Reaction of Nitroxides with 1,5-Dihydroflavins and $N^{3,5}$-Dimethyl-1,5-dihydrolumiflavin

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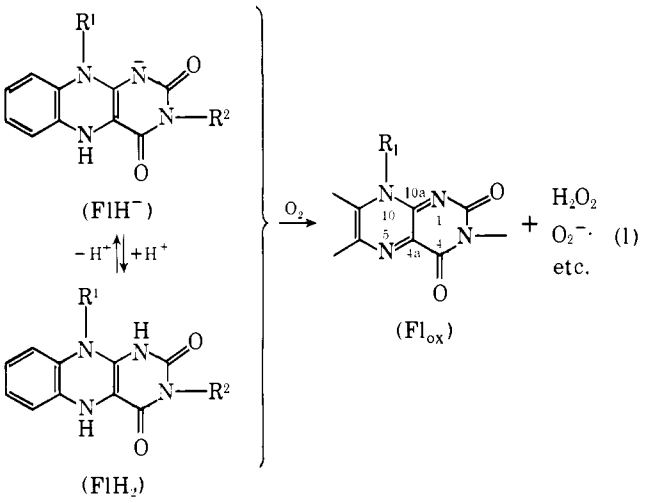
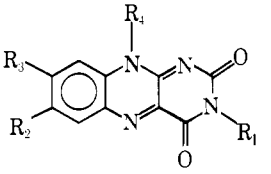
Abstract: Kinetic studies of the $1e^-$ transfer from 1,5-dihydroflavins (FlH_2 = IIH_2 to $VIIIH_2$) and 3,5-dimethyl-1,5-dihydrolumiflavin ($FlHCH_3$) to the nitroxides IX, X, and XI are reported. Under the pseudo-first-order conditions of [nitroxide = X] > [$FlHCH_3$], the oxidation to $FlHCH_3$ proceeds in two one-electron transfer steps yielding 3,5-dimethyllumiflavin ($Fl_{ox}{}^+CH_3$) with the intermediacy of the flavin radical species $FlCH_3\cdot$ (λ_{max} 585, 505 nm). In the process, two X are converted to the corresponding hydroxylamine. The rate of formation of $FlCH_3\cdot$ ($0.78\ M^{-1}\ s^{-1}$) exceeds that of its disappearance ($3 \times 10^{-2}\ M^{-1}\ s^{-1}$) by 26-fold at pH 8.9. When employing a number of nitroxides with $FlHCH_3$, the logarithm of the second-order rate constants for the formation of $FlCH_3\cdot$ is found to be a linear function (slope 6.0) of the $E_{1/2}$ of nitroxide. The reaction of the nitroxide XI with 1,5-dihydroisoalloxazines (IIH_2 to $VIIIH_2$) at pH 9 was found to be first order in appearance of oxidized flavin (Fl_{ox}) when nitroxide was in excess. Below pH 9 and in the absence of a great excess of nitroxide, the spectral time course of the reaction suggests the accumulation of FlH· as its disproportionation dimer [i.e., ($FlH_2\cdot Fl_{ox}$)]. The slope of the plot of log k_2 vs. nitroxide $E_{1/2}$ for $1e^-$ transfer from 1,5-dihydroflavin ($VIIIH_2$) to nitroxides is found to be the same as found for $1e^-$ transfer from 3,5-dimethyl-1,5-dihydroflavin to nitroxides. A plot of flavin $E_{1/2}$ vs. logarithm of the second-order rate constants for reaction of nitroxide XI with a series of 1,5-dihydroflavins is linear. The data points for the 9a, 10a sterically hindered dihydroflavins fit this line. It is concluded that $1e^-$ transfer does not involve the 9a and 10a positions.

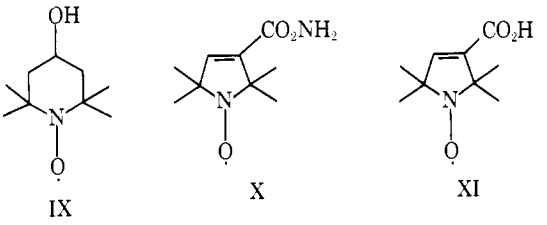
The oxidation of 1,5-dihydroisoalloxazines (FlH_2 and FlH^-) by O_2 (eq 1) has drawn attention[2] owing to its considerable biochemical importance and because it has been considered to represent a rapid electron transfer from a singlet (FlH_2 or FlH^-) to a triplet species ($^3O_2$). In preceding studies we have shown that the reaction of $FlHCH_3$ with O_2 in methanol yields the corresponding 4a-hydroperoxyflavin ($FlHO_2H$).[2] Hamilton[3] has pointed out that the reaction of dihydroflavin with $^3O_2$ should involve a $1e^-$ transfer rather than a $2e^-$ transfer because the latter is a spin-forbidden process. The logical conclusion[2] to Hamilton's arguments would be that any formation of $FlHO_2H$ would result from the recombination, within an intimate radical pair, of FlH· and $O_2^-\cdot$. Thermodynamic calculations establish[2b] that the flavin radical $O_2^-\cdot$ species is a reasonable intermediate. It has been pointed out[4] that the central 1,4-dihydropyrazine ring of the 1,5-dihydroisoalloxazine molecule confers to the latter a propensity to act as a $1e^-$ donor in both the thermodynamically more stable butterfly conformation and the higher free energy containing planar conformation. When in the bent conformation, Hoffman[5a,b,c] orbital splitting of the lone pair electrons on the N(10) and N(5) positions may be expected to place one pair of electrons in a higher energy orbital, the splitting being relieved on radical formation. When in the planar conformation, the 1,4-dihydropyrazine ring may be recognized as being antiaromatic.[5d] This destabilizing feature is also relieved on $1e^-$ abstraction.



