A STUDY OF SOLVENT EFFECTS ON THE PHOSPHORESCENCE PROPERTIES OF FLAVINS

G. R. BRUNK, K. A. MARTIN, *and* A. M. NISHIMURA

From the Department of Chemistry, Wichita State University, Wichita, Kansas 67208

ABSTRACT A combination of zero field triplet state techniques are used to study the excited electronic states of a series of flavin and flavin related molecules both in single crystals and glass matrices. Particular attention is given to the effects of solvent interaction on the triplet state properties of the flavin molecules. The total phosphorescence decay rate constants at 1.4°K are reported for the flavin molecules in polar and nonpolar solvents. The rate constants are then correlated to the degree of solvent interaction. Results indicate possible complex formation between the isoalloxazine and adenine groups in FAD. Finally, the results and possible interpretation on the study of a flavoenzyme, L-amino acid oxidase are presented.

INTRODUCTION

The effect of light on flavins and the resulting photochemical reactions important in light-induced biological responses in living organisms has been of interest to researchers (Beinert, 1960; Yagi, 1968; Kamin, 1971; Yagi, 1975). Previous studies have included the phototropic response of plants arising from the photochemistry of riboflavin on plant hormones and the participation of riboflavin as a photosensitizer in reactions involving the oxidation of indoleacetic acid (Galston, 1950). These studies were motivated by the observed photo-oxidation in vitro of many biologically significant molecules, such as amino acids and estrogens (Nye et al., 1953). The flavins have also been shown to participate in enzymatic reactions involving the absorption and emission of light. These reactions include photosynthetic phosphorylation, the photoreduction of nitrate, and bioluminescence (Totter and Cormier, 1955; Stoy, 1956; Wessels, 1957).

Since these reactions involve the electronic states of flavins as intermediate species, a more complete understanding of the nature of the excited states and the mechanisms for the relaxation processes of flavins is needed. Flavins exhibit unique phosphorescence in the visible range of the optical spectrum. This phosphorescent emission from the flavin coenzyme might be used as a monitor for electronic changes during reactions at the active site of a flavoenzyme. The typically long lifetimes of the phosphorescent metastable electronic state yield a large steady-state population detectable by optical techniques. The sensitivity of optical detection techniques can be used to probe a molecule unique to the active site since the molecular environment at the active site of an enzyme is strongly influenced by the electronic potential energy of the molecule. Phosphorescence microwave double resonance (PMDR) provides a method

by which these molecular interactions at the active site may be probed and measured (Schmidt et al., 1969; Tinti et al., 1969; Tinti and El-Sayed, 1971). In addition to yielding kinetic parameters for the electronic relaxation process, PMDR technique measures dipolar interactions of the two unpaired electrons in the triplet electronic state arising from anisotropic coupling and spatial delocalization along the principal axes of the molecule.

Moore and Kwiram (1974) recently reported a study of the triplet state of several flavin molecules in ethylene glycol-water solution. In this paper we report an extensive study of a series of flavin-related molecules in which the effects of intermolecular solvent interactions on the triplet state are considered.

EXPERIMENTAL SECTION

Materials

Pteridine was synthesized using the procedure of Albert and Yamamoto (1968). (Structures of pteridine and other flavin molecules are shown in Fig. 1.) The crude yield was subjected to multiple sublimations at 110°C and 0.01 torr to extract yellow crystals with melting range consistent with reported values for pteridine (Albert et al., 1951). NMR spectra of the product were compared with those previously reported for pteridine and found to be correspondingly similar (Matsuura and Goto, 1963). Pyrido[2, 3-*b*]pyrazine (PP) was obtained from Aldrich Chemical Co., Milwaukee, Wisc., and purified by multiple vacuum sublimation. 4-Hydroxypteridine (HPT) was purchased from Aldrich Chemical Co., recrystallized from water and dried. Lumazine in the hydrated form was recrystallized from water and dried at 100°C. IR spectra of the dried lumazine revealed the absence of an OH vibration. Alloxazine was purified by crystallization from ethanol. The following compounds were purified by recrystallization from water or by paper chromatography. Reduced forms of lumichrome and riboflavin were prepared following the procedure of Beinert (1956) in which a reducing solution was prepared by mixing 0.25 g of sodium dithionite in 25 ml of boiled distilled water and 1 drop of 6 M NaOH in a saturated solution of the molecule to be reduced. Reduction was confirmed by absorption spectra taken on a Beckman DG spectrophotometer (Beckman Instruments, Fullerton, Calif.). Separation of the reduced species was achieved by paper chromatography in the reducing solution. Flavin mononucleotide (FMN), flavin adenine dinucleotide (FAD), and L-amino acid oxidase and adenosine were purchased from Sigma Chemical Co., St. Louis, Mo. Adenosine diphosphate (ADP) and adenine were purchased from Calbiochem, San Diego, Calif.

