Electron Paramagnetic Resonance Studies of the Triplet State of Flavin and Pteridine Derivatives*

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ABSTRACT: By electron paramagnetic resonance (epr) techniques, the $\Delta m = 1$ and $\Delta m = 2$ spectra of the lowest triplet state of riboflavin derivatives were investigated. In rigid glasses at 77°K the derivatives of alloxazine, isoalloxazine, leucoflavin, and pteridine were photoexcited into the triplet state. The effect of substituents at different positions of the molecular ring was investigated to get information on the electronic structure of the triplet state of the flavins. This highly delocalized triplet state has a spin distribution similar to that of the flavin semiquinone radical. The leucoflavins did not give rise to an epr triplet signal. This may be correlated with previous work which indicates a bent configuration. Epr and phosphorescence measurements provide information on the amphoteric behavior of flavin mononucleotide in the lowest triplet state. By these measurements the anionic form of this molecule cannot be distinguished from the neutral one, in contrast to the cationic form. For the cationic–neutral equilibrium the pK values in the ground state and in the triplet state are shown to be comparable, and much lower than that of the lowest excited singlet state. Riboflavin derivatives sensitize the photodecomposition of solvent molecules. This probably involves a biphotonic process with the lowest triplet state of the solute as intermediate.

Riboflavin shows phosphorescence in rigid media at low temperatures (Dhere and Castelli, 1938). By epr[1] techniques the strong $\Delta m = 2$ triplet signal was observed when illuminating riboflavin in rigid solutions at 77°K (Smaller, 1963). Shiga and Piette (1964, 1965) detected an epr triplet signal of flavin derivatives. Furthermore, they studied the pH dependence of the triplet state. The maximum triplet yield was obtained in neutral solution.

The following epr studies of the triplet states of substituted flavins and pteridines were undertaken to get more information on the electronic properties of riboflavins. Such an investigation of riboflavins seems to be desirable in order to understand better photochemical reactions occurring *via* the triplet state (for a review see, Oster *et al.*, 1962; Holmström, 1964). The triplet state probably plays an important role, too, when riboflavin acts as a photosensitizer and an electron donor in biological processes.

Radda and Calvin (1964) found that FMN in the triplet state is a better electron acceptor than in the ground state. Radda (1966) examined the inhibitory action of electron donors on the photoreduction rates of flavins. He proposed a quenching mechanism involving a complex between the inhibitor and the flavin in its triplet state.

Whether higher excited states are involved in photosensitization processes of flavins has not been carefully investigated. In the case of naphthalene, for instance, it was possible to demonstrate that free radicals are produced by solvent decomposition at a rate proportional to the rate of the appearance of the epr signal due to the naphthalene triplet state (Siegel and Eisenthal, 1965). A biphotonic absorption process has been suggested to explain photosensitization of alcohol, when irradiating nucleic acid derivatives in alcoholic solutions at 77°K (Hélène *et al.*, 1966). Similar phenomena have been observed in the case of aromatic amino acids (Douzou and Ptak, 1964). Thus it seemed reasonable to look after such processes in the case of flavins.

In the course of our experiments, the epr spectrum of the triplet state of the cationic form of FMN could be distinguished from the neutral and anionic ones. This enabled us to demonstrate that the pK value of the triplet state is of the same order of magnitude as that of the ground state.

Epr Investigation of the Lowest Triplet State of Organic Molecules. The following section summarizes the basic concepts of epr spectroscopy of the lowest triplet state of organic molecules (Hutchison and Mangum, 1961; de Groot and van der Waals, 1960; Kottis and Lefebvre, 1963, 1964; Wasserman *et al.*, 1964). Microwave transitions occur between the three energy levels

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[1] Abbreviations used: epr, electron paramagnetic resonance; FMN, flavin mononucleotide; ZFS, zero-field splitting.

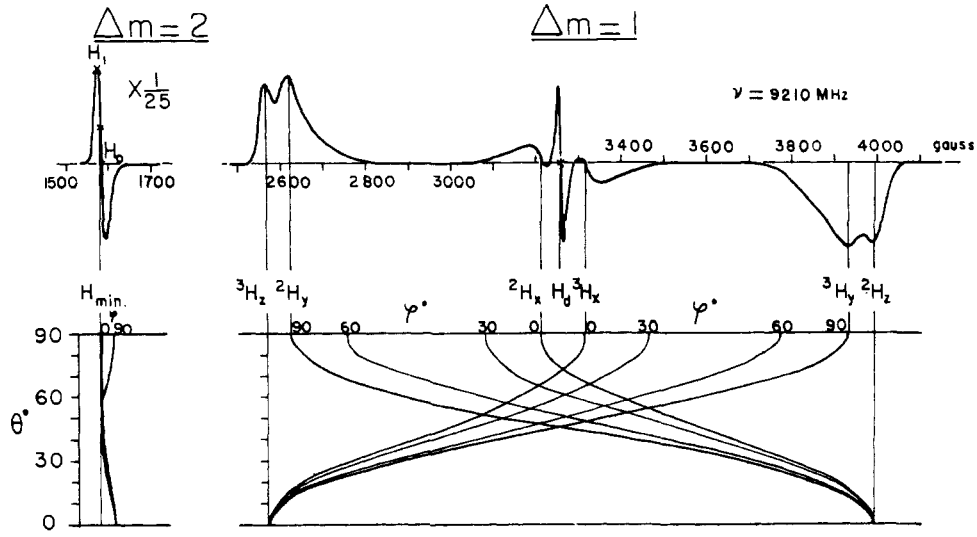

FIGURE 1: Epr spectrum of the lowest triplet state of FMN. This is the cationic form photoexcited in a rigid solution of water–propylene glycol–6 N HCl, at 77°K. The resonance field distribution is given in the lower part.

of the molecule in the triplet state. These energy levels depend upon the following main features.

