

Excited States and Reactivity of 5-Deazaflavine. Comparative Studies with Flavine[†]

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ABSTRACT: Electronic structure and reactivity of riboflavin (RF) and 5-deazaflavine (DF) have been discussed on the basis of molecular orbital and spectroscopic results. Photochemical reactivity has also been compared in terms of the photooxidation of NADH by RF and DF. It has been shown that the reactivity of position 5 is approximately independent of the identity of atom (N or C). This seems to be true also for the photoreactivity of flavines with respect to the oxidation of

NADH, in spite of the fact that the triplet state of DF has less n, π^* character than that of RF. Thus, the unique reactivity of N(5) can be characterized as being "orbital-controlled" in a nonconcerted reaction. The role of N(5) has also been discussed in terms of thermodynamic parameters (ionization potential, electron affinity, and stability of semiquinone intermediates).

Chemical perturbations and modifications of catalytic groups and coenzymes have been valuable in elucidating the reaction mechanisms of a number of enzymatic processes. In the case of flavoenzymes, modification at N(5) has been of particular interest in recent years. For example, Edmondson *et al.* (1972) ascertained that N(5) is crucial in stabilizing flavine semiquinones by the bound proteins, on the basis of their studies with 5-deazaflavine analogs in which N(5) is replaced by C(5). Brüstlein and Bruice (1972) have been able to establish the *in vitro* reaction mechanism of the NADH oxidation by flavine in terms of a hydride transfer to N(5), again based on the study with a 5-deazaflavine analog. The aim of the present work is to evaluate the chemical reactivity of 5-deazaflavine in comparison with the naturally occurring flavine. It is hoped that the present reactivity study provides a new insight into the electronic mechanism of flavine-catalyzed reactions, including photochemical reactions which play an important part in photobiology.

Experimental Section

Materials. Riboflavin and related flavines are the same as those used in our earlier work (Sun *et al.*, 1972). 3,10-Dimethyl-5-deazaalloxazine (DF)¹ was a gift from Professor T. C. Bruice. β -NADH was obtained from Sigma Chemical Co. and was used without further purification. The solvents used (including water) were purified by distillation and/or alumina column chromatography to remove luminescent trace impurities. The phosphoric acid (85%) employed was analytical grade from Matheson Coleman and Bell.

Spectroscopic Methods. Absorption spectra were recorded on a Cary 118C spectrophotometer at 298°K. For low temperature measurements, a specifically designed dewar compartment was used. Luminescence (fluorescence and phosphorescence), the phosphorescence lifetime, and polarization

were measured as described previously (Sun *et al.*, 1972). Corrected spectra were recorded on a Perkin-Elmer spectrofluorometer (Model MPF-3).

Photolysis and Quantum Yields of the NADH Oxidation. Photolysis of NADH and flavine or the DF analog was carried out by irradiating an anaerobic solution in a 10-mm light path cuvette (flushed for 20 ~ 30 min with prepurified nitrogen) at 405 nm. The cuvette was serum-rubber-stoppered. A full recovery (100%) of the original absorbance after air oxidation of the photolyzed solution ensured the anaerobic condition. The monochromatic source was provided by a Bausch & Lomb xenon light source (150 W) with a high-intensity grating monochromator (Model 33-86-07). Identical slit widths were used for all samples which had an absorbance of 1 (90% light absorbed initially). The band pass used was fixed at 10 nm, and the intensity of the light output was monitored by the rate of photobleaching of riboflavin. The constancy of the light output from the high-intensity monochromator was also checked by ferrioxalate actinometry (Song and Moore, 1968). The quantum yield was evaluated from the initial rate in reference to the quantum yield (0.006) for the photobleaching of RF in water (Holmström and Oster, 1961).

Theoretical Section

Molecular Orbital Computations. Both Hückel MO and ω -SCF MO ($\omega = 0.5$) were used in obtaining MO energy levels (k_i) and LCAO expansion coefficients (c_i). Since results based on the latter method are essentially parallel to those from the former, they are not presented in this paper. Details of these and other reactivity data are available upon request. Coulomb (α) and resonance (β) integrals employed were the same as those recommended by Pullman and Pullman (1963). The SCF MO CI Pariser-Parr-Pople method (Pariser and Parr, 1953; Pople, 1953) was used for calculations of certain quantities such as spectroscopic parameters and frontier densities. Pople-Nesbet unrestricted DODS (different orbitals for different spins) method (Pople and Nesbet, 1954) was employed to calculate the spin density distribution for the triplet states of flavines. Reactivity indices calculated include frontier orbital density (F_r^{OD}), superdelocalizability for nucleophilic attack (S_r^{DN}), frontier electron density (F_r^{ED}), superdelocaliza-

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¹ Abbreviations used are: RF, riboflavin; DF, 3,10-dimethyl-5-deazaalloxazine; DFH⁺, protonated 5-deazaflavine; RFH⁺, protonated riboflavin; DFH₂, reduced 5-deazaflavine; RFH₂, reduced riboflavin; F, flavine; FH₂, reduced flavine.