The solvents, methanol and propylene glycol, were purified by multiple vacuum distillation while ethanol (red label from U.S. Industrial Chemical Co., New York) and *p*-xylene (research grade Phillips Petroleum Co., Bartlesville, Okla.) were used without further purification. There was no emission in the visible region exhibited by the solvents. *p*-Dichlorobenzene (DCB), durene, and naphthalene were extensively zone refined under an inert atmosphere for an equivalent of over 200 passes.

Methods

Single crystals of DCB, durene, and naphthalene containing appropriate guest molecules were grown in a standard Bridgeman furnace. Pieces from the single crystals were then cut, mounted inside a slow wave helix or a quartz ampule, and immersed in liquid helium. The guest concentration for all samples was approximately 0.5% mol/mol.

For experiments requiring a continuous wave (cw) excitation source, a 100 W xenon-mercury high pressure arc lamp was used with filtering through a solution containing $NiSO_4$ and

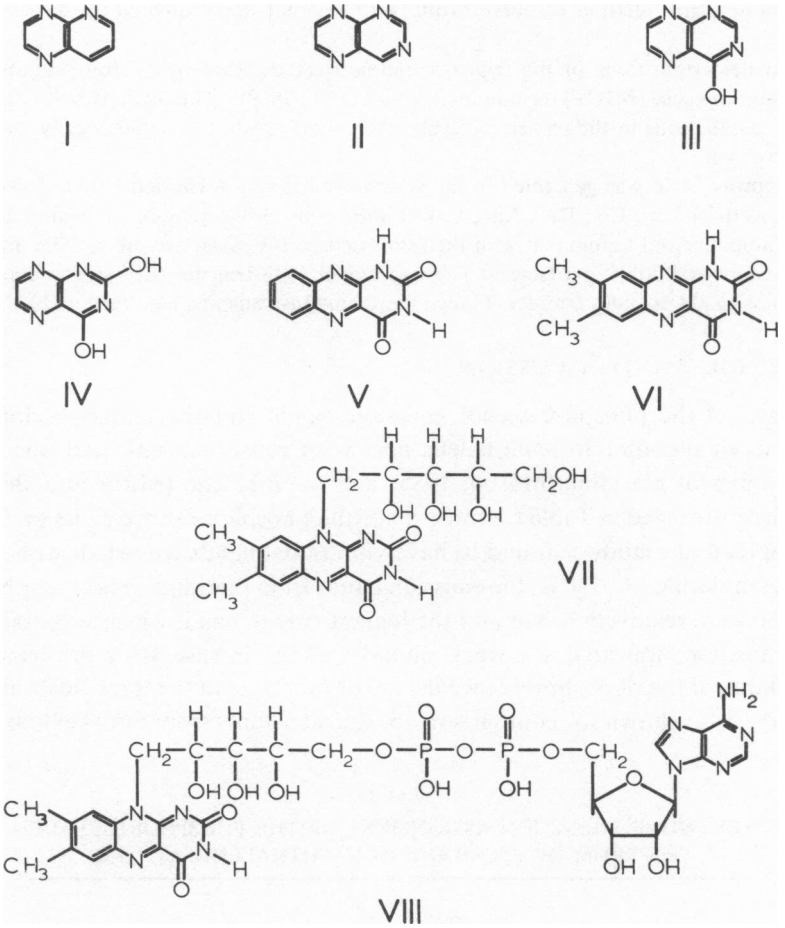

FIGURE 1 Some flavin molecular structures. (I) Pyrido[2-3-*b*]pyrazine, (II) pteridine, (III) 4-hydroxypteridine, (IV) lumazine, (V) alloxazine, (VI) lumichrome, (VII) riboflavin, (VIII) flavin adenine dinucleotide.

