_9H of the flavinylalanines ($V_{2,5}$) coalesce to one peak in going from high to low concentration (*cf.* Figure 1A and accompanying discussion), the reference properties in connection with these protons are far from ideal. In addition, since flavin C_6H and C_9H experience a specific shielding due to the intramolecular interaction between flavin and aromatic amino acid, the upfield-shift values as a measure for such interactions, obtained by subtraction of the chemical shifts of flavinyl aromatic amino acids from those of the flavinylalanine, may be misleading.

To obtain the actual peak separation of the flavin C_6H and C_9H, the chemical shifts of which would correspond to an arrangement of the flavin relative to alanine portion without interfering side-chain interaction, the molecule can be made to assume an unfolded conformation by increasing the concentration of dimethyl sulfoxide. It can be seen from Figure 6 that the resonance peaks of the flavin C_6H and C_9H of the flavinylalanine (V_5) are fully separated in 71% aqueous dimethyl sulfoxide with a chemical-shift difference of 7 cps. The short-chain flavinylalanine (V_2) shows a similar trend, with a chemical-shift difference of 8 cps at extensive peak separation. Sarma *et al.* (1968) reported a similar shift difference (10 cps) between the flavin C_6H and C_9H in riboflavin 5′-phosphate at an extrapolated zero flavin concentration. To obtain the chemical shift values of the flavin C_6H and C_9H, assuming no alanine side-chain interaction, 7 cps for V_5 and 8 cps for V_2 were added to the chemical-shift value of the two-proton peak at zero flavin concentration.

Data in Table II and Figure 7 summarize the effects of

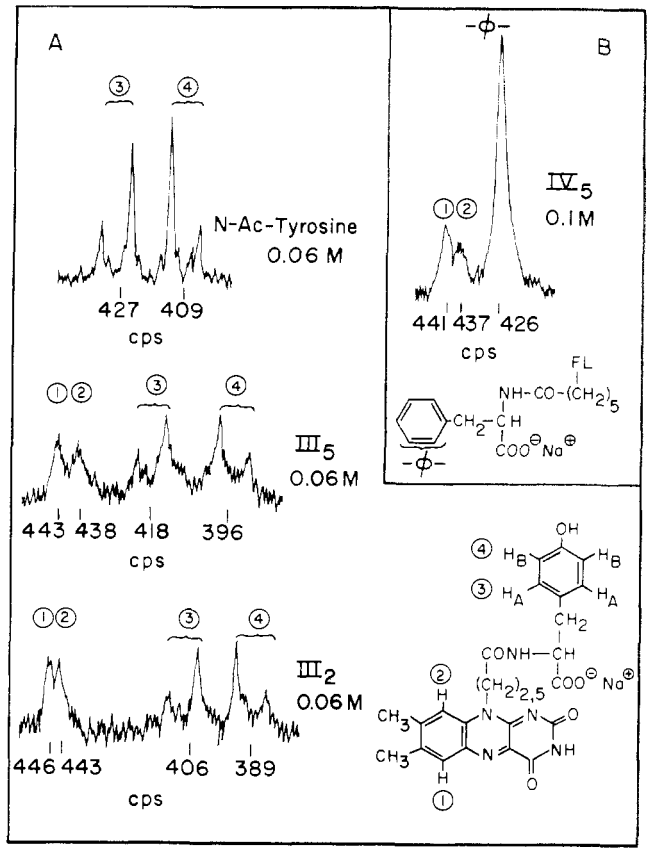


FIGURE 7: Spectral studies. (A) 60-MHz proton spectra of 0.06 M flavinyltyrosines ($III_{2,5}$, R = R_1) and *N*-acetyltyrosine, 37°; dimethylsilapentanesulfonate was added as internal standard. (B) Proton magnetic resonance spectrum of 0.1 M flavinylphenylalanine (IV_5, R = R_1).