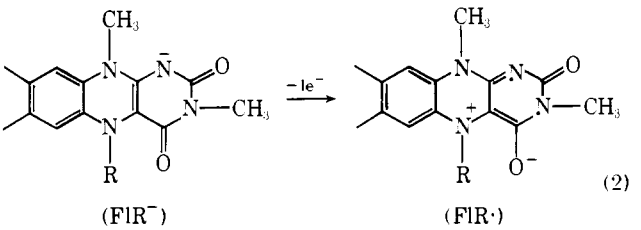
Like ground-state oxygen, the nitroxides possess an unpaired electron and undergo $1e^-$ transfer reactions. We have chosen to investigate the kinetics of the reaction of 3,5-dimethyl-1,5-dihydrolumiflavin ($FlHCH_3$ and $FlCH_3^-$) and the dihydro forms of isoalloxazines II-VIII (i.e., IIH_2-$VIIIH_2$) with the nitroxides IX-XI. 3,5-Dimethyl-1,5-dihydrolumiflavin



	nm	R_1	R_2	R_3	R_4
I	440	CH_3	H	CN	CH_3
II	426	CH_3	CN	H	CH_3
III	443	CH_3	Cl	H	CH_3
IV	443	CH_3	H	H	2',6'-dimethylphenyl
V	435	CH_3	H	H	2'-methylphenyl
VI	435	CH_3	H	H	CH_3
VII	443	CH_3	CH_3	CH_3	CH_3
VIII	443	$CH_2CO_2^-$	CH_3	CH_3	CH_3



($FlHCH_3$ and $FlCH_3^-$) yields the stable radical $FlCH_3\cdot$ on loss of $1e^-$ and flavinium cation $Fl_{ox}{}^+CH_3$ on $2e^-$ oxidation. The



(2)

λ_{max} 504 and 585 nm

chemistry of $FlCH_3\cdot$ and $Fl_{ox}{}^+CH_3$ has been described previously.[6]

Experimental Section

Materials. Nitroxides. 2,2,6,6-Tetramethyl-4-oxopiperidine hydrochloride and 4-hydroxy-2,2,6,6-tetramethylpiperidine were purchased from the Aldrich Chemical Co. 3-Carboxy-2,2,5,5-tetramethyl-3-pyrrolinyl-1-oxy (XI) was purchased from Eastman and recrystallized from ethyl acetate, mp 210–211 °C (lit.[7] mp 210–211 °C). 4-Hydroxy-2,2,6,6-tetramethylpiperidine-1-oxy (IX) was prepared by oxidation of 4-hydroxy-2,2,6,6-tetramethylpiperidine, using hydrogen peroxide, EDTA (disodium salt), and sodium tungstate in aequeous solution.[8] The product was recrystallized three times from a mixture of ethyl ether and hexane, mp 72–73 °C (lit.[8] 71.5 °C). 3-Carbamoyl-2,2,5,5-tetramethylpyrroline was prepared from 2,2,6,6-tetramethyl-4-oxopiperidine hydrochloride by the method of Rozantsev.[9] The substance was subsequently used without purification. 3-Carbamoyl-2,2,5,5-tetramethylpyrrolinyl-1-oxy (X) was prepared from 3-carbamoyl-2,2,5,5-tetramethylpyrroline by the method of Rozantsev and Krinitskaya,[7] mp 203–204 °C (lit.[7] mp 203–204 °C). 2,2,6,6-Tetramethyl-1,4-piperidinol was prepared by reduction of 4-hydroxy-2,2,6,6-tetramethylpiperidine using 85% hydrazine[10] (Matheson Coleman and Bell), mp 158–159 °C (lit.[10] 158 °C).