$CoSO_4$. Additional filtering was accomplished using a Corning 7-54 filter (Corning Glass Works, Corning, N.Y.). The lamp was placed in a 45° configuration to a 3/4 m Czerny-Turner spectrometer with 100-cm^{-1} slits and a thermoelectrically cooled EMI 6256B photomultiplier tube (Electronics for Medicine, Inc., White Plains, N.Y.) was used. In some cases, a $\frac{1}{4}$ m monochromator was used equipped with a 1P28 photomultiplier. Temperatures as low as 1.4 K were routinely obtained by pumping the vapor above the liquid helium. These temperatures were determined by vapor pressure measurements of the liquid helium.

For phosphorescence decay measurements, vibronic bands of the phosphorescence were isolated (usually the 0,0 band) with the spectrometer and the excitation source was extinguished in about 2 ms by an electronically controlled mechanical shutter, or alternatively, the decay was measured following a xenon flash lamp excitation of 10 μs duration. The signals were repetitively accumulated in a Tracor-Northern NS-570 signal averager (Tracor-Northern, Middleton, Wisc.). The resulting data was computer fitted to an equation describing the time-dependent decay of the phosphorescence intensity (*vide infra*). The program used the Newton-

Raphson nonintegral method of curve fitting with internal approximated gradients (Powell, 1965).

The zero field transitions of the triplet sublevels were detected by microwave-induced delayed phosphorescence (MIDP) technique (Schmidt et al., 1969). The method relies on the differences in populations in the triplet sublevels when the transition is adiabatically swept with microwave power.

The microwave field was generated in the slow wave helix by a Hewlett-Packard sweep generator (Hewlett-Packard Co., Palo Alto, Calif.) with appropriate plug-in unit, amplified by a 1 W TWT amplifier and terminated with the semi-rigid 50 Ω coaxial assembly. The microwave sweep was calibrated utilizing a Hewlett-Packard model 540B transfer oscillator in conjunction with a Philips 6630 frequency counter (Philips Electronic Instruments, Mt. Vernon, N.Y.).

RESULTS AND DISCUSSION

The energies of the phosphorescence emission bands and the tentative vibrational assignments of pteridine in naphthalene host were consistent with previous IR and Raman studies of pteridine (Mason, 1955) and naphthalene (Mitra and Bernstein, 1959). (These are listed in Table I.) Integrity of the phosphorescence emission from all other samples under study as found to have vibrational bands consistent with that for this parent molecule. At 1.4°K, the emission bands from pteridine in both naphthalene and durene were relatively broad and the highest energy band, which was assigned to the 0,0 transition, appeared as a weak shoulder of the intense 468.2 nm band. The rate constants of the phosphorescence decay for pteridine in the three hosts are given in Table II. Also shown for comparison are rate constants from our previous studies

TABLE I

TENTATIVE VIBRATION ASSIGNMENT OF THE PHOSPHORESCENCE SPECTRUM OF PTERIDINE IN NAPHTHALENE AT 1.5°K

$\bar{\nu}$	$\Delta\bar{\nu}$(in cm^{-1} from 0.0 band)	Assignment
cm^{-1}		
22,000		
21,360	640	Skeletal vibrations
21,050	950	Skeletal vibrations
19,050	2950	C - H stretching
18,870	3130	C - H stretching
18,520	3480	640 + 2950 = 3590
18,350	3650	640 + 3130 = 3770
16,000	6000	2 x 2950 = 5900
15,950	6050	2 x 3130 = 6260

TABLE II

TOTAL RATE CONSTANTS FOR THE SPIN COMPONENTS OF THE PHOSPHORESCENT TRIPLET STATE OF AZANAPHTHALENES (IN s^{-1})

Sample	0,0 Energy	Rate Constants		
		k_z	k_y	k_x
	cm^{-1}			
Quinoxaline in Durene[a]	21,250	11.1	0.59	0.40
1,3-Diazanaphthalene in Durene[b]		4.0	1.1	0.42
1,5-Diazanaphthalene in Durene[b]	23,180	4.4	0.52	0.31
1,8-Diazanaphthalene in Durene[b]	23,580	6.3	0.46	0.10
Pyrido[2,3-b]pyrazine in Ethanol	22,150	2700	190	--[c]
4-Hydroxypteridine in Ethanol	24,300	14	4.0	1.4
4-Hydroxypteridine in p-Dichlorobenzene	21,880	18	5.0	1.4
Pteridine in Propylene Glycol	22,000	12	2.0	0.51
Pteridine in Naphthalene	21,360	10	2.4	0.38
Pteridine in Durene	20,460	11	1.8	0.46
Pteridine in Ethanol	23,670	11	3.2	1.3

[a] From Schmidt et al. (1971).