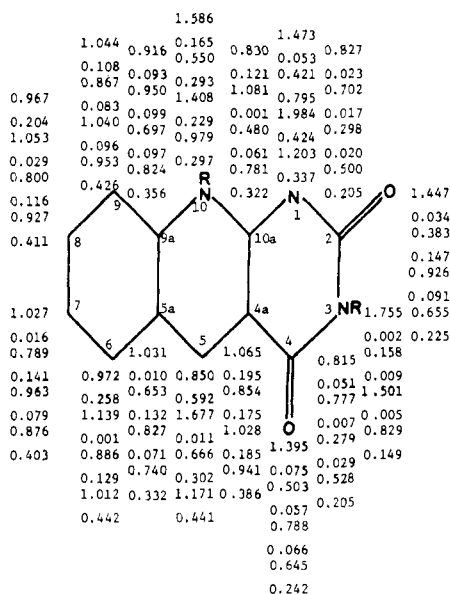


FIGURE 1: The reactivity map for DF calculated by using the Hückel MO method. Numbers from top to bottom at each position are: P_{rr} (π -electron density), F_r^{OD} , S_r^{DN} , F_r^{ED} , S_r^{DE} , F_r^{RD} , S_r^{RD} , and π_{rr} .

bility for electrophilic attack (S_r^{DE}), frontier radical density (F_r^{RD}), superdelocalizability for radical attack (S_r^{RD}), and atom-atom polarizability (π_{rr}). Frontier densities and superdelocalizabilities are reactivity indices derived from the frontier electron theory (Fukui *et al.*, 1954) and perturbation theory (Fukui *et al.*, 1957), respectively, while atom-atom polarizability is derived by Coulson and Longuet-Higgins (1947) on the basis of perturbation theory. It should be noted that frontier density, F_r , is a static measure of the stability of the transition state complex between the r th atom of the flavine and reagent (nucleophile, electrophile, and radical). The superdelocalizability, S_r , is a measure of the stabilization energy *via* π -hyperconjugative interactions between the substrate π orbital and pseudo π orbital of the r th reaction center-reagent complex in its transition state. These reactivity indices are defined as²

$$F_r^{OD} = 2(c_r^{\text{LEMO}})^2$$

$$S_r^{DN} = 2 \sum_j^{\text{unocc}} \frac{(c_r^j)^2}{(-k_j)}$$

$$F_r^{ED} = 2(c_r^{\text{HOMO}})^2$$

$$S_r^{DE} = 2 \sum_j^{\text{occ}} \frac{(c_r^j)^2}{k_j}$$

$$F_r^{RD} = (c_r^{\text{HOMO}})^2 + (c_r^{\text{LEMO}})^2$$

$$S_r^{DR} = \sum_j^{\text{occ}} \frac{(c_r^j)^2}{k_j} + \sum_j^{\text{unocc}} \frac{(c_r^j)^2}{(-k_j)}$$

$$\pi_{rr} = \left(\frac{\partial P_{rr}}{\partial \alpha_r} \right) = \left(\frac{1}{\beta} \right) \sum_j^{\text{occ}} \sum_l^{\text{unocc}} n_j (2 - n_l) \left[\frac{(c_r^j)^2 (c_r^l)^2}{k_j - k_l} \right]$$

$$\delta E_\pi = P_{rr} \delta \alpha_r + \frac{1}{2} \pi_{rr} (\delta \alpha_r)^2 + \dots$$

² Useful applications of frontier orbitals to the chemical reactivity have been described at an elementary level (Bradley and Gerrans, 1973).

TABLE I: The Preferentially Reactive Positions (in order of reactivity) toward Attacking Reagents.