proximity of the tyrosine and phenylalanine on the chemical shift of the corresponding flavinyl peptides. The analysis of the AA′BB′ pattern arising from the aromatic protons of the tyrosine molecule was performed in a prescribed manner (Bovey, 1969). It can be seen from Figure 7A that the resonances of the aromatic protons of the tyrosyl residue (H_B *ortho* and H_A *meta* to the phenolic OH group) are, relative to those of *N*-acetyltyrosine, increasingly upfield shifted as the chain length separating flavin and tyrosine is shortened. The upfield shift of the aromatic protons, H_A and H_B, of the tyrosyl residue of III_2 amounts to 20 cps for both of the proton groups. The protons *ortho* to the phenolic hydroxyl group in the long-chain peptide (III_5) are slightly more shielded than the tyrosine *meta* protons (13 *vs.* 9 cps), but with a smaller overall upfield shift compared with those of the 2-methylene flavinyltyrosine. The upfield shifts of the flavin aromatic protons, C_6H and C_9H, show a similar behavior in relation to the chain length (17 and 12 cps for III_2 and 8 and 10 cps for III_5).

These results also suggest an association between flavin and tyrosine in the peptides ($III_{2,5}$) by vertical stacking and that the geometry for the mode of association is similar to that of the flavinyltryptophans ($II_{2,5}$). Considering the enhanced upfield shift of the flavin C_6H compared with that of C_9H and the upfield shifts of the phenolic protons of the tyrosyl residue (20 cps), the presented chemical-shift data allow the construction of a molecular model of the short-chain flavinyltyrosine,

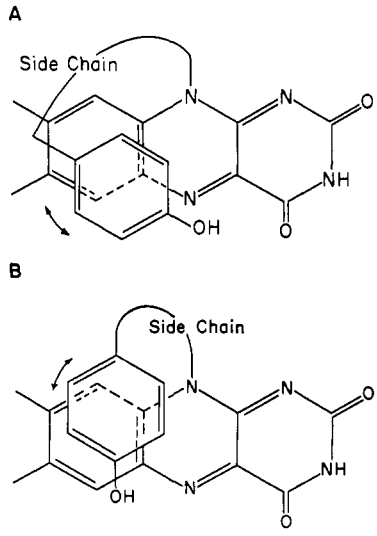


FIGURE 8: Models illustrating proposed conformation for flavinyltyrosines: (A) III_2 and (B) III_5.

depicted in Figure 8A. This illustration shows that on the average, mainly the benzenoid and part of the heteroaromatic portion of the flavin nucleus are involved in the association with the aromatic part of the tyrosine molecule. The long-chain tyrosine (III_5) shows a slightly different conformational arrangement which is shown in Figure 8B, taking into account that the flavin C_9H and tyrosyl-H_B are more shielded than the flavin C_6H and tyrosyl-H_A.

The proton magnetic resonance spectrum of the aromatic region of the flavinylphenylalanine (IV_5, R = R_1) is shown in Figure 7B. The resonance peaks of the flavin C_6H and C_9H and that of the phenyl protons of the phenylalanyl residue follow a similar tendency by appearing at a higher field with the short-chain peptide than those of the corresponding long-chain analog, shown in Table II. As was demonstrated with the short-chain flavinyltryptophan (II_2) and -tyrosine (III_2) the flavin C_6H of IV_2 is more shielded than the C_9H (13 *vs.* 10 cps). However, the upfield shift of these protons of the long-chain peptide (IV_5) shows the same value (9 cps). In addition, the resonance peak of the phenyl protons of IV_2 shifts upfield 16 cps and that of IV_5 10 cps relative to the resonance position of the protons of *N*-acetylphenylalanine. It can be concluded from these data that the flavinylphenylalanines ($IV_{2,5}$) intramolecularly associate in a manner similar to the corresponding flavinyltyrosines ($III_{2,5}$) shown in Figure 8.