1. ZEEMAN EFFECT. The splitting of the energy levels is proportional to the field strength of an external static magnetic field. The magnetic field removes the degeneracy of the zero-field triplet state. The three energy levels are associated with the magnetic quantum numbers, $m = -1, 0$, and $+1$, respectively.

2. MAGNETIC DIPOLE–DIPOLE INTERACTION between the two electrons contributing to the triplet state. This interaction removes the degeneracy of the triplet state, even in the absence of an external magnetic field. The corresponding energies are denoted as ZFS energies. The dipole–dipole interaction is an anisotropic one, described by a spin–spin coupling tensor, the diagonalized form of which determines the coordinate system of the magnetic axes. In the system of the magnetic axes the ZFS energies are given by the following relation

$$X = -\tfrac{1}{2}(g\beta)^2 \left\langle \frac{r_{12}^2 - 3x_{12}^2}{r_{12}^5} \right\rangle \qquad (1)$$

and similarly the Y and Z energies by cyclic change of the variable x_{12}. The distance between the two electrons is denoted by r_{12}. The vector $\vec{r}_{12} = \vec{r}_2 - \vec{r}_1$ has the components x_{12}, y_{12}, and z_{12}, respectively. The bracket denotes an average over the triplet electron wave function. β is Bohr's magneton, and the g factor is assumed to be isotropic.

From eq 1 it follows that

$$X + Y + Z = 0 \qquad (2)$$

In the literature there also exist the following notations

$$D = -\tfrac{3}{2}Z \text{ and } E = -\tfrac{1}{2}(X - Y) \qquad (3)$$

with the ZFS values chosen as $|X| < |Y| < |Z|$. The mean-square ZFS parameter is defined as

$$D^* = (D^2 + 3E^2)^{1/2} = \tfrac{3}{2}(X^2 + Y^2 + Z^2)^{1/2} \qquad (4)$$

The magnetic dipole–dipole interaction has three consequences for epr spectroscopy of randomly distributed triplet molecules. (a) The resonance field values are strongly dependent upon the orientation of the molecule with respect to the direction of the external steady magnetic field. Using the X-band microwave frequency range, the detection of triplet states ($\Delta m = \pm 1$ transitions) in randomly oriented molecules is rather difficult since the epr signal is spread over several thousands of gauss. This difficulty can be overcome by orienting the triplet molecules in a host matrix of a single crystal, like durene or biphenyl. Up to now, this technique has been applied to study the triplet state of symmetric molecules like naphthalene, phenanthrene, and phenoxazine (Hutchison and Mangum, 1961; Brandon *et al.*, 1964; Lhoste *et al.*, 1966). (b) The three spin states are no longer pure quantum states. The $\Delta m = \pm 2$ transitions become allowed and are only slightly anisotropic compared to the $\Delta m = \pm 1$ transitions (de Groot and van der Waals, 1960). The $\Delta m = 2$ transition represents the lowest resonance field, H_{min}. From the value of H_{min} it is only possible to calculate the mean-square ZFS. (c) The resonance field values for the $\Delta m = 1$ transitions are stationary when the steady magnetic field is parallel or nearly parallel (within a cone of about 5–10°) to one of the three magnetic axes. These canonical orientations appear as peaks in the derivative epr spectrum. With

favorable molecules they can be detected although the intensity is two orders of magnitude lower than the H_{min} line intensity. The positions of these canonical peaks determine the three ZFS energies according to eq 7 (Kottis and Lefebvre, 1963, 1964; Wasserman *et al.*, 1964).

In the $\Delta m = 1$ region there appears a sharp absorption line. This line is due to the direct transition from the lowest to the highest level, induced by a simultaneous absorption of two microwave quanta. This double-quantum transition is only weakly dependent upon the orientation of the molecules; its position in the epr spectrum, H_d, gives a value of D^*.

The ZFS energies can be derived from the resonance field values by means of

$$D^{*2} = \frac{3}{4}[\delta^2 - 4(g\beta)^2 H^2_{min}] \quad (5)$$

$$D^{*2} = 3[\delta^2 - (g\beta)^2 H_d^2] \quad (6)$$

$$X = \frac{1}{6\delta}(g\beta)^2[{}^3H_x^2 - {}^2H_x^2] \quad (7)$$

and similar for Y and Z, respectively. δ is the microwave quantum. ${}^3H_x$ and ${}^2H_x$ are the stationary resonance field values in the $\Delta m = 1$ region.

As an illustration (Figure 1), the resonance field distribution is plotted for the epr spectrum of the triplet state of the cationic form of flavin mononucleotide. Another, well elaborated example, has been done for phenoxazine (Lhoste *et al.*, 1966), which is a molecule akin to riboflavin. According to Kottis and Lefebvre (1963, 1964), the resonance field distribution has been numerically calculated as a function of the molecular orientation. The ZFS energies were derived from the $\Delta m = 1$ spectrum. The magnetic axes were assumed to form a Cartesian coordinate system, its axes, x, y, z, being associated with the ZFS energies, X, Y, Z, respectively. The angles θ and φ are the polar coordinates of the magnetic field direction in this coordinate system. The magnetic resonance field distribution is plotted for any value of the θ angle, taking different values for the angle φ (Figure 1).