Reagent	Compd	Reactivity Map	Rel Reactivity
Nucleophile	DF	Figure 1 ^a	C(5), C(6), C(8), C(10a), C(4a)
		<i>b</i>	C(5), C(4a), C(8), C(6), C(10a)
	DFH ⁺	Figure 2 ^a	C(5), C(10a), C(6), C(8)
	RF	Not shown ^a	N(5), C(4a), C(6), C(8), C(10a)
Electrophile	DF	Figure 1 ^a	N(5), C(4a), N(10), C(10a), C(8)~C(6)
		<i>b</i>	N(5), C(10a), C(6), C(8), C(4a)
	RF	Not shown ^a	N(1), N(10), N(3), C(9), C(7)~C(4a)
	<i>b</i>		N(1), C(7), C(9a), C(5a), C(8)
Radical	DF	Figure 1 ^a	N(1), C(5), N(10), C(6), C(4a)
		<i>b</i>	C(5), N(1), C(4a), N(10)~O(2), C(8)
	RF	Not shown ^b	N(5), C(4a), N(1), C(9a), C(8)

^a Relative reactivity in order of the sum ($F_r + S_r + \pi_{rr}$)

^b Relative size of the frontier density (F_r) calculated from the SCF MO CIP-P-P method.

where HOMO and LEMO stand for highest occupied and lowest empty molecular orbitals, respectively, P_{rr} is the π -electron density, and n_j and n_l are occupation numbers (number of π electrons) of the j th and l th molecular orbitals, respectively.

Results

Reactivity. Figures 1–3 show reactivity maps calculated for DF, DFH⁺, and RFH⁺.¹ Table I compares reactive positions between these two compounds. Of particular significance is the fact that position 5 is the most reactive toward incoming nucleophiles regardless of whether the position is occupied by nitrogen (as in the naturally occurring form) or carbon. It should also be noted that the N(5) nitrogen is surprisingly less basic than the N(1). This difference has been noted earlier by Pullman and Pullman (1963), and was confirmed in an all-valence-electron calculation (Song, 1968). A major difference between the reactivity patterns of 7,8-dimethylflavine and DF appears to be the relative reactivity of C(4a) toward nucleophiles. Otherwise, relative reactivity patterns of these two flavines show substantial similarity. Thus, N(1) is predicted to be most reactive toward electrophiles and radical reagents, followed by positions 10 and 5, respectively.

Figure 2 presents the reactivity map for N(1)-protonated deazaflavine. The most significant effect of the N(1) protonation is predicted to be the enhancement and suppression of the

TABLE II: The Preferentially Reactive Positions (in order of reactivity) toward Attacking Reagents.

Reagent	Compd	Reactivity Map	Rel Reactivity
Nucleophile	DFH ₂	Figure 4 ^a	C(9), C(6), C(7), C(9a), C(10a)
		<i>b</i>	C(10a), C(4), O(4), C(5a), C(9)
	RFH ₂	Not shown ^a	C(10a), C(9a), C(7), C(5a), C(8)~C(9)
		<i>b</i>	C(10a), C(4a), C(4), O(4), C(9a)
Electrophile	DFH ₂	Figure 4 ^a	C(4a), N(10), N(1), C(10a), C(5a)~C(9)
		<i>b</i>	C(4a), N(10), O(4), C(10a), C(7), C(9)
	RFH ₂	Not shown ^a	N(5), C(4a), N(10), N(3), C(10a)~N(1)
		<i>b</i>	C(10a), C(4a), O(4), C(9a), N(3)~N(1)
Radical	DFH ₂	Figure 4 ^a	C(4a), N(10), C(10a), C(9), C(7), N(1) C(9)~C(7)
		<i>b</i>	C(10a), C(4a), N(10), O(4), C(4), C(7), C(9)
	RFH ₂	Not shown ^a	N(5), C(4a), N(10), N(3), C(7), N(1)~C(9)
		<i>b</i>	C(10a), N(5), C(4a), N(10), C(9a)

^{a, b} See corresponding footnotes of Table I.

reactivity at C(5) and C(4a), respectively, toward nucleophiles, in comparison with the neutral DF. This trend was also found in the case of RF and its N(1)-protonated form.

Figure 4 shows the reactivity map for reduced DF (DFH₂). Table II lists relative reactivity in preferential order based on these reactivity maps, along with the relative reactivity ordering for reduced RF (RFH₂) (maps not shown). The C(10a) position in the latter is most reactive toward nucleophile reagents, while C(9) in the former is most reactive. Toward electrophiles, C(4a) is most reactive in DFH₂, but no definite choice can be made in the case of RFH₂, although N(5), C(4a), and C(10a) are predicted to be highly reactive in two different sets of reactivity data. Radical reagents are predicted to preferentially attack C(4a) in DFH₂, whereas N(5), C(4a), and C(10a) in RFH₂ are predicted to be the preferred sites of attack by radical reagents.