All of the foregoing results based on the dependence of chemical shifts upon variation in concentration and solvent are extended by data on the shifts of the flavin C_6H and C_9H upon change in temperature as shown in Figure 9. It can be seen that both C_6H and C_9H of the long-chain carboxypentylflavin (VI_5) are downfield shifted upon increase in temperature; the effect may be slightly greater upon C_6H. A similar trend is found with the flavinyl peptides. As already pointed out, the position (counts per second) of both flavin aryl protons in the flavinyltryptophans, $II_{2,5}$, is essentially the same and remains nearly so throughout the temperature range (10–50°) studied. However, separation of these two peaks becomes greater upon increasing temperature with the flavinyltyrosines ($III_{2,5}$) and -phenylalanines ($IV_{2,5}$) wherein the effect upon C_6H is greater

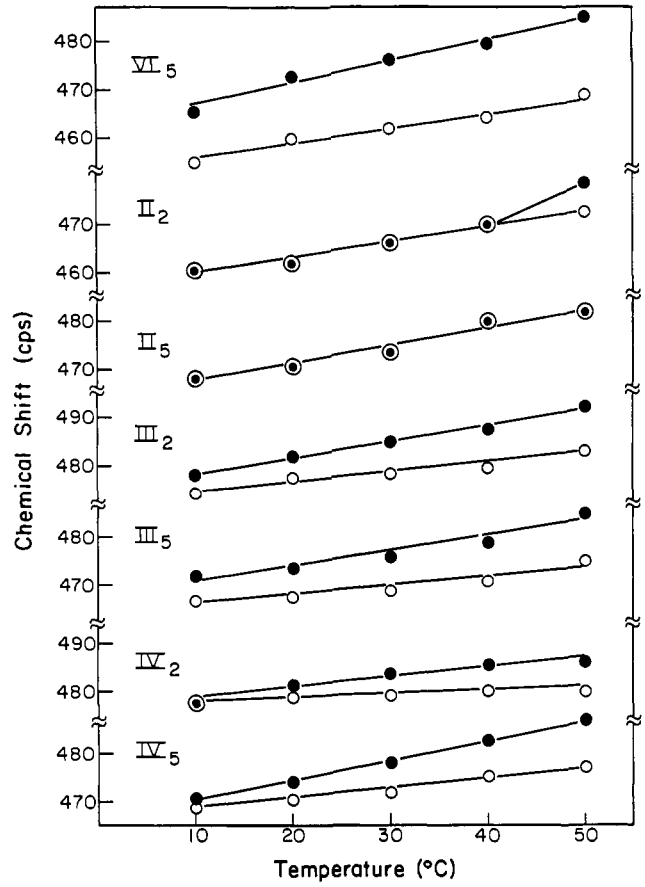


FIGURE 9: The effect of temperature upon the chemical shift of flavin C_6H (●) and C_9H (○) in carboxypentylflavin (VI_5), flavinyltryptophans ($II_{2,5}$), flavinyltyrosines ($III_{2,5}$), and flavinylphenylalanines ($IV_{2,5}$). Flavins were 0.03 M in D_2O (pD 7.0) with tetramethylsilane as external standard.

than upon C_9H. Overall, the data from variation in temperature complement the results obtained by change in solvent, whereupon the intramolecular complexes are more disrupted at higher temperature or concentrations of dimethyl sulfoxide. The more tightly intramolecularly complexed flavinyltryptophans are less effected by such change in temperature than the less tightly complexed flavinyltyrosines or weakly complexed -phenylalanines, in agreement with their fluorescent properties (MacKenzie *et al.*, 1969). Again, as stated earlier, the greater effects observed upon C_6H rather than C_9H emphasize the intramolecular contribution in the folded peptides.