Flavins. 8-Cyano-3,10-dimethylisoalloxazine and 7-cyano-3,10-dimethylisoalloxazine were prepared according to a synthetic scheme which will be published in a separate manuscript dealing with the properties of these two flavins. 7-Chloro-3,10-dimethylisoalloxazine (III), 10-(2′,6′-dimethylphenyl)-3-methylisoalloxazine (IV), 10-(2′-methylphenyl)-3-methylisoalloxazine (V), and 3,10-dimethylisoalloxazine (VI) were from a previous study.[11] 3,5-Dimethyl-1,5-dihydrolumiflavin and its oxidized form were prepared by the method of Ghisla et al.[12] 3,7,8,10-Tetramethylisoalloxazine (VII, 3-methyllumiflavin) was prepared according to the *N*-oxide method of Yoneda et al.[13] Thin layer chromatography (TLC) on silica established the compound to be pure and homogeneous using two solvent systems, mp 297 °C dec (lit.[13] mp 298–301 °C dec). 3-Carboxymethyl-7,8,10-trimethylisoalloxazine (VIII, lumiflavin 3-acetate) was synthesized utilizing the method of Hemmerich.[14,15] TLC on silica gel exhibited a single spot with chloroform as the solvent. A chloroform/methanol solvent systems showed a trace impurity that could be attributed to unhydrolyzed ester from the last step of the synthesis. The material was used without further purification, mp 300 °C dec.

Methods. All melting points are uncorrected. Spectra were recorded on a Cary 118C spectrophotometer. Kinetic measurements were made on a Cary 16, Cary 118C, or a Durrum-Gibson Model 13001 stopped-flow spectrophotometer which was enclosed in a glovebox under nitrogen atmosphere. Polarographic half-wave potentials were determined at 25 °C in borate buffer solution (0.1 M, pH 9.05, μ = 1 with KCl, 2% acetonitrile) using a PAR Model 174 polarographic analyzer equipped with a saturated calomel reference electrode. pH measurements were taken using a Radiometer Model 26 pH meter equipped with a standardized Model EA-125 Metrohm or GK-2302C Radiometer electrode at 30 °C. Rates were determined at 30 °C by following the appearance of oxidized isoalloxazine at the wavelengths indicated. All anaerobic solutions were prepared, stored, and transferred under a nitrogen atmosphere in a glovebox. All such solutions were deoxygenated by bubbling argon for 45 min.

Kinetic Measurements for Reaction of $FlHCH_3$ with Nitroxides. Anaerobic stock solutions of $FlHCH_3$ (20–50 μL, 0.005–0.01 M in 0.1 M aqueous KOH) were added to the side arm of a Thunberg cuvette containing 3 mL of a buffered solution of nitroxide (2×10^{-2} to 4×10^{-4} M). The solutions were deaerated by bubbling vanadous ion scrubbed argon for 30–45 min. The Thunberg cell was closed and transferred to the cell compartment of a spectrophotometer thermostated at 30 °C. After temperature equilibration the contents of the Thunberg cuvette were mixed and changes in optical density at 585 nm ($FlCH_3\cdot$) were followed.

Kinetic Measurements for the Oxidation of Dihydroisoalloxazine (FlH_2) by Nitroxides. Stock solutions (0.002 M) of isoalloxazines II–VIII were made up in acetonitrile (spectral grade, Matheson Coleman and Bell). The stock solution (1 mL) and 1 mL of an EDTA solution (0.05 M) were mixed and diluted to 25 mL with the appropriate buffer solution. The resulting solution was deaerated by bubbling with argon for 30 min. A solution of a nitroxide in the same buffer was deaerated in the same manner. Both solutions were transferred into the anaerobic box containing the stopped-flow spectrophotometer. The isoalloxazine solution was photoreduced inside the storage syringe just prior to mixing with the nitroxide solution. The concentration of isoalloxazines employed in the kinetic studies was ca. 4×10^{-5} M and concentration of the nitroxides ca. 4×10^{-4} to 2.5×10^{-2} M.

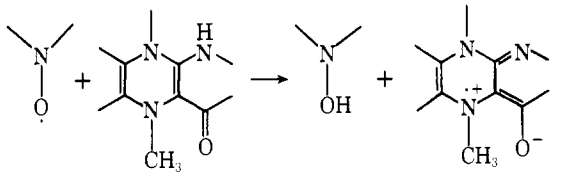
Fluorescence quenching studies were carried out at 22 °C on a recording Hitachi Perkin-Elmer MDF-2A spectrofluorometer using wavelengths of 440 (excitation) and 510 nm (maximum emission). The nitroxide X was used as quencher in MOPS buffer solution (pH 7.0, μ = 1.0, 6 vol % acetonitrile) containing the isoalloxazine VII (ca. 5×10^{-5} M).

Product Analysis. In the reaction of $FlHCH_3$ with nitroxides, the formation of $FlCH_3\cdot$ was characterized by the spectrum of the radical (λ_{max} 504, 585 nm). In the reaction of dihydroisoalloxazines (I–VIII) with nitroxide, the product of reaction showed the characteristic spectra of the corresponding isoalloxazine. To determine the fate of the nitroxide, 1 mL of the isoalloxazine (VII) stock solution (0.002 M) and 1 mL of EDTA solution (0.05 M) were mixed and diluted to 25 mL with water. The solution was deaerated as previously. An aqueous solution of IX (0.018 M) was also deaerated. Both solutions were transferred into a glovebox under nitrogen. The isoalloxazine solution was photoreduced with visible light and then 100 μL of the nitroxide solution was added. The solution was removed from the box and the water removed by lyophilization. The solid residue was dissolved in methanol and analyzed by TLC employing silica gel and alumina chromatographic sheets (Eastman) with chloroform–ethanol (10:1) and chloroform–2-propanol (10:1) as eluents. Authentic samples of the reactant, e.g., 4-hydroxy-2,2,6,6-tetramethylpiperidine-1-oxy and the proposed product 2,2,6,6-tetramethyl-1,4-piperidinol, were used as reference. In each case, two major spots were observed on the chromatogram which were identical in position with that of 3-methyllumiflavin and 2,2,6,6-tetramethyl-1,4-piperidinol. The experiment was repeated with $FlHCH_3$ and again the hydroxylamine was found to be the product from the nitroxide moiety.