[b] From Nishimura et al. (1971).

[c] Rate constant not observed.

on 1,3-, 1,5-, and 1,8-diazanaphthalenes (Nishimura et al., 1971) as well as total decay rates of quinoxalene (Schmidt et al., 1971).

The kinetic parameters for the phosphorescence decay process were measured by a combination of methods described by Tinti and El-Sayed (1971) at low (~1.4°K) and high (≥4.2°K) temperatures. The rate parameters were obtained by considering the total decay at very low temperatures and assuming that the spin-lattice relaxation processes can be neglected. The decomposition of the decay of phosphorescence upon shuttering the excitation light yields three first-order exponential rate parameters since the emission intensity decays according to:

$$I(t) = \sum_i k_i^r N_i(0) \exp(-k_i t),$$

where k_i^r is the radiative rate constant, $N_i(0)$ is the steady-state population, and k_i is the total rate constant. The subscript refers to the individual sublevels in the triplet state. The decay of the phosphorescence intensity following excitation with a xenon flash directly yields the rate constants of the fastest emitting sublevel.

Even at liquid nitrogen temperatures, the three spin sublevels of the triplet state are thermally coupled by the high density of occupied phonon states leading to the thermalization of the spin population. However, at very low temperatures, such as 4.2°K and lower, the occupied density of phonon states available for the thermal spin relaxation process is very low and the triplet sublevels effectively become isolated. In all cases studied, at 4.2°K, the spin-lattice relaxation process was found to be slow as compared with other relaxation processes. The required higher temperatures were obtained by allowing the helium level to fall below the sample where the temperature was estimated to be ~10°K. At this temperature the decay constant was found to be consistent with the individual rate constants extracted at 1.4°K.

Pteridine has only a reflection symmetry element and can be described by the C_s point group. The axes system was chosen such that the spin axes were coincident with the molecular axes with y and z spin axes corresponding to the in-plane short and long axes, respectively. As described in our earlier work on 1,5- and 1,8-diazanaphthalenes, the intersystem crossing process which is consistent with the group theoretical analysis was the $^1n, \pi < \approx\approx > {}^3\pi, \pi^*$ mechanism (Müller and Dörr, 1959; Nishimura and Vincent, 1972). Although the triplet spin τ_y and τ_z in pteridine belong to identical representations in C_s, the molecule topologically resembles quinoxaline for which the uppermost τ_z spin state is dominantly radiative. The observation is consistent with the selection rule derived from group theoretical arguments from spin-orbit interaction in nitrogen substituted aromatics which predict the spin state coincident with the in-plane long axis of the molecule to be radiative for phosphorescence originating from a π, π^* state. For quinoxaline, selection rules forbid phosphorescence from τ_y whereas radiation from τ_x requires mixing via spin-orbit interaction between two π, π^* states which is naturally expected to be weak. Since we observe that τ_y is more radiative than τ_x, τ_y must gain its radiative property via asymmetric vibration which couple $^3\pi, \pi^*$ with states of n, π^* and/or σ, π^* types. In pteridine, with the low symmetry caused by the nitrogen in the 3 position, τ_y is expected to steal radiative strength from τ_z. The enhancement of the radiative rate constant for τ_y relative to that for τ_z is not dramatic, presumably since the nonbonding electrons of the nitrogen in the 1, 5, and 8 positions are roughly coincident with the y spin axis.

The compound PP in ethanol gave a phosphorescence spectrum characteristic of azanaphthalenes and vibrational assignments were found to correspond to those for pteridine as shown in Table I. The stability of PP was monitored by NMR before and after experiments were carried out and found unchanged. Although PP grew substitutionally in durene and several other hosts, no emission was observed. The lifetime of PP in ethanol was found to be extremely short, $< 1 \times 10^{-4}$ s (cf. Table II).