Only the Hutchison and Mangum technique (1961) enables one to relate the magnetic axes, and thus the ZFS energies, to the molecular geometry. Furthermore, this type of experiment may provide a detailed picture in terms of spin densities if the anisotropic hyperfine structure arising from the interaction of the electron spin with the nuclear spins can be resolved. Such data have been obtained with molecules akin to the basic three-ring system of riboflavin, *viz.*, with phenoxazine and phenothiazine oriented in a single crystal of biphenyl (J. M. Lhoste and M. Ptak, to be published). With the flavins and pteridines studied, this technique was not applicable. Therefore, the electronic configuration of the triplet state has to be investigated from randomly oriented molecules. Pertinent data are furnished by the ZFS energies which become modified with respect to different ionic, reduced, and substituted forms of the flavin and pteridine rings.

Experimental Section

Samples. Riboflavin and FMN were purchased from Calbiochem; pteridine derivatives from Aldrich Co. The derivatives of riboflavin, lumiflavin, and leucoflavin were prepared (Dudley *et al.*, 1964; Hemmerich *et al.*, 1965a). Lumazine and methylated lumazines were generously supplied by Dr. Pleiderer, Technische Hochschule, Stuttgart, Germany.

The solvents used are indicated in Table I. They gave noncracking transparent rigid glasses at 77°K. It was verified that the pure solvents did not phosphoresce and gave no epr signal.

The solutions used were 10^{-3}–10^{-2} M for epr measurements, and 5×10^{-4} M for phosphorescence studies. They were contained in quartz tubes for 3-mm i.d. Both degassed and nondegassed samples were used, with similar results.

Optical Measurements. Absorption spectra were measured with a Cary 15 spectrometer. These spectra were also used to check the concentration and the purity of the compounds. Low-temperature spectra were recorded using a cooling system built in the laboratory. Phosphorescence spectra at 77°K were measured with an Aminco-Keirs spectrophosphorimeter equipped with a 1P28 photomultiplier.

Epr Spectra. They were recorded at 77°K with a Varian X-band spectrometer equipped with an optical transmission cavity and the low-temperature gas-flow system. The magnetic field strength was calibrated with a Varian F-8 proton resonance flux meter, the frequency of which was monitored by a Hewlett-Packard frequency counter. The microwave frequency was calibrated with a Silverlab wavemeter.

The light of an Osram HBO 500W mercury arc was focused on the sample by a quartz lens. An aqueous solution of $CuSO_4$ avoided warming up of the sample and a glass filter cut off the short-wavelength ultraviolet light. Different light intensities were obtained with neutral density filters.

The $\Delta m = 2$ line and parts of the $\Delta m = 1$ spectrum were recorded in the usual way. The central region of the $\Delta m = 1$ spectrum, being superimposed to the free-radical signal, was plotted as the difference spectrum in the signal intensity before and during illumination; fixed magnetic field values were used. The experiments were performed by irradiating the samples only for a short time. The samples were frequently replaced in order to eliminate triplet signals from photochemical degradation products.

Results

Epr Spectra of the Flavin Derivatives. A typical epr spectrum of the lowest triplet state of a flavin derivative is presented in Figure 1. The $\Delta m = 1$ line shape is somewhat unusual since two ZFS energies have comparable absolute values (but opposite signs),

TABLE I: Epr Properties of the $\Delta m = 2$ Transitions in Rigid Solvents at 77°K.[a]

	Solvent[b]	H_0 (gauss)	D^*/hc (cm^{-1})[c]	λ_{max} ($m\mu$)
Isoalloxazines				
Riboflavin	Et	1602	0.062	605
Lumiflavin	Et	1602	0.062	605
Tetraacetylriboflavin	Et	1603	0.061	610
3-Benzyllumiflavin	Et	1602	0.062	605
O-(2)-Methyllumiflavin	Et	1596	0.066	598
2-Thiolumiflavin	Et	1596	0.066	585
4-Phenyliminolumiflavin	Et–DMF	1599	0.064	620
8-Morpholino-8-norlumiflavin	Et–DMF	1585	0.072	620
FMN neutral	Pg–W	1602	0.062	608
anionic	Pg–W–NaOH	1602	0.062	608
cationic	Pg–W–HCl	1582	0.074	555
Alloxazines				
Lumichrome	Et	1578	0.077	545
1,3-Dimethyllumichrome	Et	1581	0.075	555
1,3-Dimethylalloxazine	Et	1575	0.078	530
Leucoflavins				
1,3,10-Trimethylleucoflavin	Et	No signal[d]		545[d]
1,3,10-Trimethyl-5-benzylleucoflavin	Et	No signal		470
5-Acetylleucoflavin	Et	No signal		443
5-Isopropylleucoflavin	Et	No signal[d]		540[d]
10-Isopropylleucoflavin	Et	No signal		435
Pteridines				
Lumazine	Et	1564	0.086	470
1,3-Dimethyllumazine	Et	1569	0.083	470
8-Methyllumazine	Et	1578	0.079	460
6,7-Dimethyl-4-oxopteridine	Et–DMF	1535	0.098	462
2-Amino-6,7-dimethyl-4-oxopteridine	Et–DMF	1559	0.088	502
2-Ethylmercapto-4-oxopteridine	Et–DMF	1557	0.089	455
Xanthopterin	Et–DMF	No signal		465
7-Methylxanthopterin	Et–DMF	No signal		440
2,4-Diamino-6,7-dimethylpteridine	Et–DMF	1537	0.097	460
2,4,7-Triamino-6-phenylpteridine	Et–DMF	1538	0.097	540

[a] Wavelength of the maximum phosphorescence intensity of flavins and pteridines in rigid solutions at 77°K. [b] Solvents: Et = ethanol, Pg–W = propylene glycol–water (1:1), Et–DMF = Et–dimethylformamide (6:1). [c] D^* value calculated from corrected H_{min} value (see text). [d] Phosphorescence of a lumichrome derivative. A weak epr signal with the H_0 value of lumichrome was observed.