III. Conformation of Flavinyl Aromatic Acid Methyl Esters in Dimethyl Sulfoxide. It was illustrated in Figures 4 and 6 that flavinylamino acids exist in dimethyl sulfoxide in rather unfolded conformations which do not allow as extensive interactions between the aromatic ring systems of flavin and aromatic amino acid in contrast to the mode of association in D_2O. To investigate the possibility of dipolar interactions between tyrosine or tryptophan and the pyrimidine portion of the flavin nucleus in a nonaqueous solvent, the proton magnetic resonance spectra of the flavin aromatic amino acid methyl esters ($II_{1,5}$, $III_{1,5}$, IV_1, V_1) and the *O*-methyltyrosine peptides of $III_{1,5}$ (R = R_2) were measured in dimethyl sulfoxide. For

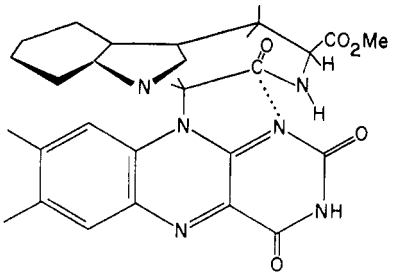


FIGURE 10: Model of flavinyltryptophan (II_1, R = R_2) showing spatial arrangement in dimethyl sulfoxide.

this particular investigation, the very short-chain flavinyl peptides were included to guarantee a very close perturbation distance between flavin and amino acid. The use of the methyl esters of the flavinyl peptides instead of the corresponding free acids diminishes possible changes in conformation due to the interaction of the polar carboxyl group of the amino acid side chain with the isoalloxazine nucleus as may occur with the flavinylamino acids. Because of the proton acceptor properties of dimethyl sulfoxide (Katz and Penman, 1966; Newmark and Cantor, 1968), it was necessary to relate the chemical shifts of the protons of the flavinyl aromatic amino acid methyl esters to reference compounds, *i.e.*, methyl ester of flavinylalanine, *N*-acetylamino acid ethyl esters, and carboxyalkylflavins, in a standard manner. Such an experimental series additionally allows one to distinguish between intermolecular hydrogen bonding of the solvent molecules with amide, imide, and phenolic hydrogens of the flavinyl peptides and possible intramolecular hydrogen bonds with the amide proton and the 2- or 4-carbonyl function of the flavin nucleus.

Chemical shifts were measured in counts per second from liquid tetramethylsilane sealed in capillary tubing within the nuclear magnetic resonance tube, and the reported shifts have not been corrected for bulk susceptibility. No large effect of concentration on the chemical shifts of the flavinyl aromatic amino acid could be observed over a concentration range of 0.12–0.05 M.

The chemical-shift data of the flavinyl amino acid methyl esters are summarized in Tables III and IV. It can be seen that the chemical shifts of the protons of long-chain flavinyl peptides and the corresponding *N*-acetylamino acid ethyl esters exhibit essentially the same values. The flavin C_6H, the imide NH of the flavin portion, and the proton of the phenolic hydroxyl group in the short-chain flavinyl analogs do not show pronounced chemical-shift differences compared with their flavin and amino acid references. However, the resonance peaks of the flavin C_9H and C_8CH_3, and the amide NH of the short-chain flavinyl aromatic amino acid methyl esters exhibit pronounced chemical-shift differences. Thus, the flavin C_9H resonance peak is shifted upfield on the average 26 cps and that of flavin C_8CH_3 (7 cps). Protons of the amide functions of these peptides follow an inverse trend in exhibiting resonance peaks at a field lower (31–39 cps) than do their long-chain flavinyl analogs and the corresponding *N*-acetylamino acid esters.