TABLE III

COMPARISON OF PHOSPHORESCENCE DECAY RATE CONSTANTS (IN s^{-1})[a] AND INTERCEPTS[b] FOR LUMAZINE IN SEVERAL SOLVENTS AT 1.4°K USING CONTINUOUS WAVE AND FLASH EXCITATION SOURCES

Host	Excitation Source	k_z	k_y	k_x	I_z	I_y	I_x
Durene	cw	13	3.9	0.93	8.0	3.8	1.0
Durene	flash	-	1.8	-			
Propyleneglycol	cw	-	3.7	1.3	∿0	1	1.3
Propyleneglycol	flash	14	3.6	0.90	5.9	4.8	1.0
Ethanol	cw	-	2.5	1.1	∿0	2.1	1.0
p-Dichlorobenzene	cw	25	7.5	1.1	2.5	3.3	1.0

[a] ±5%.

[b] ±5%. Intercepts of phosphorescence decays extrapolated to time zero. Cf. text.
- dash line indicates rate constant not observed.

Shown in Table III are the total rate constants for the phosphorescence decay as determined from cw and flash excitation sources for lumazine in three hosts. As evident from the table, the nature of the lowest triplet state of lumazine is π, π^*, a fact well documented (Sun et al., 1972).

The zero field splittings (zfs) of several flavin molecules are reported in Table IV as determined by MIDP, a typical decay trace of which is shown in Fig. 2. The zfs shown in Table IV were consistent with the $^3\pi, \pi^*$ state assignment for the pteridine derivatives.

TABLE IV

OBSERVED ZERO FIELD SPLITTING ENERGIES FOR SEVERAL PTERIDINE DERIVATIVES IN *p*-DICHLOROBENZENE HOST AT 1.4°K

Guest	Transitions[a]		
	MHz		
4-Hydroxypteridine	3000	1700	1300
Lumazine	5300	3700	--[b]
Lumichrome	5200	3600	--[b]

[a] ±100 MHz.

[b] Transition not observed.

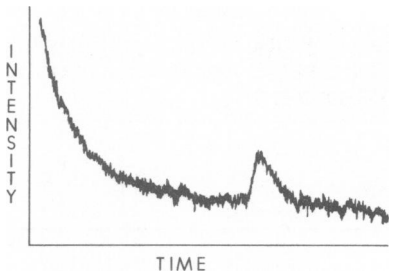

FIGURE 2 Typical intensity trace of a microwave-induced delayed phosphorescence decay at 1.4°K. This particular trace shows the total phosphorescence decay of lumazine in *p*-dichlorobenzene. The microwave frequency was swept 5.1–5.5 GHz and the total time width was 400 ms.

Shown in Table V are the rate constants for oxidized and reduced riboflavin and lumichrome. Increase in the phosphorescence decay process is presumably caused by interactions of the chromophore with the ribitol moiety. The small increase in rate constants for the reduced forms can be attributed to partial delocalization through nonbonding orbitals of the heteroatoms resulting in enhanced spin-orbit effect (Siegel

TABLE V

PHOSPHORESCENCE DECAY RATE CONSTANTS IN s^{-1} (±5%) FOR FLAVINS IN ETHANOL AT 1.4°K

Guest	k_z	k_y	k_x
Alloxazine	9.0	2.2	0.5
Lumichrome			
oxidized	25	3.7	1.2
oxidized[a]	27	5.7	2.4
reduced	35	3.6	0.90
Riboflavin			
oxidized	13	2.4	0.80
reduced[b]	24	2.6	0.90
Adenine	1.6	0.50	0.30
Adenosine	2.5	0.70	0.30
Adenosine diphosphate	8.0	0.70	0.20

[a] Solvent - 64% ethanol and 36% water.

[b] Solvent - methanol.

TABLE VI

PHOSPHORESCENCE DECAY RATE CONSTANTS IN s^{-1} (±5%) FOR FLAVIN ADENINE COMPLEXES AND FLAVIN COENZYMES

Guest	Host	Wavelength	k_z	k_y	k_x
		Å			
Lumichrome (oxidized)-Adenine	Ethanol	5550	--	4.5	2.3
Riboflavin (oxidized)-Adenosine	Ethanol	4000	--	9.8	0.41
		7000	--	50	15
Flavin adenine dinucleotide	Ethanol[a]	7000	--	83	34
Flavin mononucleotide	Ethanol[a]	6500	--	30	8.7

[a] Solvent - 95% ethanol and 5% water.

