Radical Mechanisms for 1,5-Dihydro-5-methylflavine Reduction of Carbonyl Compounds

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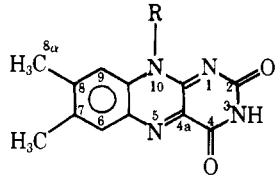
Abstract: The reaction of 1,5-dihydro-3,5-dimethyllumiflavine (FlMeH $\rightleftarrows$ $FlMe^-$ + H^+; $[FlMeH_T]$ = [FlMeH] + $[FlMe^-]$) with $CH_3COCO_2C_2H_5$, C_6H_5COCHO, Cl_3CHO (Ch), barbituric acid, benzoquinone (BQ), naphthoquinone (NQ), and ninhydrin (NIN) provides the blue (λ_{max} 580, 502 nm) zwitterionic 3,5-dimethyllumiflavine radical (FlMe·). The kinetics of the reaction have been studied with Ch, BQ, NQ, and NIN. Of the two mechanisms available for the formation of FlMe· [i.e., two-electron transfer to yield oxidized flavine ($FlMe_{ox}^+$) followed by comproportionation of $FlMe_{ox}^+$ + FlMeH $\rightarrow$ 2FlMe· and a one-electron transfer to provide FlMe· directly] the one-electron transfer can be shown to be correct for Ch, BQ, and NIN and by inference is general. Thus, reduction of Ch by FlMeH does not yield the expected two-electron reduction product 2′,2′,2′-trichloroethanol, the rate of formation of FlMe· on reaction of FlMeH with BQ exceeds the rate constant anticipated for the comproportionation reaction, and in the reaction with ninhydrin (at $[FlHMe_T] \geq [NIN]$) the ratio of $[NIN]_{t=0}/[FlMe\cdot]_{t=\infty}$ = 1:1. The rate constants for reaction of FlMeH and $FlMe^-$ with Ch are 1.5×10^{-5} M^{-1} min^{-1} and 1×10^{-3} M^{-1}, respectively. The reaction with BQ is biphasic yielding in the first step ($k_{rate} \geq 10^8$ M^{-1} sec^{-1}) FlMe· and in the second step (k_{rate} = 4.0 sec^{-1}) $FlMe_{ox}^+$. The reaction of BQ with FlMe· to yield the oxidized N(5)-methylflavinium salt ($FlMe_{ox}^+$) is independent of [BQ] when $[BQ] \geq 10 \times [FlMe\cdot]$ and also independent of pH. A mechanism involving complexation of BQ with FlMe· ($K_c \geq 2 \times 10^3$ M^{-1}) followed by intracomplex electron transfer is suggested. The reaction of naphthoquinone with $FlMeH_T$ is also biphasic involving the intermediate formation of FlMe·. The rate of appearance of FlMe· is independent of pH and thus the mole fraction of FlMeH and $FlMe^-$, $k_{rate} = 4 \times 10^4$ M^{-1} sec^{-1}. The reaction of NIN with $FlMeH_T$ can be characterized by a rate constant for reaction with FlMeH (2.2×10^3 M^{-1} sec^{-1}) and $FlMe^-$ (4.25×10^5 M^{-1} sec^{-1}). 1,5-Dihydrolumiflavine-3-acetic acid (FlH_2) undergoes a one-electron transfer to Malachite Green in a reaction second order in Malachite Green ($k_3' = 2.6 \times 10^5$ M^2 sec^{-1}). These results are discussed in conjunction with our previous suggestion of one-electron transfer in the dihydroflavine reduction of carbonyl compounds, i.e.

$$FlH_{2T} + >C{=}O \rightleftarrows FlH\cdot\ >\dot{C}(O^-) \underset{-H^+}{\overset{+H^+}{\rightleftharpoons}} FlH\cdot\ >\dot{C}(OH) \rightleftarrows Fl_{ox} + HC(OH)<$$

$$FlH_{2T} + >C{=}O \rightleftarrows FlH\cdot\ >\dot{C}(O^-) \underset{-H^+}{\overset{+H^+}{\rightleftharpoons}} FlH\cdot\ >\dot{C}(OH) \rightleftarrows Fl_{ox} + (-)C(OH)< + H^+$$

The stabilizing influence of N(5)-methylation upon flavine radical is discussed in terms of lone-pair lone-pair splitting of the nonbonding electrons at the N(5) and N(10) positions of dihydroflavine.