A possible spatial arrangement, compatible with the above and a space-filling model of flavinyltryptophan methyl ester as an example, is shown in Figure 10. One can see that the aromatic ring of the amino acid methyl ester moiety (whether tryptophan, tyrosine, or phenylalanine) is sufficiently near the flavin C_9H and C_8CH_3 that conformational interactions at these sites could result in altered ring-current shifts. Furthermore, in such a conformational arrangement, a dipolar interaction between N-1 of the flavin, which exhibits the highest locus of basicity in the flavin nucleus (Dudley *et al.*, 1964; Tollin, 1968a,b), and the carbonyl function of the side-chain amide group could occur. Such side-chain to ring interactions under similar conditions have been reported (Föry *et al.*, 1968). Further support for the unfolded type of conformation proposed, is that line widths of the resonance peaks of the flavin and amino acid protons involved are not significantly affected and that the resonance positions of the imide NH are essentially the same for all flavinyl peptides. An overlap by vertical stacking between the aromatic amino acid part and the pyrimidine portion would cause an increase in line width of the peaks of the aromatic amino acid protons. Secondly, the imide N_3 proton of the flavin molecule would, also in disagreement with the experimental data, experience a shielding from the overlapping aromatic amino acid part.

It has to be emphasized that the results obtained are not sufficiently conclusive to propose a specific conformation of the flavinyl aromatic amino acid methyl esters in this nonaqueous environment. Besides the conformational arrangement illustrated in Figure 10, there are other possibilities of intermediate unfolded conformation. In this connection, one may even assume a somewhat random process of conforma-

TABLE III: Chemical Shifts of Flavin Proton Peaks of Flavinyl Amino Acid Methyl Ester (R = R_2) in Dimethyl Sulfoxide.[a,b]

Compound	NH_E	FC_6H	FC_9H	FC_8CH_3	FC_7CH_2
Carboxyalkylflavins					
VI_1	704	499	489	*c*	167
VI_5	700	494	488	*c*	166
Flavinylalanine Methyl Ester					
V_5	703	495	488	*c*	167
Flavinyltryptophan Methyl Ester					
II_1	703	496	462	165[d]	165[d]
II_5	700	495	486	*c*	166
Flavinyltyrosine Methyl Ester					
III_1	703	498	461	167[d]	167[d]
III_5	700	495	486	*c*	166
Flavin-*O*-methyltyrosine Methyl Ester					
III_1 (*p*-OCH_3)	704	497	461	166[d]	166[d]
III_5 (*p*-OCH_3)	700	494	486	*c*	166
Flavinylphenylalanine Methyl Ester					
IV_1	703	496	461[e]	166[d]	166[d]

[a] Chemical shifts in cps downfield from external tetramethylsilane at 37°; flavin concentrations were 0.12 M. [b] For peak assignments, see Figures 3 and 7. [c] This peak appears at resonance position of D_5H-dimethyl sulfoxide (multiplet), 172 cps. [d] One peak of 6 protons from combined FC_8CH_3 and FC_7CH_2. [e] This peak appears at resonance position of phenyl protons.

TABLE IV: Chemical Shifts of Amino Acid Proton Peaks of Flavinyl Amino Acid Methyl Esters (R = R_2) in Dimethyl Sulfoxide.[a-c]

Compound	NH_F	C_6H_5-OH	NH_D	$C_{1',4'}H$[d]	C_6H_5-H	$C_{5'}H$	$C_{2',3'}H$[d]	H_A	H_B	H_C[d]	C_6H_5-OCH_3	$H_{A'}H_B$[d]	CH_3
						Flavinylalanine Methyl Ester							
V_5			515							282[e]			96[f]
						N-Acetyltryptophan Ethyl Ester							
	672		515	464		450	444			293		208	
						Flavinyltryptophan Methyl Ester							
II_1	673		554	464		456	442			298		212	
II_5	673		516	463		452	443			*g*		209	
						N-Acetyltyrosine Ethyl Ester							
		573	515					442	424	284		193	
						Flavinyltyrosine Methyl Ester							
III_1		573	546					443	424	290		190	
III_5		571	514					442	424	*g*		192	
						O-Methyltyrosine Methyl Ester·HCl							
								453	436		248		
						Flavinyl-*O*-methyltyrosine Methyl Ester							
III_1 (*p*-OCH_3)			548					450	434	293	247	198	
III_5 (*p*-OCH_3)			515					450	432	292	242	195	
						N-Acetylphenylalanine Ethyl Ester							
			518		458					290		199	
						Flavinylphenylalanine Methyl Ester							
IV_1			550		457					294		203	