- Dash line indicates rate constant not observed.

and Judeikis, 1966). The phosphorescence rate constants for adenine, adenosine, and ADP are also shown in Table V. ADP shows larger rate constants presumably due to the intramolecular perturbation from the phosphate group, but in general the triplet state of the adenine moiety is characterized by long phosphorescence decay, indicating $^3\pi, \pi^*$ lowest triplet state.

The total emission spectrum of FAD in ethanol did not show a subspectrum characteristic of adenine, indicating possible energy transfer and subsequent trapping from the adenine to flavin moiety (*vide infra*). Evidence for such complex formation was further supported by the extremely low intensity observed for the fast k_z component in which the increased relaxation via radiationless process decreased the steady-state population in the τ_z sublevel (cf. Table VI).[1]

Since the isoalloxazine group in FAD is attached to the ribitol and phosphate groups to form a long floppy molecule, the possibility exists for such complex formation of the flavin and adenine moieties (Weber, 1945; Miles and Urry, 1968; Sarma et al., 1968). For example, crystal structure studies of intermolecular complex through formation of alternating adenine and isoalloxazine ring stack has been determined by Voet and Rich (1971).

[1] It must be emphasized that the extension of the relative energy ordering and the assignment of the triplet spin axes for quinoxaline to the flavin molecules is not implied. It is difficult at best to correlate such assignments and energy orderings even between the molecules which are reported here, due to the various tautomeric, reduced and oxidized forms which severely change the symmetry and electronic structure of these molecules.

To further test the possibility of complex formation, riboflavin-adenosine, and lumichrome-adenine mixture were prepared in 1:1 (mol:mol) concentration in ethanol, the results of which are shown in Table VI. In the lumichrome-adenine solution, the adenine group yielded an intense characteristic phosphorescence subspectrum with lifetime $< 1 \times 10^{-4}$ s. For riboflavin-adenosine complex, the presence of the adenosine phosphorescence spectrum and lifetimes consistent with those of the monomer indicate possible steric hindrance to hydrogen bonding. However, the subspectra corresponding to the isoalloxazine moiety in both solutions decayed with a lifetime consistent with that of riboflavin and lumichrome.

The increase in rate constants for FMN shown in Table VI when compared with decay constants for riboflavin reflect intramolecular coupling effects, probably due to the strong hydrogen bonding with the phosphate group. Such intramolecular hydrogen bonding with ribitol and isoalloxazine groups were not as apparent in riboflavin (cf. Table V).

According to classical theories on energy transfer by multipole and exchange interactions such as those proposed by Foster (1960) and others (Robinson and Frosch, 1963), transfer is manifest by the quenching of the donor emission as were observed in several systems under study. Although our experiments do not provide evidence in support of the exact mechanism for the energy transfer process, strong hydrogen bonding could provide coupling energy between the donor and acceptor molecules. Since such interactions are strong, transfer of energy can readily occur via singlet → singlet in which the phosphorescence spectrum of the donor is not observed, as well as via triplet → triplet and triplet → singlet mechanisms.

It is curious that the acceptor phosphorescence decay from complexes under study is invariably shortened in at least one of the sublevel rate constants. An early work by Terenin and Ermolaev found the lifetimes of the acceptor molecules were not affected for analogous donor-acceptor complexes (Terenin and Ermolaev, 1956). Since crystallographic studies indicate tight pairing of adenine and alloxazine complexes (Voet and Rich, 1971), such hydrogen bonded structures will have large interaction energies. Following the transfer of energy to the acceptor molecule and consequent to the strong vibronic coupling via hydrogen bonding between donor and acceptor, the excited acceptor molecule has the possibility for relaxation via radiationless vibrational modes of the donor molecule. The net result is a shorter phosphorescence lifetime measured for the acceptor molecule since the observed phosphorescence rate constants are a sum of the rate constants for radiative and nonradiative processes. Although crystallographic data provide far more precise information on bond distances, the method used here could provide useful interaction distances of hydrogen bonded complexes in biological analogs.[2]

The enzyme L-amino acid oxidase used in this study was prepared in a buffered solu-

[2]Recently, by means of PMDR techniques, energy transfer in an enzyme-dye complex was observed to follow the Foster's mechanism and attempts were made to describe the spatial relationship of the chromophores (cf. Maki and Co, 1976).