To determine the fate of $FlCH_3\cdot$ in the reaction of $FlHCH_3$ with nitroxide, 2 mL of anaerobic solution of $FlHCH_3$ (ca. 5×10^{-5} M) in methanol was put in the lower part of a Thunberg cuvette in a nitrogen glovebox. The upper bulb of the cuvette contained 50 μL of an anaerobic solution of nitroxide IX (0.1 M) in methanol. The Thunberg cuvette was sealed and the spectrum of the flavin solution recorded on a Cary 118C spectrophotometer. The nitroxide and flavin solutions were mixed and repetitively scanned overnight. After most of the radical ($FlCH_3\cdot$) had disappeared (600 nm), a major peak at around 360 nm remained which indicated the presence of the 4a-methoxy adduct of $Fl_{ox}{}^+CH_3$.

Results

The reaction of nitroxides with $FlHCH_3$ or $FlCH_3{}^-$ involves a $1e^-$ transfer from flavin to nitroxide to yield $FlCH_3\cdot$ and a hydroxylamine (Experimental Section, eq 3). Under the



(3)

pseudo-first-order conditions of [nitroxide = X] = 4×10^{-4} to 2×10^{-2} M $\gg$ [$FlHCH_3$ + $FlCH_3{}^-$] = 2×10^{-5} to 5×10^{-5} M, and at pH values below 9, the appearance of $FlCH_3\cdot$ (585 nm) was found to follow the first-order rate law to at least 3 half-lives. When the reaction was followed by repetitive scanning from 400 to 600 nm (pH 7.2), only the appearance of $FlCH_3\cdot$ could be observed (λ_{max} 585, 505 nm). Plots of the pseudo-first-order rate constant (k_{obsd}) vs. [X] were found to be linear (Figure 1). From the slopes of the nitroxide dilution plots the second-order rate constants of Table I were calculated. For the purpose of calculation, values of k_{obsd} were determined at four or more values of [X] at each pH. Inspection of Table I reveals that the values of k_2 remain invariant with pH, buffer type, and the concentration of buffer employed. Since the pK_a associated with dissociation of $FlHCH_3$ to

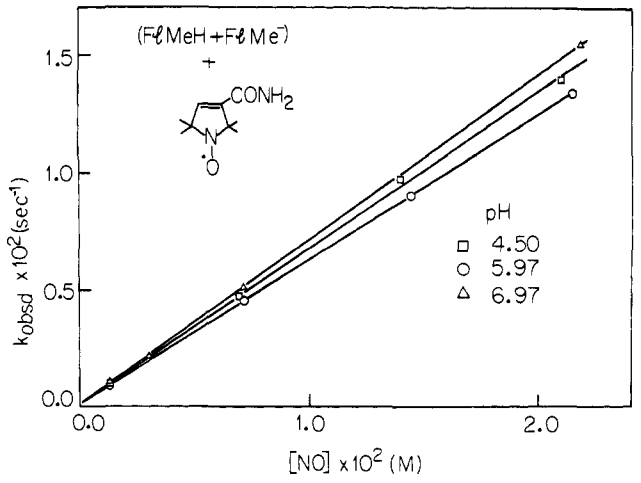


Figure 1. Dependence of the pseudo-first-order rate constant (k_{obsd}) for appearance of FlMe· upon the concentration of nitroxide (X): □, acetate buffer (0.1 M); ○, MES (0.1 M); △, MOPS (0.1 M). Reaction at 30 °C, μ = 1.0 with KCl, solvent H_2O with 2% CH_3CN (v/v).

Table I. Second-Order Rate Constants (k_2) for the Reaction of $FlHCH_3$ with 3-Carbamoyl-2,2,5,5-tetramethyl-3-pyrrolinyl-1-oxy (X) at Various pHs, 30 °C, μ = 1.0 with KCl

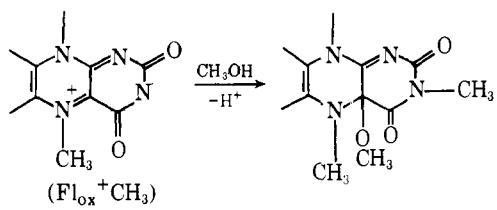
pH	Buffer	k_2, M^{-1} s^{-1}
4.50	Acetate 0.1 M	0.68
5.00	Acetate 0.02 M	0.61
	Acetate 0.08 M	0.64
	Acetate 0.1 M	0.66
	Acetate 0.4 M	0.68
	Acetate 0.8 M	0.75
5.55	Acetate 0.1 M	0.67
5.97	MES 0.1 M	0.63
6.51	Phosphate 0.1 M	0.70
6.97	MOPS 0.1 M	0.71
7.00	Phosphate 0.1 M	0.69
7.81	Phosphate 0.1 M	0.78
8.90	Borate 0.1 M	0.78