[a,b] See Table III. [c] Chemical shifts of $COOCH_3$, 237 ± 2 cps; chemical shift of M_2SO-D_5H (center of multiplet), 172 cps. [d] Center of multiplet. [e] Quartet. [f] Doublet. [g] Not resolved.

tional interactions between flavin and aromatic amino acid in dimethyl sulfoxide.

Concluding Remarks

The specific upfield shifts of the aromatic protons of the flavin and amino acid moieties of the flavinyl peptides in D_2O allow the conclusion that the aromatic and heteroaromatic portion of these compounds interact intramolecularly *via* vertical ring stacking. It was further shown that the spatial arrangements of the flavinyltryptophans, -tyrosines, and -phenylalanines are similar within a homologous series going from a long-chain to the short-chain peptide. Additionally, the magnitude of the upfield shifts of the aromatic protons of tryptophanyl > tyrosyl > phenylalanyl moieties within the flavinyl peptides is in agreement with the same order for extent of flavin fluorescence quenching and overall degree of intramolecular association (MacKenzie *et al.*, 1969).

Because of the complexity of theoretical evaluations of ring-current fields related to flavin and amino acid molecules, no attempt was made to correlate the observed ring-current shifts with the average distance separating the flavin from the aromatic amino acid, as was done by Johnson and Bovey (1958) in the case of benzene. Furthermore, for the interpretation of the specific upfield shifts in connection with the related conformational arrangements, it was assumed that the diamagnetic ring-current fields of the benzenoid and heteroaromatic portion of the isoalloxazine nucleus are to a first approximation of similar magnitude.

The possibility of charge-transfer interactions in formation of the intermolecular complex between flavin and tryptophan was suggested in numerous investigations (Fujimori, 1959; Harbury *et al.*, 1959; Szent-Györgyi, 1960; Cilento and Tedeschi, 1961; Wilson, 1966). Furthermore, Karreman (1961, 1962) proposed a spatial arrangement of the flavin–tryptophan complex, taking into account possible coulombic interactions between the indole and isoalloxazine ions, obtained by charge transfer from tryptophan to the flavin molecule. Thus, considering such an arrangement of maximal coulombic attraction, interatomic distances, and bond angles, the benzenoid and heteroaromatic portions of the indolyl residue are situated above the pyrimidine and heteroaromatic parts, respectively, of the flavin. The results for the intramolecular complexes in aqueous environment presently investigated suggest that mainly the aromatic portion of flavin and aromatic amino acids are involved in this flavin–amino acid association. Although dispersion forces are likely to be additionally involved in maintaining the proposed folded conformations, at least

for the tryptophanyl and tyrosyl peptides, charge-transfer forces are not dominant in stabilizing the ground states of the complexes of the flavin aromatic amino acids.

Experimental Section[2]

Proton mangetic resonance spectra were determined at 60 and 100 MHz on high-resolution Varian Models A-60A and HA-100 spectrometers, respectively, the latter operating in the frequency-sweep mode. All measurements were carried out at a probe temperature of 37 ± 1° except where otherwise noted. Every sample was preequilibrated at the desired temperature for at least 5 min. Chemical shifts were measured in cycles per second either from external tetramethylsilane or internal 2,2-dimethyl-2-silanepentane-5-sulfonate. No bulk susceptibility corrections have been made. The chemical shifts have not been corrected for the intermolecular effects of the aromatic flavins on the resonance position of the protons of 2,2-dimethyl-2-silanepentane-5-sulfonate (*cf.* Hand and Cohen, 1965). These effects are small compared with the chemical shifts observed for this flavin system. The accuracy of the measured shifts are well within ±1 cps, being somewhat limited by line width and reduced signal intensities at low solute concentrations. D_6-Dimethyl sulfoxide and D_2O were used as solvents. Solutions in D_2O were prepared using sodium hydroxide (NaOD) to adjust the pD[3] to 7.0. The exchangeable protons of the samples were removed by lyophilization from D_2O, and the samples were redissolved in fresh D_2O.