Spectroscopic Properties. Spectroscopic parameters are needed in order to describe the reactivity of flavine in their electronic excited states. Figure 5 shows the absorption and luminescence spectra of DF (3,10-dimethyl-5-deazaizoxazine) in ethanol at 77°K. The absorption spectrum of DF is similar to that of RF, except for the blue shift of the former with reference to the latter. The polarization characteristics of the luminescence (fluorescence and phosphorescence) are also similar to those of flavines such as RF (Sun *et al.*, 1972). The luminescence band is composed of fluorescence and phosphorescence, the latter being resolved with a phosphorescope. The vibrational resolution is lost in phosphoric acid, along

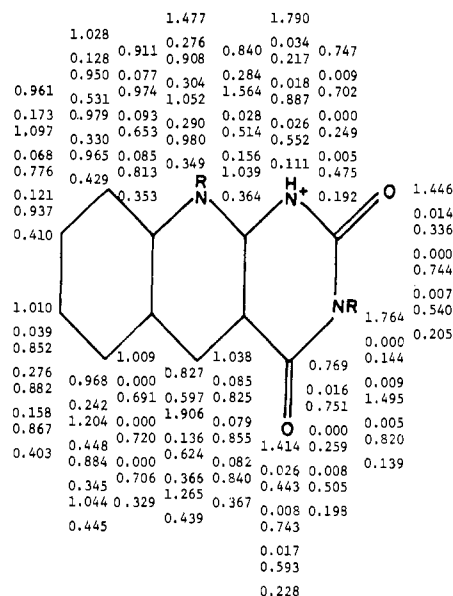


FIGURE 2: The reactivity map for N(1)-protonated DF calculated by using the Hückel MO method. See the legend of Figure 1 for identification of the reactivity indices shown.

with a substantial decrease in the degree of polarization of the phosphorescence. The fluorescence band also becomes featureless in phosphoric acid with λ_F^{\max} of 414 nm (not shown). Table III compares the spectroscopic properties of DF and RF. It can be seen that the lack of N(5) in the former is exemplified by a longer phosphorescence lifetime and a higher phosphorescence quantum yield relative to the latter.

Photochemical Reactivity and Oxidation of NADH. The photooxidation of NADH by flavines follows Stern-Volmer kinetics, as shown in Figure 6. However, there is a severe deviation at high concentrations of NADH. Values of the Stern-Volmer slopes for RF and DF are 1.168×10^{-3} and 1.16×10^{-4} , respectively. Lumichrome also photooxidizes NADH with similar kinetic response, yielding a slope of 8.72×10^{-4} (not shown). It appears that DF is most efficient in the photooxidation of NADH, compared to RF and lumi-

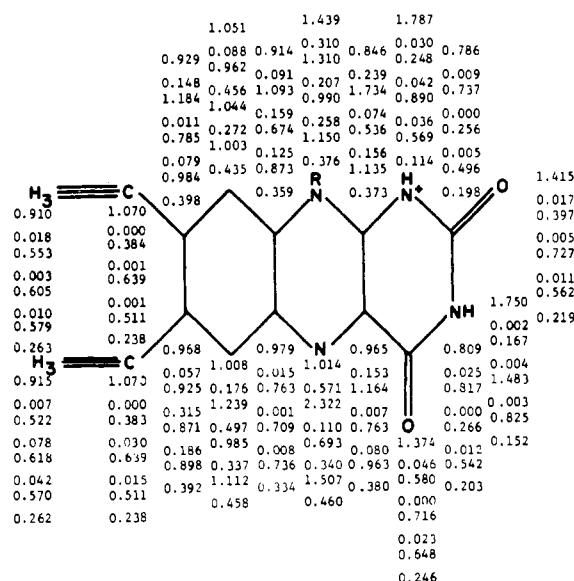


FIGURE 3: The reactivity map for N(1)-protonated RF calculated by using the Hückel MO method. See the legend of Figure 1 for identification of the reactivity indices shown.

TABLE III: Spectroscopic Characteristics of 3,10-Dimethyl-5-deaza-isoalloxazine and Riboflavine.^a

Flavine	λ_{abs} (nm)	λ_{F} (nm)	$\Phi_{\text{F}}, \text{H}_2\text{O}, 298^\circ\text{K}$	Φ_{F}	λ_{p} (nm)	Φ_{p}	$\Phi_{\text{F}}/\Phi_{\text{p}}$	τ_{p} (sec)
3,10-Dimethyl-5-deaza-isoalloxazine	422	426 (0-0)	0.37 ± 0.04	0.37 ± 0.03	493	0.38 ± 0.03	0.94	1.46
	398 (max)	433			522			(1.56) ^b
	377	458			570 (sh)			
	360 (sh)							
	323							
	313							
	266							
Riboflavine	444 (max)	~ 480 (0-0)	0.32 ± 0.02	0.60 ± 0.06	$\sim 610^c$	0.004 ± 0.001	150	0.17
		490 (max)						
		525						