tion of triethanolamine in water:ethanol, 1:1 mixture adjusted to pH 7.3 with NaOH (Wellner and Meister, 1961; DeKok and Rawitch, 1969). The buffered solution with catalase, as well as the buffered solution with catalase and L-leucine substrate did not exhibit emission in the visible spectrum. The enzyme alone showed a subspectrum characteristic of riboflavin, the lifetime of which was less than 0.1 ms and is presumably due to increased radiationless decay arising from molecular interactions with amino acid residues at the active site of the enzyme. Such quenching of emission has been observed for other flavoenzymes (Kamin, 1971). For the enzyme-substrate complex, the optical emission spectrum of a solution of the enzyme and substrate showed the absence of the subspectrum associated with the flavin, possibly due to strong quenching by substrate complex formation.

In summary, these results indicate that the total phosphorescence rate constants can be used as a qualitative measure of external solvent perturbation. Although some of the data presented here are complementary to results of studies by other methods, the zero field triplet state technique can be extended to detect solvent interactions of biological molecules in their respective environment. However, before attempts can be made to study such large molecular systems, information is needed to quantify the sensitivity to environmental interactions upon the molecule under study. Further studies are currently in progress using PMDR techniques in such large biological molecules as L-amino acid oxidase and other flavoenzyme systems.

Acknowledgment is made to the donors of the Petroleum Research Fund, administered by the American Chemical Society, for partial support of this work. We also thank the National Institutes of Health for supporting this research.

Received for publication 24 June 1976 and in revised form 30 August 1976.

REFERENCES

ALBERT, A., D. J. BROWN, and G. CHEESEMAN. 1957. Pteridine studies. Part I. Pteridine and 2- and 4-amino- and 2- and 4-hydroxy-pteridines. *J. Chem. Soc.* 474.

ALBERT, A., and H. YAMAMOTO. 1968. Pteridine studies. Part XXXVI. The action of acid and alkali on pteridine. *J. Chem. Soc.* 2289.

BEINERT, H. 1956. Spectral characteristics of flavins at the semiquinoid oxidation level. *J. Am. Chem. Soc.* **78**:5323.

BEINERT, H. 1960. Electron transferring flavoprotein. *In* The Enzymes, Vol. 1. P. D. Boyer, H. Lardy, and K. Myrback, editors. Academic Press, Inc. New York. 467.

DEKOK, A., and A. B. RAWITCH. 1969. Studies on L-amino acid oxidase. II. Dissociation and characterization of its subunits. *Biochemistry.* **8**:1405.

FOSTER, T. 1960. Excitation transfer. *In* Comparative Effects of Radiation. M. Burton, J. S. Kirby-Smith, and J. L. Magee, editors. John Wiley & Sons, New York. 300.

GALSTON, A. W. 1950. Riboflavin, light, and the growth of plants. *Science (Wash. D.C.).* **111**:619.

KAMIN, H. 1971. *In* Flavins and Flavoproteins. University Park Press, Baltimore.

MAKI, A. H., and T. Co. 1976. Study of triplet-singlet energy transfer in an enzyme-dye complex using optical detection of magnetic resonance. *Biochemistry.* **15**:1229.

MASON, S. F. 1955. Pteridine studies. Part VI. The ultraviolet and infrared absorption spectra of the monosubstituted pteridines. *J. Chem. Soc.* 2336.

MATSURA, S., and T. GOTO. 1963. Pteridine studies. Part I. Nuclear magnetic resonance studies of pteridine and methylpteridines. *J. Chem. Soc.* 1773.

MILES, D. W., and D. W. URRY. 1968. Reciprocal relations and proximity of bases in flavin-adenine dinucleotide. *Biochemistry.* **7**:2791.

MITRA, S. S., and H. J. BERNSTEIN. 1959. Vibrational spectra of naphthalene-d_0, -α-d_4, and -d_8 molecules. *Can. J. Chem.* **37**:553.

MOORE, T. A., and A. L. KWIRAM. 1974. An investigation of the triplet state of flavins and flavoproteins by optical detection of magnetic resonance. *Biochemistry.* **13**:5403.

MÜLLER, R., and F. DÖRR. 1959. Absorptions und Phosphorezenzspektren der Mono- und der Diazanaphthaline. *Z. Electrochem.* **63**:1150.