$FlCH_3^-$ is ca. 6.4,[4] these results dictate the kinetic expression

$$k_{obsd} = k_2[FlHCH_3 + FlCH_3^-][\text{nitroxide}] \quad (4)$$

At pH values of about 9 or greater, the initially formed $FlCH_3$· reacts further so that the time course for [$FlCH_3$·] follows the A → B → C kinetic expression.[16] Computer fitting (Figure 2) of [$FlCH_3$·] vs. time at four concentrations of X (pH 8.9) to the consecutive pseudo-first-order A → B → C process provided the [X]-dependent apparent first-order rate constants for appearance and disappearance of $FlCH_3$·. Plots of these rate constants vs. [X] (insets A and B of Figure 2) were linear and the second-order rate constants of eq 5 could be calculated

$$\underset{A}{FlCH_3^-} \xrightarrow{0.78[X]\ s^{-1}} \underset{B}{FlCH_3\cdot} \xrightarrow{3\times10^{-2}[X]\ s^{-1}} \underset{C}{Fl_{ox}{}^+CH_3} \quad (5)$$



(6)

from the slopes. Formation of $Fl_{ox}{}^+CH_3$ as the final product may only be presumed since this species is not sufficiently

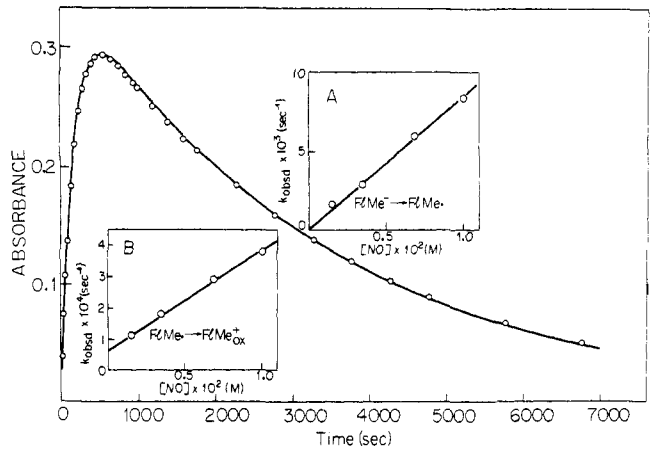


Figure 2. Computer fit of the data points for FlMe· appearance and disappearance to the consecutive first-order (A → B → C) rate equation in the reaction of $FlMe^-$ (ca. 7×10^{-5} M) with nitroxide X at 6.87×10^{-3} M [pH 9.0, 0.1 M borate buffer, μ = 1.0 with KCl, solvent H_2O with 2% CH_3CN (v/v) 30 °C]. Insert A: apparent rate constants (k_{obsd}) for *appearance* of FlMe· vs. [X]. Rate constants determined from the computer fit of appearance and disappearance of FlMe·. Insert B: apparent rate constants for *disappearance* of FlMe· vs. [X]. Intercept corresponds to spontaneous reaction of FlMe· (see ref 6).

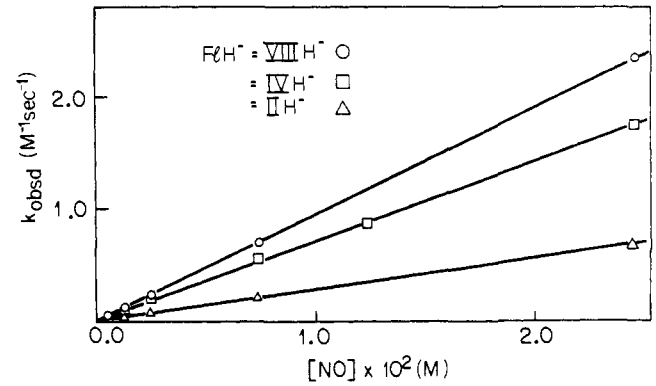
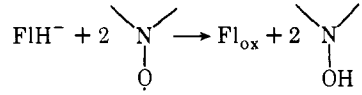


Figure 3. Dependence of the pseudo-first-order rate constants for the reaction of three 1,5-dihydroflavins (ca. 7×10^{-5} M) vs.[nitroxide = XI] [pH 9.0, 0.1 M borate buffer, μ = 1.0 with KCl, solvent H_2O-CH_3CN 2% (v/v), 30 °C].

stable in aqueous solutions at pH values of 9 and above.[6] However, when X was allowed to react with $FlHCH_3$ in methanol (anaerobic) the final product could be identified spectrally as the 4a-methoxy pseudobase adduct of $Fl_{ox}{}^+CH_3$.