and therefore the corresponding pairs of peaks coalesce. The energies X, Y, Z, were calculated from eq 7 using stationary field values from Figure 1. The narrow line, H_d, corresponds to the double-quantum transition. From eq 4–6 the value of H_{min} may be calculated with a precision determined by that of the data of the $\Delta m = 1$ spectrum. In the derivative epr spectrum, the value of H_{min} is related to the position of the first maximum of the derivative line, H_1, and to H_0, the intersection of the derivative line with the gauss axis (Figure 1). As suggested by Kottis and Lefebvre (1963, 1964) it is given by

$$H_{min} = H_1 + nh \quad (0 \leqslant n \leqslant 1) \qquad (8)$$

Here, $h = H_0 - H_1$. For flavins and pteridines n was experimentally found to be equal to 0.6. The higher the parameter n, the lower the value of D^*. The reason for this fact is that for triplet states with low ZFS energies the peak-to-peak linewidth of the $\Delta m = 2$ line is mainly due to hyperfine interaction and small variations of the D^* value resulting from matrix effects.

Tables I and II list the experimental value of the ZFS energies derived from the $\Delta m = 2$ and $\Delta m = 1$ spectra. A weak signal observed with two leucoflavins (Figure 2) is not reported in Table I, since the position of this line coincides with that of lumichrome. Being also confirmed by phosphorescence emission, this

TABLE II: Zero-Field-Splitting Energies Derived from the $\Delta m = 1$ Epr Spectra of Flavins and Pteridine Triplet States in Rigid Solutions at 77°K.[a]

	Solvent	X	Y	Z	D	E	D^*
Isoalloxazines							
Riboflavin	Et						
3-Benzyl-lumiflavin	Et	±0.0034	±0.0336	∓0.0369	±0.0553	±0.0150	0.0611
FMN neutral	PG–W						
anionic	PG–W–NaOH						
cationic	PG–W–HCl	0.0034	0.0404	0.0440	0.0660	0.0185	0.0733
Tetraacetylriboflavin	Et	0.0067	0.0309	0.0376	0.0564	0.0121	0.0602
Alloxazines							
Lumichrome	Et	0.0035	0.0424	0.0460	0.0690	0.0194	0.0768
1,3-Dimethyllumichrome	Et	0.0026	0.0415	0.0440	0.0660	0.0194	0.0741
1,3-Dimethylalloxazine	Et	0.0075	0.0406	0.0481	0.0721	0.0165	0.0776
Pteridines							
Lumazine	Et	0.0028	0.0480	0.0508	0.0763	0.0226	0.0858
1,3-Dimethyllumazine	Et	0.0056	0.0447	0.0503	0.0755	0.0196	0.0828

[a] The values are divided by the constant factor hc and given in reciprocal centimeters.

arises from the fact that leucoflavins are oxidized in the presence of light.

Phosphorescence Spectra of the Flavin Derivatives. The wavelengths, given in Table I, are derived from the peak of the phosphorescence spectrum. It is corrected with respect to the spectral sensitivity curve of the photomultiplier. The exact phosphorescent lifetime, being for most of the compounds tested shorter than 0.1 sec, was not accessible with our equipment. Shiga and Piette (1964, 1965) measured for riboflavin a mean half-life of 9–27 msec, depending on experimental conditions at 77°K. Being corrected for the spectral emission of the light source, the excitation spectra were compared to the absorption spectra to eliminate the presence of impurities. Most of the absorption spectra are reported in the literature (Dudley *et al.*, 1964).

The Acid–Base Properties of the FMN Triplet State. Aqueous solutions of FMN were used, the pH of which was adjusted with sodium hydroxide and hydrochloric acid. These solutions were then mixed with an equal volume of propylene glycol in order to get a glassy solvent at 77°K. Among the three ionic states of FMN (Figure 3), the neutral and the anionic ones could not be distinguished, neither by taking phosphorescence spectra nor by the position of the $\Delta m = 2$ epr line. The cationic form showed differences in the emission spectrum (Figure 4) as well as in the epr spectrum (Tables I and II).

Monitoring the H_{min} line intensity and using the corrected emission intensity of these two ionic forms, the protonation in the triplet state was studied as a function of the concentration of HCl added. The reaction was performed in the propylene glycol matrix at 77°K (Figure 5a). For the ground state the same reaction was studied, with the same solvent at 300°K and at 77°K, respectively (Figure 5b). Here, the percentage of the two forms was measured from their absorption spectra. These spectra are different (Dudley *et al.*, 1964) and, besides becoming narrower, the absorption bands remain unchanged at low temperatures.

Solvent Sensitization Reactions of Flavins. During the ultraviolet irradiation of ethanolic solutions at 77°K of any flavin or pteridine tested, there arises in the epr spectrum a free-radical signal which increases rapidly with the time of illumination. The line shape of that signal corresponds (Siegel and Eisenthal, 1965) to that of the free-radical $CH_3\dot{C}HOH$, superimposed on a single line with a lower g value. With flavin derivatives as solutes, this single line was found to have the same g value and a line width similar to that of the signal obtained, when irradiating a frozen aqueous solution of FMN. Using a glass cut-off filter ($\lambda > 3100$ A), the epr signal remained unchanged under conditions where a long irradiation of pure ethanol produced no epr signal. The relative intensity of the two components of the free-radical signal remained constant with respect to any intensity or time of ultraviolet exposure.