Flavinyl Amino Acids ($II_{2,5}$, $III_{2,5}$, IV_5, $R = R_1$). A mixture of 1 mmole of $I_{2,5}$, 1.25 mmoles of the appropriate L-amino acid, and 0.12 g of triethylamine in 20 ml of dimethyl sulfoxide was stirred at room temperature for 4 hr and then poured into a mixture of 600 ml of diethyl ether and 200 ml of petroleum ether (bp 30–60). The solvent was decanted and the oily residue was washed three times with ether. The crude material was dissolved in 30 ml of 10% sodium bicarbonate, treated with decolorizing carbon, and filtered, and the filter residue was washed with 10 ml of water. The combined filtrates were extracted three times with chloroform. The organic phases were washed twice with 10 ml of 5% sodium bicarbonate. The combined aqueous solutions were extracted twice with 500 ml of ether and adjusted to pH 1 with concentrated hydrochloric acid. The precipitate was collected on a filter and washed three times with 10 ml of cold water. The crude flavin was twice reprecipitated from 20 ml of 5% sodium bicarbonate with concentrated hydrochloric acid; yields obtained were 50–60%.

II_2 [R = R_1]; yield; 50%. *Anal.* Calcd for $C_{26}H_{24}N_6O_5$: C, 62.39; H, 4.83; N, 16.79. Found: C, 62.31; H, 4.72; N, 16.65.

II_5 [R = R_1]; yield: 55%. *Anal.* Calcd for $C_{29}H_{30}N_6O_5$: C, 64.19; N, 5.57; N, 15.49. Found: C, 64.07; H, 5.65; N, 15.29.

III_5 [R = R_1]; yield: 58%. *Anal.* Calcd for $C_{27}H_{29}N_5O_6$: C, 62.42; H, 5.63; N, 13.48. Found: C, 62.73; H, 5.34; N, 13.53.

IV_5 [R = R_1]; yield: 60%. *Anal.* Calcd for $C_{27}H_{29}N_5O_5$: C, 64.40; H, 5.80; N, 13.91. Found: C, 64.46; H, 5.99; N, 14.36.

Flavinyl Amino Acid Benzyl Esters (III_2, IV_2, $V_{2,5}$, $R = R_3$). To 30 ml of dimethyl sulfoxide were added 1 mmole of $I_{2,5}$, 1.25 mmoles of the appropriate L-amino acid benzyl ester *p*-tosylate, and 0.8 g of anhydrous sodium bicarbonate. The mixture was stirred for 4 hr at room temperature and then poured into 170 ml of *n*-butyl alcohol. The butanol solution was extracted twice with 160 ml of water, then successively with 100 ml each of 0.5 N hydrochloric acid, water, and 5% sodium bicarbonate. The aqueous solutions were washed twice with 100 ml of ethyl acetate. The combined organic phases were dried over sodium sulfate and evaporated to a small volume (6–10 ml), and the benzyl ester was precipitated by slow addition of ether. The flavinyl peptide benzyl ester appeared as one spot in *n*-butyl alcohol–2 N ammonium hydroxide–ethanol (3:1:1) or acetone on thin-layer chromatography.[4] Yields obtained were 72–94%.