^a Data obtained in ethanol at 77°K, unless indicated otherwise. ^b In phosphoric acid. ^c Uncorrected value. The corrected $\lambda_{\text{p}}^{\text{max}}$ ranges from 600 to ~ 610 nm, depending on correction procedures.

chrome. Contrary to the reported independence of the quantum yield on the NADH concentration (Radda and Calvin, 1964), Figure 6 clearly shows not only the Stern-Volmer dependence but also deviation at higher concentrations of NADH, as noted above. The quantum yield for the photooxidation of NADH (4×10^{-5} M) by RF (9×10^{-5} M) was reduced sevenfold (from 0.021 to 0.003) in the presence of KI (3×10^{-4} M). At this concentration of KI, the excited singlet state population is practically unaffected in terms of the measured fluorescence intensity. This result suggests that the triplet state of RF, which is quenched by the heavy atom salt, is the reactive state for the photooxidation of NADH.

Discussion

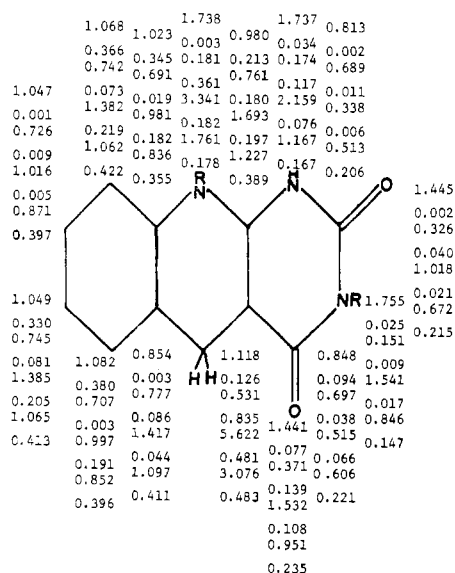
The lone pair of electrons of pyridine, as a Lewis base, add to a Lewis acid such as trimethylboron and sulfur trioxide, forming donor-acceptor complexes. Contrary to the general behavior of the pyridinyl nitrogen, N(5) in flavines undergoes completely opposite type of reactions in that nucleophiles

such as sulfite ion (Massey *et al.*, 1969) and phosphine (Müller, 1972) add to N(5), which is acting as if it is a Lewis acid instead of the more conventional Lewis base. This unique feature of the reactivity in flavines has little precedent in other heterocyclic systems.³

It is now possible to understand the unique features of the reactivity at N(5), as well as C(4a) toward nucleophiles, in terms of reactivity maps shown in Figures 1-3 and summarized in Table I. Namely, N(5) carries the highest frontier orbital density and superdelocalizability for the attack by a nucleophile. These quantities are a measure of the availability of the lowest empty molecular orbital at N(5) for electrons of an incoming nucleophile and stabilization of the transition state of the N(5)-nucleophile complex. Furthermore, the N(5) reactivity in flavines is not only maintained at the same position in the DF irrespective of the identity of the atom (N or C), but is somewhat enhanced in the latter. In view of the predicted reactivity pattern and recent experimental finding of the NADH oxidation by DF, the use of DF in D₂O as a flavine model may prove to be beneficial in elucidating the electronic mechanisms of amino acid oxidases, glucose oxidase, and succinic dehydrogenase, etc. This is particularly promising in determining the differential reactivity of N(5) *vs.* C(4a) toward nucleophilic substrates and intermediates such as carbanions in the amino acid and glucose oxidase reactions (Porter *et al.*, 1973).

Under acidic conditions, both N(1) and N(5) may be protonated. For such a protonated species, the reactivity at C(4a) toward nucleophiles increases. For example, using method (b) in Table I, frontier orbital densities for the protonated RF are 0.496 for N(5), 0.409 for C(4a), 0.159 for C(10a), and 0.088 for N(1). This trend is consistent with the acid-catalyzed nucleophilic attack at C(10a) (Bruice *et al.*, 1971).

DFH₂ shows C(4a) to be most reactive toward electrophile and radical (Table II). Thus, the oxygenation of the DFH₂ may likely proceed preferentially at C(4a). Such a reaction can be compared with the oxygenation of reduced flavines in bacterial bioluminescence (Eberhard and Hastings, 1973), mono-oxygenase (Hamilton, 1971), and oxygenation of reduced flavines (Massey *et al.*, 1969; Walker *et al.*, 1970).