The free-radical production was compared to the triplet state population of the solute as a function of time, and for different intensities of incident light. The triplet population of the solute was measured by setting the steady magnetic field at the resonance value, corresponding to the first maximum H_1 of the $\Delta m = 2$ line. The free-radical population was determined in a second independent run, with a new sample and under similar conditions of irradiation. In that case the field value for resonance was adjusted on a hyperfine component of the free-radical signal of ethanol.

A typical experiment obtained with riboflavin in

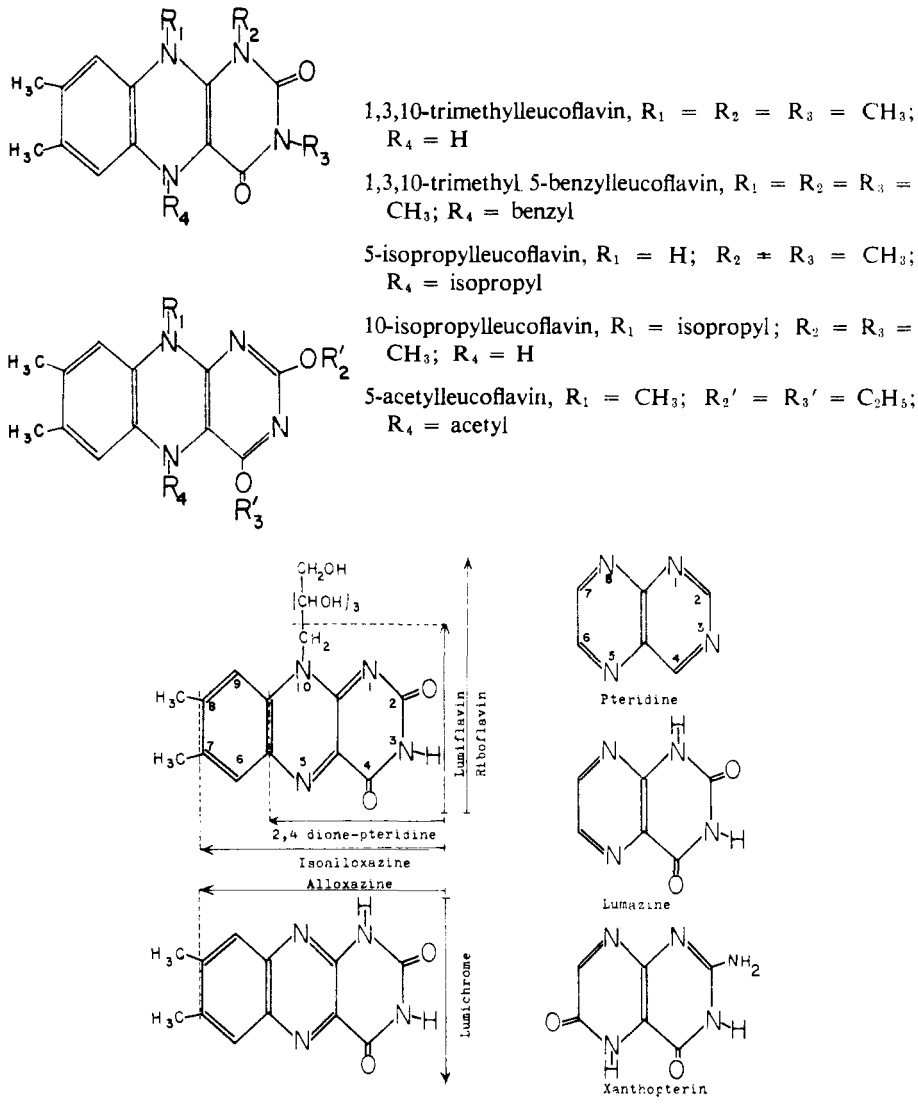

FIGURE 2: Nomenclature and numbering of the flavins and pteridines.

ethanol is presented in Figure 6. Within experimental precision, the free-radical production at any time was found to be proportional to the triplet population of the solute, for different light intensities used. The initial slopes of the free-radical production curves (tan α, Figure 6) and of the steady-state triplet population curves (tan β, Figure 6) were related to the relative incident light intensity, I. They are proportional to I^a and I^b, respectively; the values of a and of b depend on the nature of the solute and the absolute light intensity (Table III).

Discussion

Nature of the Lowest Triplet State. The ZFS energies of the lowest triplet state of flavins and pteridines are among the smallest ones observed for phosphorescent bicyclic and tricyclic aromatic molecules. Equation 1 shows that these low values correspond to a high delocalization of the two electrons contributing to the triplet state. We may conclude that the two electrons are probably spread over π orbitals of the $\pi-\pi$ triplet state. The radiative lifetime of the phosphorescent triplet state of riboflavin is of the order of 20 msec (Shiga and Piette, 1964, 1965). Such a mean half-life may also be attributed to a $n-\pi$ triplet state (Ermolaev, 1963). It has to be mentioned that the quinoid state of flavins is probably planar. The hydroquinoid state (leuco form) can be aplanar, depending on the environmental conditions (Hemmerich *et al.*, 1965b). For that form we could not detect any epr triplet signal.

For planar $\pi-\pi$ triplet states the ZFS energy associated with the axis perpendicular to the molecular plane is one of the Z (or Y) energies (Table II), and it

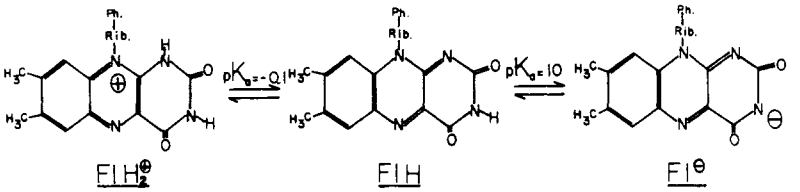

FIGURE 3: The three ionic species of FMN as a function of pH.