Reaction of Nitroxides with Dihydroisoalloxazines IIH_2-$VIIIH_2$. At pH 9.05 the reactions of nitroxide XI with dihydroflavins (IIH_2-$VIIIH_2$) were found to be first order in the production of Fl_{ox} when the nitroxide concentration (4×10^{-4} to 2.5×10^{-2} M) exceeded that of FlH^- (4×10^{-5} M). Plots of k_{obsd} vs. [nitroxide] were found to be linear for each flavin studied (Figure 3). Employing four concentrations of XI, the second-order rate constants were obtained as the slopes of plots of [XI] vs. k_{obsd} for each FlH^- species (Table II). For each reaction the dihydroflavin was shown spectrally to yield Fl_{ox}. The reaction of $VIIH_2$ with IX was shown to yield in addition to Fl_{ox} the hydroxylamine derived from IX (eq 7). This



(7)

suggests two $1e^-$ reduction steps. The reactions were not studied at pH values above 9 because of competing hydrolysis of the Fl_{ox} product. On lowering the pH to the neutral and

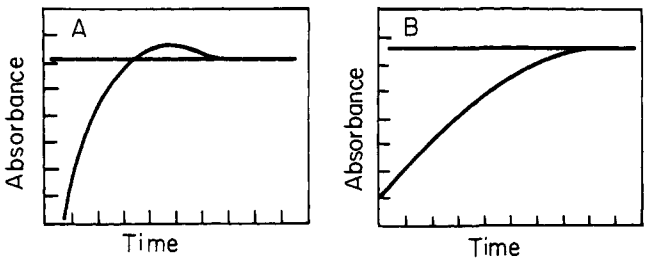


Figure 4. Stopped-flow spectrophotometric absorbance vs. time traces for the reaction of FlH_2 with the nitroxide IX at pH 7.2. Absorbance is 0.01 per division and time 1 s per division. A. ($VIIIH_2$ + $VIIIH^-$) at 3.4×10^{-5} M and IX at 1.9×10^{-3} M. B. ($VIIH_2$ + $VIIH^-$) at 1.0×10^{-5} M and IX at 2.5×10^{-4} M.

Table II. Second-Order Rate Constants (k_2) for the Reaction of FlH_2 with 3-Carboxy-2,2,5,5-tetramethyl-3-pyrrolinyl-1-oxy (XI) at pH 9.05 (0.1 M Borate, 30 °C, μ = 1 KCl) and $E_{1/2}$ Values for Reduction of Fl_{ox} (Borate Buffer 0.1 M, pH 9.05, μ = 1.0, 25 °C)

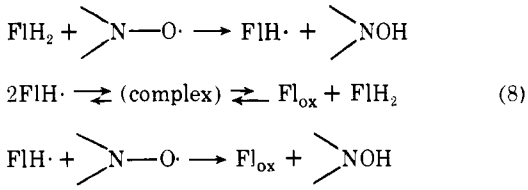
FlH_2	$E_{1/2}$ SCE, V	k_2, M^{-1} s^{-1}
IIH_2	−0.353	28.5
$IIIH_2$	−0.425	54.0
IVH_2	−0.428	73.0
VH_2	−0.428	69.5
VIH_2	−0.477	55.5
$VIIH_2$	−0.538	130.0
$VIIIH_2$	−0.538	96.5

acidic range, it was found that the reactions of nitroxides with dihydroflavins become biphasic at lower nitroxide concentrations (1×10^{-4} to 1×10^{-3} M). Thus, in the reaction of $VIIH_2$ and $VIIIH_2$ with nitroxide IX (pH 7.2), the increase in OD (380, 390 nm) reached a maximum and then leveled off to the observed OD_∞ (Figure 4A). The reactions of IH_2 and IIH_2 with IX exhibited similar spectral behavior below pH 8.0. The time course of the reaction (λ 443 nm) could be forced to fit a first-order plot for over 95% of the total change in OD if an infinity absorbance (OD_∞') well above the true infinity was employed (Figure 4B). Employing $VIIH_2$ (9.8×10^{-6} M), the fractional increase in the infinity absorbance $(OD_\infty' - OD_\infty)/(OD_\infty - OD_0)$ required to obtain good first-order kinetics to 3 "half-lives" at pH 7.2 was found to be a function of [nitroxide] (see Table III). Thus, the lower the [nitroxide] the greater the divergence of the reaction from pseudo-first-order. Repetitive scanning of reaction mixtures employing either the Cary 118C or manual point by point scanning with a stopped-flow spectrophotometer was employed with the objective of identifying metastable intermediates. Spectra (290-480 nm) obtained at 6 °C of the reaction of IIH_2 with XI (pH 7.2) exhibited tight isosbestic points at 293, 330, and 371 nm. At 30 °C spectral examination of the reaction of $VIIH_2$ with IX (pH 7.2), IVH_2 with X (pH 9.0), $VIIH_2$ with IX (pH 7.2), and $VIIIH_2$ with IX (pH 7.2) established in each case that dihydroflavin was converted to the corresponding oxidized flavin species. We have previously shown that the more stable $FlCH_3\cdot$ is complexed in aqueous solution by Fl_{ox}, $FlCH_3^-$, and $FlCH_3\cdot$. However, oxidized flavin product does not complex with nitroxide at the concentrations employed in the kinetic experiments. Thus, at concentrations of 2×10^{-5} M Fl_{ox} (VII) and 1×10^{-3} M nitroxide (X) no quenching of the fluorescence of Fl_{ox} could be observed (excitation λ 440 nm). The kinetic behavior and its dependence on [nitroxide] may find explanation in the transient formation of the dimeric complex (500-600 nm) of $FlH\cdot$ as in eq 8.