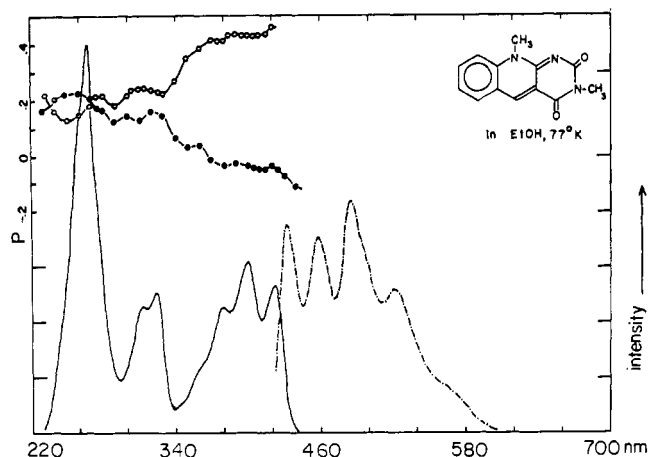


FIGURE 5: Absorption (—), corrected luminescence (fluorescence + phosphorescence, ----), and polarized excitation (with respect to $\lambda_F = 440$ nm (○), and with respect to $\lambda_F = 495$ nm (●)) spectra of 3,10-dimethyl-5-deazaalloxazine in ethanol at 77°K. The calculated energies (P-P SCF MO CI) for $S_1 \leftarrow S_0$ and $S_2 \leftarrow S_0$ transitions are 384 and 312 nm, respectively, in satisfactory agreement with the observed absorption maxima at 400 and 324 nm.

We now turn our discussion to the excited state properties of DF relative to RF. From the polarized fluorescence excitation spectrum (Figure 5), the angle between $S_1 \leftarrow S_0$ and $S_2 \leftarrow S_0$ transition moments is estimated to be in the range of 20–30°. This is also predicted by calculations which indicate a relatively small angle (27°) between these two transition moments. Thus, substitution of N(5) with C(5) does not alter the polarization characteristics of the long wavelength absorption bands (see Song *et al.*, 1972, for polarization direction of flavines).

The phosphorescence quantum yield of DF is substantially higher than that of RF (Table III). More significantly, the singlet-triplet split in DF is rather small (~ 2900 cm $^{-1}$), compared to RF (Kurtin and Song, 1969) for the (π, π^*) states involved. This suggests that the triplet state of the former distributes its spin density more toward the aromatic ring than riboflavin. The π -spin density calculations supports this argument (Figure 7). Nevertheless, the spin density at C(5) of DF is higher than at N(5) of flavine, and this difference may be an important factor for the photoreactivity of DF with respect to the NADH oxidation to be discussed later.

The phosphorescence lifetime of DF is about 10 times longer than that of RF (Table III), thus illustrating the importance of the $^1,^3(n, \pi^*)$ source at N(5) in the $T_1 \rightsquigarrow S_0$ intersystem crossing rate. The phosphorescence from DF is largely out-of-plane in character.

The spectroscopic properties of DF described above can be summarized by the characterization that the triplet state of DF reflects a more pure (π, π^*) nature than that of RF. This is an important point with regard to the photoreactivity of the compound. It is generally accepted that the more $^3(n, \pi^*)$ character the lowest triplet state possesses, the more reactive is the state for hydrogen abstraction (Turro, 1965). Surprisingly, DF is still photoreactive in the hydrogen or hydride abstraction from NADH, as will be discussed later.

Quenching of the photooxidation of NADH by paramagnetic metal ions (Radda and Calvin, 1964) and by KI is suggestive of the involvement of the triplet state as the reactive form. The intercepts and corresponding values of Φ_{ISC} (>0.5) in the Stern-Volmer plot (Figure 6) further confirm that the photoreactive state of flavines is a triplet, $^3(\pi, \pi^*)$. The

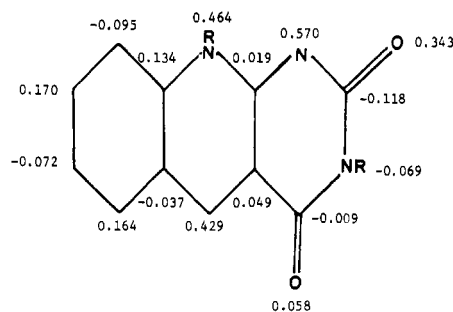
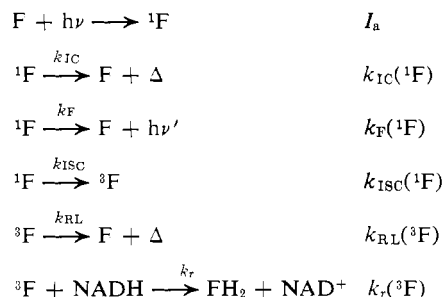