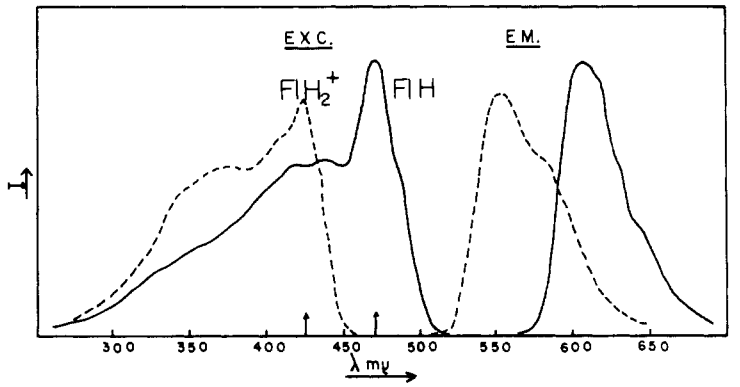

FIGURE 4: Uncorrected excitation and phosphorescence spectra of neutral and cationic FMN, 5×10^{-4} M, in a rigid solvent of water–propylene glycol–HCl, at 77°K. The relative intensity I of the phosphorescence of the cationic species is reduced by a factor of 5.

TABLE III: Analysis of the Photosensitization Reactions.

Compound	Light Intensity		n_T (t = 0)	a (free radical)	b (triplet)
	Absolute	Relative			
Riboflavin in ethanol	Medium	1	1		
		0.5	0.65	1.6 ± 0.2	2.5 ± 0.3
		0.33	0.54		
	High (Figure 6)	1	1	1.2 ± 0.2	1.55 ± 0.3
		0.33	0.77		
		1	1		
Lumichrome in ethanol	Low	0.5	0.58	1.8 ± 0.2	2.8 ± 0.3
		0.33	0.40		
		1	1		
Lumazine in ethanol	Low	0.5	0.52	1.9 ± 0.2	2.8 ± 0.2
		0.33	0.35		

is probably negative (McLachlan, 1962). That highest ZFS value defines D. The other two ZFS energies Y (or Z) and X define E, which reflects the symmetry of the spin distribution in the molecular plane. The E value is small for pteridines as well as for flavins.

Analysis of Substituent Effects. To describe the electronic structure of the triplet state in terms of spin densities, data obtained from epr studies with single crystals have to be used. Based on substituent effects, it is only possible to draw a qualitative picture of the electronic distribution in the triplet state. The interpretation of these effects has to be done carefully, since even weakly perturbing substituents, such as alkyl groups, may not mainly affect the ring atoms where the substituent is attached.

Using methylnaphthalene a lowering of the ZFS energies was observed (de Groot and van der Waals, 1963). This reflects a higher delocalization through

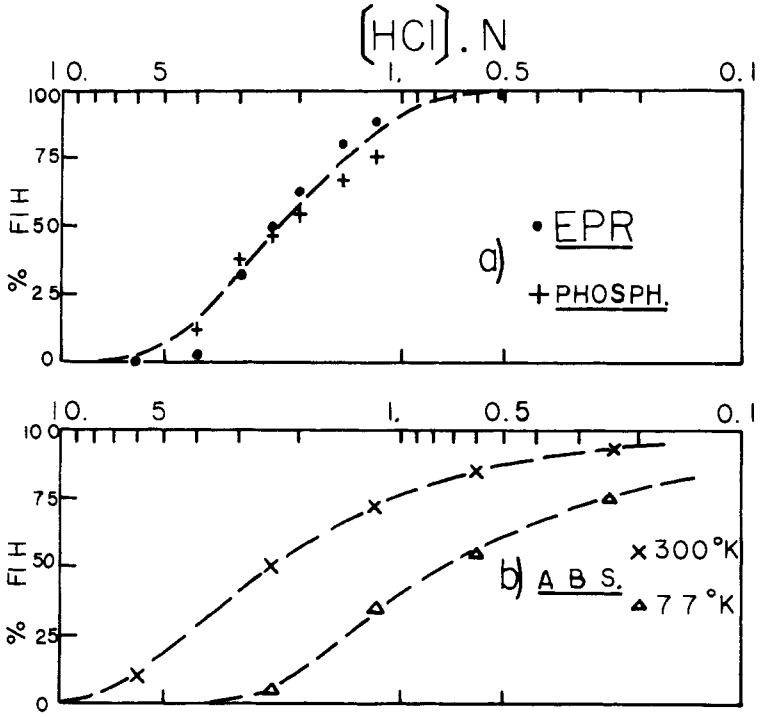

FIGURE 5: Protonation reaction of FMN in a solvent of water–propylene glycol–HCl. (a) In the triplet state the percentage of neutral FMN is measured from epr and phosphorescence spectra at 77°K. (b) In the ground state the percentage of neutral FMN is measured from absorption spectra at 300 and 77°K.