Flavinyl Amino Acids (III_2, IV_2, $V_{2,5}$, $R = R_1$). A suspension of the flavinyl amino acid benzyl ester in 270 ml of *n*-butyl alcohol was treated at 90–100° for 2–3 min and then hydrogenated over palladium black (Wieland, 1912; Willstätter and Waldschmidt-Leitz, 1921) at room temperature and 4 atm for 4.5 hr. Ammonium hydroxide (100 ml of 0.5 N) was added and the mixture was stirred for 3–4 min. The catalyst was collected on a filter and washed with 50 ml more of the ammonium hydroxide in small portions. The combined heterogeneous filtrates were adjusted to pH 7.0 and evaporated to dryness under reduced pressure at 40°. The residue was suspended in 20–30 ml of 2 N hydrochloric acid and kept overnight at 4°. The crude flavin was collected on a filter and washed with small portions of cold water. Reprecipitation from 0.5 N ammonium hydroxide with concentrated hydrochloric acid, followed by crystallization from dimethylformamide–ether, gave the flavinyl amino acids (R = R_1) in 42–79% yield.

III_2 [R = R_1]; yield: 79%. *Anal.* Calcd for $C_{24}H_{23}N_5O_6$: C, 60.37; H, 4.86; N, 14.67. Found: C, 60.90; H, 4.71; N, 14.10.

IV_2 [R = R_1]; yield: 65%. *Anal.* Calcd for $C_{24}H_{23}N_5O_5$: C, 62.46; H, 5.02; N, 15.18. Found: C, 62.49; H, 5.26; N, 15.37.

V_2 [R = R_1]; yield: 60%. *Anal.* Calcd for $C_{18}H_{19}N_5O_5$: C, 56.10; H, 4.96; N, 18.17. Found: C, 56.00; H, 5.16; N, 18.39.

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[2] Elemental analyses were performed by Schwarzkopf Microanalytical Laboratory, Inc. (Woodside, N. Y.).

[3] pD = pH (meter reading) + 0.40 (Glasoe and Long, 1960).

[4] Thin-layer chromatograms were run on MN silica gel S-HR (Brinkmann). Spots were detected by visual examination under ultraviolet light.

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Aminomalonic Acid. Spontaneous Decarboxylation and Reaction with 5-Deoxypyridoxal*

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ABSTRACT: The spontaneous decarboxylation of aminomalonic acid has been studied as a function of pH at constant temperature and ionic strength. It has been found that the neutral species, $^{+}NH_3CH(COOH)COO^{-}$, is about 2.5 times more reactive than the protonated form, $^{+}NH_3CH(COOH)_2$, and approximately 3600 times more reactive than malonic acid monoanion, $CH_2(COOH)COO^{-}$ An anionic mechanism for the spontaneous decarboxylation is suggested. The interaction of aminomalonate with 5-deoxypyridoxal in the pH range 2.9–6.7 at 30° results in the formation of β-(2,5-dimethyl-3-hydroxypyridyl-4-)serine by an aldol-like condensation and decarboxylation. At zero buffer concentration (extrapolated), this appears to be the only reaction. However, in the presence of buffers, there is observed in addition to the condensation reaction, buffer catalysis of the 5-deoxypyridoxal-catalyzed decarboxylation of aminomalonate.

There have been a number of isolated investigations concerned with a possible biochemical role for aminomalonic acid. As early as 1914, the possibility was raised that aminomalonic acid might be an intermediate in protein metabolism, especially in the serine–glycine conversion (Knoop, 1914). Shemin (1946), using a double-labeling technique, ruled out this pathway but the theoretical basis for Shemin's experiments was questioned by Ogston resulting in the well-known "3-point attachment" hypothesis for interaction between enzyme and substrate (Ogston, 1948). More recently, several reports have offered suggestive evidence that aminomalonate may play an as yet unidentified role in biological systems. Thus, an aminomalonic decarboxylase has been found in silkworm glands (Shimura *et al.*, 1956), rat liver (Thanassi and Fruton, 1963), and in several microorganisms (Matthew and Neuberger, 1963a). Ketomalonic acid, the α-keto analog of aminomalonic acid, has been found in several different species (Hammen and Lum, 1962; Heick and Stewart, 1964);

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