FIGURE 7: The spin density of deazaflavine in its triplet state (using Pople-Nesbet SCF MO).

kinetic data shown in Figure 6 can then be accounted for by using the following scheme.



where F stands for flavine (RF or DF), and FH₂ is reduced flavine. In the absence of NADH



where F'H₂ is reduced flavine(s) with a modified side chain. For initial reaction, *i.e.*, $(d(\text{FH}_2)/dt)_{t=0}$, the following reactions are neglected under the present experimental conditions

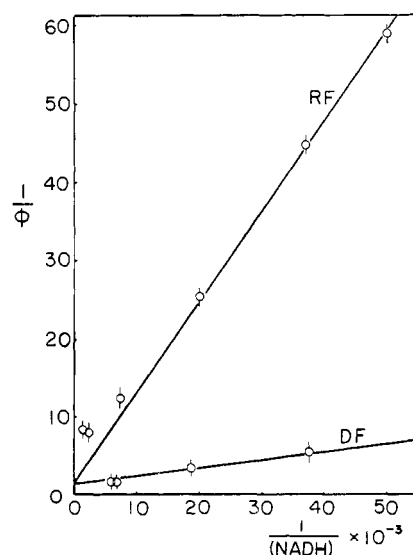
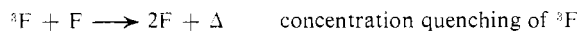
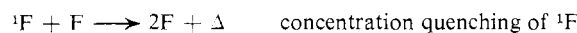


FIGURE 6: The Stern-Volmer plot of reciprocal quantum yield for the photooxidation of NADH by riboflavin (RF) and 3,10-dimethyl-5-deazaalloxazine (DF) against reciprocal concentration of NADH in water at pH 6.8 and 298°K. Within experimental errors, the quantum yields were independent of the flavine concentrations.

TABLE IV: Comparison of the Rate Constants for Photoreduction Processes of RF and DF with and without NADH at 298°K in Aqueous Solution.

	Φ_{ISC}	k_{RL}/k_r (mol l. ⁻¹)	k_{RL} (sec ⁻¹)	k_r' (sec ⁻¹)	k_r (l. mol ⁻¹ sec ⁻¹)
RF	0.70	115.7	1.4×10^4 ^a	1.21×10^2	
RF (+NADH)	0.70	8.176×10^{-4}	1.4×10^4		1.71×10^7
DF	0.63	419	$\sim 1.63 \times 10^3$	~ 3.89	
DF (+NADH)	0.63	7.31×10^{-5}	$\sim 1.63 \times 10^3$		$\sim 2.23 \times 10^7$

^a Vaish and Tollin (1970).

We then obtain the expression

$$\frac{1}{\Phi} = \frac{1}{\Phi_{ISC}} + \frac{1}{\Phi_{ISC}} \frac{k_{RL}}{k_r(NADH)} \quad (1)$$

which fits the experimental data (Figure 6) satisfactorily, except for the region where (NADH) is high. The deviation at higher (NADH) is due to the quenching of the flavine triplet or complexing with NADH.

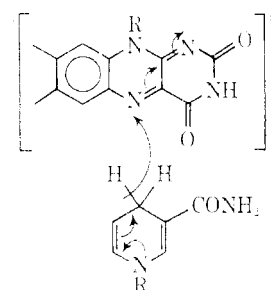
From the steady-state equation (eq 1) and Figure 6, it is possible to obtain the rate constant, k_r , provided k_{RL} is known. The quantity Φ_{ISC} is already known from Figure 6 or by assuming $\Phi_{total} \simeq \Phi_F + \Phi_{ISC} \simeq 1$, i.e., no $S_1 \rightarrow S_0$ internal conversion. Taking $\Phi_{ISC} = 0.7$ and 0.63 for RF and DF, respectively, and using $k_{RL} = 1.4 \times 10^4 \text{ sec}^{-1}$ (Vaish and Tollin, 1970), we obtain $k_r' = 1.21 \times 10^2 \text{ sec}^{-1}$ and $k_r = 1.71 \times 10^7 \text{ l. mol}^{-1} \text{ sec}^{-1}$ in the case of RF. For DF, k_{RL} has not been measured. However, the following approximations enable us to obtain a value of k_{RL} for DF

$$\left(\frac{\tau_P^{RF}}{\tau_P^{DF}} \right)_{27^\circ K, EtOH} = \left(\frac{k_P^{DF} + k_{RL}^{DF}}{k_P^{RF} + k_{RL}^{RF}} \right)_{27^\circ K} = \frac{0.17}{1.46} \simeq \left(\frac{k_P^{DF} + k_{RL}^{DF}}{k_P^{RF} + k_{RL}^{RF}} \right)_{293^\circ K} \simeq \frac{k_{RL}^{DF}}{k_{RL}^{RF}} \quad (2)$$

since $k_P \ll k_{RL}$ in solution at room temperature. Then, we obtain $k_{RL}^{DF} = 1.63 \times 10^3 \text{ sec}^{-1}$. Using this value, k_r' and k_r for DF have been calculated (Table IV).