hyperconjugation. The electronic structure of the triplet state of phenothiazine is known in details from single crystal experiments (J. M. Lhoste and M. Ptak, to be published). In this molecule the D^* parameter becomes smaller when alkyl groups are substituted on atoms at which the spin density is high in the nonsubstituted compound. (a) The nature of the substituent at position 10 of the flavins does not affect the ZFS energies, even for FMN dissolved in an aqueous rigid solvent. The only exception is tetraacetylriboflavin. (b) Alkylation (3-benzyllumiflavin) or ionization (anionic form of FMN) of N-3 does not modify the ZFS energies, but the *O*-alkylation in position 2, corresponding to the flavoquinone iminol form, increases the D^* parameter. By substitution of a sulfur atom in position 2 (2-thiolumiflavin), the lifetime of the triplet state decreases because of spin–orbit coupling; moreover a red shift of the absorption spectrum was observed. The D^* values remain practically unaltered. (c) The alloxazine–isoalloxazine tautomerism has a noticeable effect on the ZFS energies causing lower values for the isoalloxazine derivatives. The same effect is also observed with pteridines comparing 1,3-dimethyllumazine with 8-methyllumazine. A noticeable spin density in the N-10 region of the flavin ring is also indicated by the charge effect, observed for the cationic form of FMN. Comparing the ZFS energies of lumichrome and 1,3-dimethyllumichrome (the alkylation in N-3 being ineffective), the spin density at N-1 is lower at N-10. (d) Role of the benzenoid ring of flavins. Substitutions on the pteridinoid part of the flavins act similar to those of the pteridine ring. Different substitutions in positions 6 or 7 of the pteridine ring (6,7-dimethyl- and 6-phenyl-7-amino derivatives of the 2,4-diaminopteridine) do not influence the D^* value. The lack of an epr signal for xanthopterines is probably due to the low solubility of these compounds. The D^* values of the flavins are about 0.02 cm^{-1} smaller than these of the pteridines. This indicates that the triplet electrons are delocalized over the benzenoid ring. The amount of delocalization is not large, which is shown by comparing lumazine and lumichrome. A noticeable spin density in the C-7–8 region of the flavin ring becomes evident by the effect of different substituents at these two atoms (lumiflavin has a smaller D^* value than 8-morpholino-8-norlumiflavin; there are different ZFS energies for 1,3-dimethylalloxazine and 1,3-dimethyllumichrome). The methyl or chlorine substitution in C-7 and/or C-8 affects the photochemical behavior of flavins (Radda, 1966). This fact agrees with our experiments and suggests a different role for these two positions.

The qualitative picture of the spin distribution in the lowest flavoquinone triplet state is rather similar to that of the semiquinone free radical (Ehrenberg *et al.*, 1965). The electrons, responsible for these states, occupy preferentially the quinoxaline moiety of the

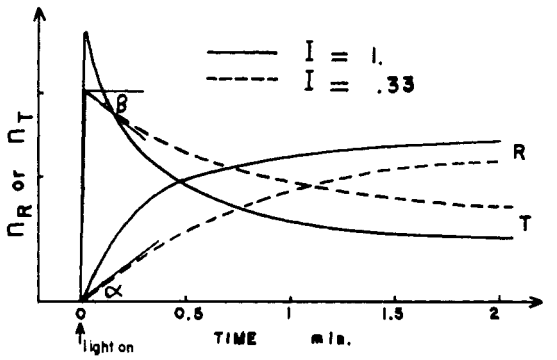

FIGURE 6: Photosensitization reaction of riboflavin, 10^{-3} M, in a rigid matrix of ethanol at 77°K. The free-radical population of the solvent, n_R, and the triplet population of the solute, n_T (arbitrary units) are plotted as a function of the time of illumination, with two different intensities, I, of incident light.

flavin ring. In acid solution the flavin radicals showed epr spectra of about the same complexity as in neutral solution. For the neutral as well as for the cation radicals, isotopic substitution in lumiflavin showed that there is no detectable hyperfine coupling to the pyrimidine nitrogens. The anionic radicals of a number of flavins all exhibit epr spectra with an even number of hyperfine lines, and the highest spin density is located at N-5. Any substitution at N-5 suppresses the epr triplet signal (see below).

The similarity of the spin distribution in the lowest triplet state and in the radical state arises from the close correspondence between their electronic structures. The two unpaired electrons of the triplet state, as well as the single electron in a radical, they both produce an exchange polarization of the spins of the closed shell (McLachlan, 1962). Apart from spin correlation factors for the triplet state, this statement holds for most alternant molecules due to the pairing of bonding and antibonding molecular orbitals. The normalized spin densities in the lowest triplet state of naphthalene are practically the same as those observed in the negative ion (Hutchison and Mangum, 1961; Carrington *et al.*, 1959). The epr measurements of Vincent and Maki showed that the fine structure of the triplet state of quinoxaline is practically identical with that of the naphthalene triplet state (Vincent and Maki, 1963). The arguments for this similarity must be considered with great care in the case of flavin derivatives because of the low symmetry and the number of heteroatoms involved. The emission and epr behavior of flavins forming chelates with diamagnetic ions is presently investigated.

The Reduced Form of Flavin. Leucoflavins showed no detectable epr triplet signal but phosphorescence emission. The intensity of phosphorescence was comparable to that of the oxidized flavins. The blue shift in the phosphorescence peak ran parallel to the shift of the long-wavelength absorption band, when going from the oxidized flavins to the leucoflavins. Shiga and Piette (1964, 1965) did not observe an epr triplet signal when illuminating chemically reduced FMN. It appears likely that the lowest triplet state has still π–π character but a higher spin–orbit coupling because of a nonplanar configuration.

McClure (1952) has shown that the long lifetimes of the phosphorescent triplet state of planar aromatic molecules are due to extremely weak spin–orbit coupling for pure π-electronic states. This observation confirms the spectroscopic evidence that flavins exhibit a bent structure in their totally reduced form (Hemmerich *et al.*, 1965b). The change to the "butterfly wing configuration" corresponds to a modification in the hybridization of the N-5 orbitals. In the reduced molecule these orbitals have more sp^3 character, and thus there results a lower conjugation of the two lateral wings. Such a nonplanar configuration may not only lower the lifetime of the triplet state, but it may also increase the zero-field splitting. The latter may become so large that the 3-cm microwave quanta are too small to induce a transition.