NISHIMURA, A. M., D. S. TINTI, and J. S. VINCENT. 1971. Phosphorescence microwave double resonance studies of 1, 3-, 1, 5-, and 1, 8-diazanaphthalene. *Chem. Phys. Lett.* **12**:360.

NISHIMURA, A. M., and J. S. VINCENT. 1972. Optical detection of electron spin resonance in the triplet state of 1,5-naphthyridine. *Mol. Cryst. Liq. Cryst.* **17**:30.

NYE, J. F., H. B. FRIEDGOOD, J. B. GARST, and D. M. MARON. 1953. Photooxidation of crystalline estrogens in the presence of flavins. *J. Am. Chem. Soc.* **75**:907.

ROBINSON, G. W., and R. P. FROSCH. 1963. Electronic excitation transfer and relaxation. *J. Chem. Phys.* **38**:1187.

POWELL, M. J. D. 1965. A method for minimizing a sum of squares of nonlinear functions without calculating derivatives. *Comput. J.* **7**:303.

SARMA, R. H., S. DANNIES, and N. KAPLAN. 1968. Investigations of inter- and intramolecular interactions in flavin-adenine dinucleotide by proton magnetic resonance. *Biochemistry.* **1**:4359.

SCHMIDT, J., W. S. VEEMAN, and J. H. VAN DER WAALS. 1969. Microwave induced delayed phosphorescence. *Chem. Phys. Lett.* **4**:341.

SCHMIDT, J., D. A. ANTHENUNIS, and J. H. VAN DER WAALS. 1971. The dynamics of populating and depopulating the phosphorescent triplet state as studied by microwave induced delayed phosphorescence. *Mol. Phys.* **22**:1.

SIEGEL, S., and H. S. JUDEIKIS. 1966. Triplet state zero-field splitting of some structurally related aromatic hydrocarbon and heterocyclic molecules. *J. Phys. Chem.* **70**:2201; A magnetophotoselection study of the polarizations of the absorption bands of some structurally related hydrocarbons and heterocyclic molecules. *J. Phys. Chem.* **70**:2205.

STOY, V. 1956. Riboflavin-catalyzed enzymic photoreduction of nitrate. *Biochim. Biophys. Acta.* **21**:395.

SUN, M., T. A. MOORE, and P. S. SONG. 1972. Molecular luminescence studies of flavins. I. The excited states of flavins. *J. Am. Chem. Soc.* **94**:1730.

TERENIN, A., and V. ERMOLAEV. 1956. Sensitized phosphorescence in organic solutions at low temperature. *Trans. Faraday Soc.* **53**:1042.

TINTI, D. S., M. A. EL-SAYED, A. H. MAKI, and C. B. HARRIS. 1969. Phosphorescence microwave double resonance (PMDR) spectroscopy. *Phys. Lett.* **3**:343; Optical detection of phosphorescent triplet state ENDOR in zero field. *Phys. Lett.* **4**:409.

TINTI, D. S., and M. A. EL-SAYED. 1971. New techniques in triplet state phosphorescence spectroscopy: application to the emission of 2,3-dichloroquinoxaline. *J. Chem. Phys.* **54**:2529.

TOTTER, J. R., and M. J. CORMIER. 1955. The relation of bacterial luciferase to alternative pathways of dihydroflavin mononucleotide oxidation. *J. Biol. Chem.* **216**:801.

VOET, D., and A. RICH. 1971. The crystal and molecular structure of an intermolecular complex between riboflavin and an adenosine derivative. *Proc. Natl. Acad. Sci. U.S.A.* **68**:1151.

WEBER, G. 1945. Fluorescence of riboflavin and flavin-adenine dinucleotide. *Biochem. J.* **40**:363.

WELLNER, D., and A. MEISTER. 1961. Studies on the mechanism of action of L-amino acid oxidase. *J. Biol. Chem.* **236**:2357.

WESSELS, J. S. C. 1957. Studies on photosynthetic phosphorylation. I. photosynthetic phosphorylation under anaerobic conditions. *Biochim. Biophys. Acta.* **25**:97.

YAGI, K., ed. 1968. *In* Flavins and Flavoproteins. University Park Press, Baltimore.

YAGI, K., ed. 1975. *In* Reactivity of Flavins. University Park Press, Baltimore.