It is clear from Table IV that the presence of a $^3(n, \pi^*)$ state (arising from N(5)-nonbonding electrons), which admixes with the lowest $^3(\pi, \pi^*)$ state, is not only unimportant for the intermolecular hydrogen transfer, but DF seems to have a slightly higher reactivity than RF. Thus, it can be concluded that the loss of N(5) in DF is not important for the intermolecular photooxidation of NADH, contrary to the general assumption that the admixing of $^3(n, \pi^*)$ character into the lowest $^3(\pi, \pi^*)$ or reversal of the $^3(n, \pi^*)$ and $^3(\pi, \pi^*)$ states is favorable for hydrogen-transfer reactions. Lumichrome, showing a substantial mixing of the $^3(\pi, \pi^*)$ with $^3(n, \pi^*)$ state, has essentially identical photoreactivity ($k_r \simeq 2 \times 10^7 \text{ l. mol}^{-1} \text{ sec}^{-1}$).

The following mechanism for the photooxidation of NADH by triplet flavine (3F) is proposed on the basis of the favorable σ overlap between the $2p_\pi$ (N(5) or C(5)) frontier orbital and the $1s$ hydrogen orbital of NADH.



In the above mechanism (not meant to be a concerted mechanism), the hydrogen or hydride of NADH is likely to be transferred from above the plane of the flavine to the position 5 through a σ -bonded complex. Although S_r^{DN} , S_r^{DR} , F_r^{OD} , and F_r^{RD} are high at N(5) and C(5), and they refer to the ground-state reactivity, these indices are also qualitative measures of the photoreactivity. This is because these quantities are defined in terms of empty molecular orbital energies and orbital sizes. Relative photoreactivity of N(1) and N(5) (or C(5)) cannot be determined until photolysis is carried out in D_2O or T_2O .

It is not possible to discriminate from the present study whether the hydrogen transfer from NADH to the triplet RF and DF proceeds *via* two-step one-electron (or hydrogen atom transfer) or one-step two-electrons (or hydride transfer). However, there is evidence that the latter mechanism is likely to be operative in the case of photoreduction of DF with EDTA (Edmondson *et al.*, 1972). In the ground-state reaction, there is evidence that the oxidation of NADH by flavine also proceeds *via* the one-step two-electron route (Suelter and Metzler, 1960; Fox and Tollin, 1966). In any case, the photochemical data provide evidence for the electrophilicity of N(5) being "orbital-controlled."

In view of the foregoing observations, the biological importance of N(5) must also be assessed in terms of thermodynamic parameters in addition to reactivity indices at position 5. The Koopmans theorem ionization potentials and electron affinities of flavines have been calculated (Song, 1972) and they are compared with those of DF and its reduced form in Table V. From Table V, it can be argued that a delicate balance of I_P and E_A for smooth electron transfer is better met by the RF-RFH₂ couple than by the DF-DFH₂ couple. The slowness of the oxidation of DFH₂ by oxygen is partly attributable to higher I_P and lower reactivity indices at the oxygenation site (C(4a), see Table II and Figure 4), compared to RFH₂. The standard redox potential of the DF-DFH₂ couple has not been reported. However, the oxidation of DFH₂ by oxygen can be facilitated by the photolysis of DFH₂. Edmondson *et al.* (1972) also ascertained that N(5) is crucial in stabilizing semiquinone intermediates by protein.

TABLE V: Ionization Potential (I_P) and Electron Affinity (E_A).

Flavine	I_P , eV	E_A , eV
RF	8.43	2.93
RFH ₂	6.93	1.32
DF	9.11	3.01
DFH ₂	8.40	1.48

In conclusion, it is safe to point out that the reactivity of position 5 (whether it be nitrogen or carbon) toward nucleophiles including hydride can be attributed to the large frontier orbital size at this position to accommodate incoming reagents in the transition state. In particular, theoretical results presented in this paper support the direct hydrogen-transfer mechanism in the oxidation of NADH by DF (Brüstlein and Bruice, 1972). We have also shown that photochemical oxidations of NADH by both RF and DF proceed equally well. Although several reactivity features of flavines have been described satisfactorily, it is fair to point out that theoretical reactivity indices presented in this paper can only be used as a qualitative guide.

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