The Triplet State of the Ionic Forms of FMN. For flavins in the ground state the isoelectronic range is a very broad one (Figure 3). The triplet state of the anionic FMN could not be distinguished from that of the neutral form, neither by the emission spectrum nor by the ZFS energies. The only effect observed in the high pH region is a reduction in the mean half-life (Shiga and Piette, 1964, 1965). This may arise from changes in the glassy matrix. The stability of the flavins toward electronical changes induced by ionization or substitution on N-3 (in the triplet state as well as in the ground state) is important for the photochemical behavior of the flavoproteins. The early steps of the photoexcitation of the coenzyme do not seem to alter the linkage with the apoenzyme. It is assumed that the apoenzyme is connected *via* a hydrogen bond to N-3. In the flavin nucleus, N-3 is the single site whose properties are approximately oxidation–reduction invariant.

Shiga and Piette (1964, 1965) did not observe the cationic form of FMN because the pH range studied was still too high. Considering the lifetime of the triplet state they proposed for the protonation equilibrium a $pK = 4$. Furthermore, the neutral form exists in the pH region 2–7. With an increase in acidity they observed in this range a continuous shortening of the triplet lifetimes. Compared to the alkaline pH range, this effect may be due to higher nonradiative decay constants with increasing acidity.

The triplet state of the cationic form of FMN can be easily distinguished both by its emission and by its epr spectrum (Figure 4, Tables I and II). The blue shift of the phosphorescence emission is comparable to the shift of the highest absorption band. The H_{min} line is shifted by 20 gauss, the spin–spin coupling being higher for the cationic form. The $\Delta m = 1$ spectrum indicates a slightly lower symmetry of the triplet state, due to the influence of charges. In this connection it is necessary to emphasize that the rigid glasses of water–propylene glycol were good at high ionic strength.

In the discussion before, the pH and pK data are known from measurements at room temperature. There exist no data on the ionic mobility in rigid glasses. Therefore, the equilibrium value of the protonation was measured for both the triplet and the ground state as a function of the initial concentration of hydrochloric acid. Between 300 and 77°K the absorption spectra of the two flavin forms remain unchanged, but the 50% protonation requires a concentration of HCl which is 3.3 times less in the rigid glass (Figure 5b). In the triplet state at 77°K the 50% protonation requires a concentration of hydrochloric acid which is three times higher than that of the ground state (Figure 5a). This indicates a slightly lower pK value for the triplet state. This significant difference shows that in rigid glasses the time constant of the proton-exchange reaction is short compared with the lifetime of the triplet state.

A similar observation has been made by Maling *et al.* (1965) studying the ionization of tyrosine. A high ionic mobility and different pK values for the ground and the triplet states were observed by absorption and epr measurements with rigid solutions.

Thus, phosphorescence and/or epr studies with rigid solutions are convenient to compare the pK values of the ground state and the triplet state, respectively. Of course, it is necessary that the absorption spectra, the phosphorescence spectra and/or epr spectra are distinguishable. These methods are experimentally easier to perform than to measure the acidity constant of the triplet state in liquid solution, using triplet–triplet absorption by flash photolysis techniques (Jackson and Porter, 1961). Investigating seven aromatic molecules, these authors obtained comparable pK values for the ground and triplet states, respectively.

The cationic form of FMN does not fluoresce (Weber, 1950), but it was shown that the pK value of the lowest excited singlet state is much higher than that of the ground state. Within a series of methyl derivatives of lumazine there was also no fluorescence in the highly acid region. Therefore, Lippert and Prigge (1960) calculated the pK value from the red shift of the long-wavelength absorption band, when protonating the neutral molecule. They found a higher pK value for the lowest excited singlet state compared to the ground state. Jackson and Porter (1961) investigated five aromatic molecules and found that the pK value of the first excited singlet state is rather different from that of the ground and the triplet state, respectively.

Role of the Triplet State in Solvent Sensitization Reactions. In this section we discuss briefly that pteridines and flavins sensitize the decomposition of solvent molecules. This mechanism involves a biphotonic absorption process, where the lowest excited triplet state of the solute molecule acts as an intermediate state.

Our data fit well with the kinetic model proposed by Siegel and Eisenthal (1965). They studied the photosensitized decomposition of ether and ethanol *via* the triplet state of naphthalene. Here, the absorption of a second photon, populating a higher excited triplet state of naphthalene, is followed by an energy-transfer process to the solvent molecule which is then photochemically altered. To describe the latter process several mechanisms have been proposed. A direct energy transfer to the solvent molecule may occur from the higher triplet state of the solute molecule, or photoionization of the solute molecule in the higher excited triplet state, or absorption of a second photon by a complex between a solute molecule (in the lowest triplet state) and a solvent molecule. Different mechanisms are possible for different solutes.

In the case of flavins and pteridines, Table III shows the kinetic analysis for high and weak light intensities. In the calculation the only assumption is that the solute molecule, which was involved in the production of a solvent free radical, cannot be promoted once more to a triplet state. For naphthalene, a quenching of the triplet state by surrounding free radicals was proposed (Siegel and Eisenthal, 1965). With riboflavins and lumichrome, the presence of a second signal arising from solute molecules suggests that a photochemical degradation of the solute molecules occurs. This degradation follows the same rate as the solvent sensitization reaction. Whether the decomposition of the solute is a consequence of photosensitization or of other competing processes cannot be concluded from these preliminary experiments.

In any case, the observation of biphotonic processes is of importance to understand photobiological mechanisms. Such processes have been investigated with aromatic amino acids and with nucleic acid derivatives (Douzou and Ptak, 1964; Hélène *et al.*, 1966).

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