The reaction of Fl_{ox} with FlH_2 to provide $2FlH\cdot$ via a thermodynamically more stable intermediate complex is well documented.[17] If a complex of $(FlH\cdot)_2$ were to accumulate at low [nitroxide] it would not be expected to absorb appreciably greater than (FlH_2 + FlH). For this reason isosbestic points would be anticipated. The scheme of eq 8 finds some support from the observations that at pH 7.2 a weak transient absorption (550-630 nm) is observed in the reaction between VII (3.4×10^{-5} M) and nitroxide IX (3.3×10^{-4} M).

Table III. Dependence of the Infinity Absorbance Value Required to Provide First-Order Behavior in the Reaction of Nitroxide IX with Dihydroflavin at 9.8×10^{-6} M (pH 7.21)

[Nitroxide = XI], M	$(OD_\infty' - OD_\infty)/(OD_\infty - OD_0)$
2.5×10^{-4}	24
5.0×10^{-4}	13
2.5×10^{-3}	11
5.0×10^{-3}	4
1×10^{-2}	0-3



$$FlH_2 + \rangle N{-}O\cdot \longrightarrow FlH\cdot + \rangle NOH$$

$$2FlH\cdot \rightleftharpoons (\text{complex}) \rightleftharpoons Fl_{ox} + FlH_2 \qquad (8)$$

$$FlH\cdot + \rangle N{-}O\cdot \longrightarrow Fl_{ox} + \rangle NOH$$

Discussion

In aqueous solution in the middle pH range (pH 4.5-9.0) the N^5-methylflavin radical ($FlCH_3\cdot$) is formed in a $1e^-$ transfer (eq 2) from $FlHCH_3$ or $FlCH_3^-$ to nitroxide (IX and X) which is reduced to the corresponding hydroxylamine. Further reaction of $FlCH_3\cdot$ with nitroxide occurs in a much slower reaction to yield $Fl_{ox}{}^+CH_3$ and an additional 1 mol of hydroxylamine (eq 5). The reaction of nitroxide with ($FlHCH_3$ + $FlCH_3^-$) is pH independent. The nitroxides also oxidize dihydroisoalloxazines (FlH_2 + FlH^-) to yield oxidized flavins and hydroxylamines via two consecutive $1e^-$ transfer reactions.

The pH independence (Table I) of the second-order rate constant (k_2) for the generation of $FlCH_3\cdot$ on reaction of nitroxide X with ($FlHCH_3$ + $FlCH_3^-$) stands in contrast to the $1e^-$ reduction of ninhydrin in which case $FlCH_3^-$ is 2×10^2 times more reactive than $FlHCH_3$.[4] To explain the pH independence of the reaction of nitroxide X with $FLHCH_3$, one has to consider the pH dependence of the redox potentials ($\Delta E_{1/2}/\Delta pH$) for both flavin and nitroxide. For nitroxide, $\Delta E_{1/2}/\Delta pH$ is ca. −0.06 V unit below pH 6.0 and zero from pH 6.0 to 9.0.[18] By analogy with FMN and FAD,[19] $\Delta E_{1/2}/\Delta pH$ for $FlHCH_3$ is estimated to be −0.06 V up to the pK_a (6.4) for $FlHCH_3$ and zero above the pK_a. In Figure 5 there is plotted the values of log k_2 for the reactions of $FlCH_3^-$ and FlH^- (VIIIH) with nitroxides IX, X, and XI vs. the $E_{1/2}$ of the nitroxides. Good linear correlations are obtained with slopes of 6.0. In Figure 6 log k_2 for the reaction of nitroxide XI with dihydroflavins IIH_2-$VIIIH_2$ is plotted against the $E_{1/2}$ values for II-VIII. The plot reveals again a linear free energy relationship of potential and $\Delta G^{\ddagger}$. From Figures 5 and 6 one can estimate the change of log k_2 for an increment of 0.06 V in $E_{1/2}$ to be approximately 0.30 for nitroxide and a change from 0.15 to 0.30 for $FlHCH_3$. Because the changes in $E_{1/2}$ values of nitroxides and flavins with pH are in the same direction and comparable in magnitude, it is not surprising that $FlHCH_3$ and $FlCH_3^-$ react with the nitroxide at comparable rates. The same kind of pH-rate independence was observed in the reaction of 1,4-naphthoquinone with ($FlHCH_3$ + $FlCH_3^-$).[4] This was rationalized by assuming that preequilibrium complex formation between 1,4-naphthoquinone and the slower reacting $FlHCH_3$ species was more favorable than the complex formation involving $FlCH_3^-$.[4] However, com-

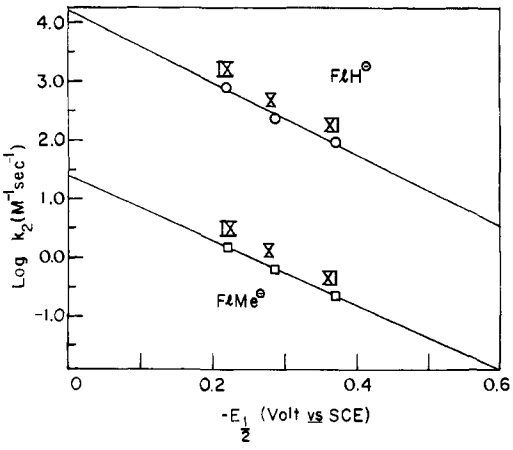


Figure 5. Logarithm of the calculated second-order rate constants (k_2) for reaction of FlH^- ($VIIIH_2$) (pH 9.0, 0.01 M borate buffer) and ($FlHCH_3 + FlCH_3^-$) (pH 7.0, 0.1 M MOPS) with nitroxides IX-XI vs. the $E_{1/2}$ of the nitroxide.

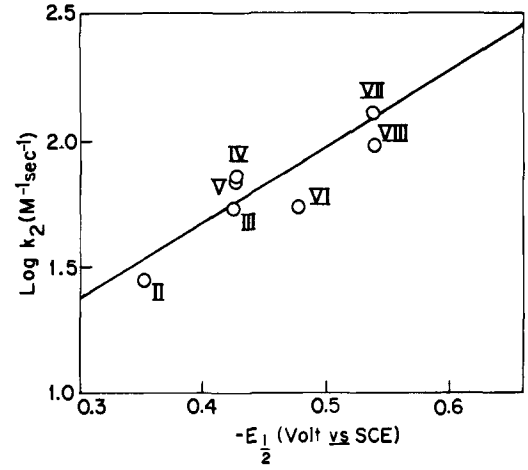
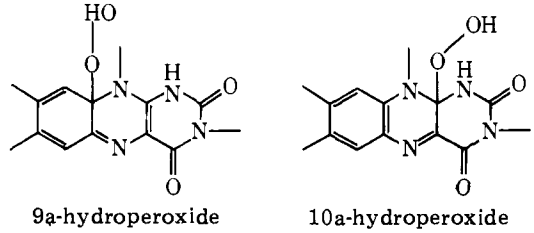


Figure 6. Plot of the logarithm of the second-order rate constants for the reaction of FlH_2 + FlH^- species with nitroxide XI (pH 9.0).

pensation involving the dependence of the $E_{1/2}$ values upon pH for the quinone and flavin is also a reasonable alternative.

The reaction of $FlHCH_3$ with nitroxides to give $FlCH_3$· involves one electron transfer. In Figure 5 the plots of the logarithm of the second-order rate constants for oxidation of FlH and $FlCH_3$ by nitroxide vs. $E_{1/2}$ (nitroxides) are linear and parallel. It is reasonable to conclude that the oxidation of FlH_2 by nitroxides also involves one electron transfer in the rate-determining step (eq 8). The 630-fold difference in rate between FlH and $FlCH_3$ (Figure 5) may be attributed mainly to the steric interaction of the methyl group in the N^5 position.

The suggestion[20,21] that the 9a- and/or 10a-hydroperoxy compounds may be formed on reaction of dihydroflavins with oxygen warrants the investigation of the importance of the steric availability of the 9a and 10a positions of 1,5- dihydroisoalloxazines to $1e^-$ transfer. In Figure 6 the points representing log k_2 for the reaction of the 9a and 10a sterically



9a-hydroperoxide 10a-hydroperoxide

hindered IVH_2 and VH_2 with nitroxide (XI) fit well on the plot of $E_{1/2}$ vs. log k_2. Clearly, electron transfer from nitroxide to reduced flavin does not involve the 9a or 10a positions of the 1,5-dihydroisoalloxazine ring. Smith and Bruice have previously shown[22] that whereas VI is susceptible to alkaline hydrolysis at the 10a and 4 positions the hydrolysis of IV is directed entirely to the 4 position. In the preceding paper,[2b] it was shown that the standard free energy for the formation of flavin radical plus superoxide anion is some 10 kcal mol^{-1} less than the free energy of activation for the oxidation of dihydroflavin. The rate-determining step in the oxidation of dihydroflavin by $^3O_2$ was suggested to be electron transfer to $^3O_3$. Then by analogy with the nitroxide oxidation of FlH^-, positions 9a and 10a are not involved in the reaction of FlH_2 with $^3O_2$.

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$$A \xrightarrow{k_1} B \xrightarrow{k_2} C$$

is $B = [(A_0k_1)/(k_1 - k_2)](e^{-k_2t} - e^{-k_1t})$.
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