Flavines I possess three oxidation states: (1) dihydro; (2)



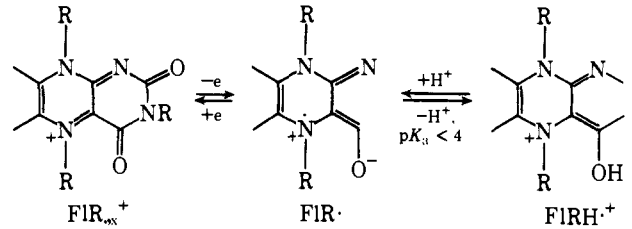
I

radical; and (3) fully oxidized. Each oxidation state may exist in solution as three ionic species.[2] Oxidized, reduced, and radical species are brought into equilibrium rapidly by the reactions of comproportionation[3,4] (eq 1 for flavine mononucleotide at 11°).[4]

$$FMN + FMNH_2 \underset{>10^6\ sec^{-1}}{\overset{>10^8\ M^{-1}\ sec^{-1}}{\rightleftharpoons}} FMN\cdot\ FMNH_2 \underset{1\times10^7\ M^{-1}\ sec^{-1}}{\overset{3.5\times10^3\ sec^{-1}}{\rightleftharpoons}} 2FMN\cdot \quad (1)$$

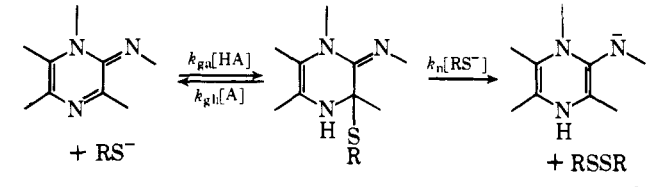
The radical species are present at an appreciable mole fraction only at the extremes of pH when the substituent R of I represents an alkyl or aryl group and either -H or an alkyl substituent resides on the N(3) position. When the N(5) and N(3) positions of I are substituted by alkyl groups to provide the *N*(5)-flavinium ion (FlR_{ox}^+), the ionic and redox equilibria of Scheme I may be obtained. The zwitterionic radical species derived from the one-electron reduction of FlR_{ox}^+ unlike its structural counterpart obtained by one-electron reduction of I is thermodynamically stable at neutrality. Thus, the comproportionation reaction (eq 1) of oxidized (i.e., Fl_{ox}) and reduced (i.e., FlH_2 + FlH^-) I favors the formation of Fl_{ox} and FlH_2 through most of the pH range. In contrast, the comproportionation reaction of oxidized $FlMe_{ox}^+$ and (FlMeH + $FlMe^-$) favors the formation of the blue (λ_{max} 502, 580 nm) zwitterionic radical species FlMe·.

Scheme I



FlR_{ox}^+ FlR· $FlRH^{\cdot+}$

In the reaction of (FlH_2 + FlH^-) with a carbonyl compound to provide Fl_{ox} plus alcohol, three types of mechanisms may be considered. These are: (1) a two-electron transfer proceeding through a covalent addition intermediate; (2) hydride transfer; and (3) two one-electron transfer reactions. An example of a two-electron transfer is the oxidation of thiol by Fl_{ox} (eq 2).[5] Suitable two-electron cova-



(2)

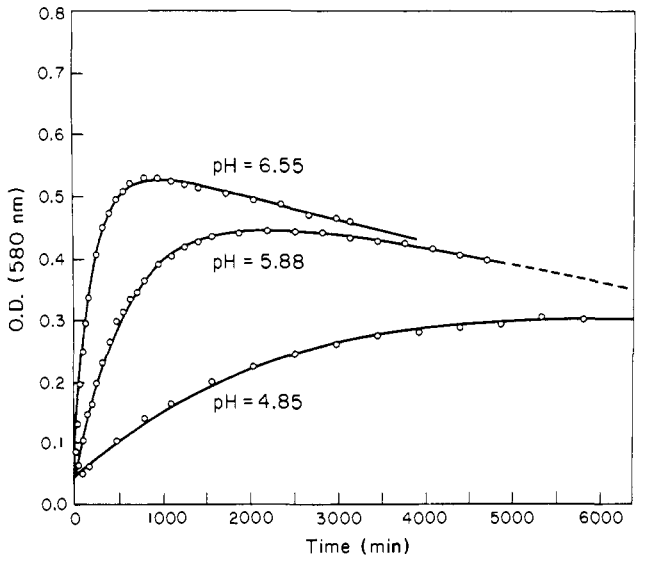
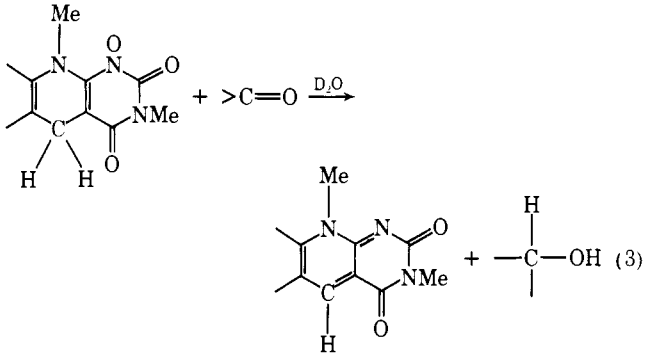
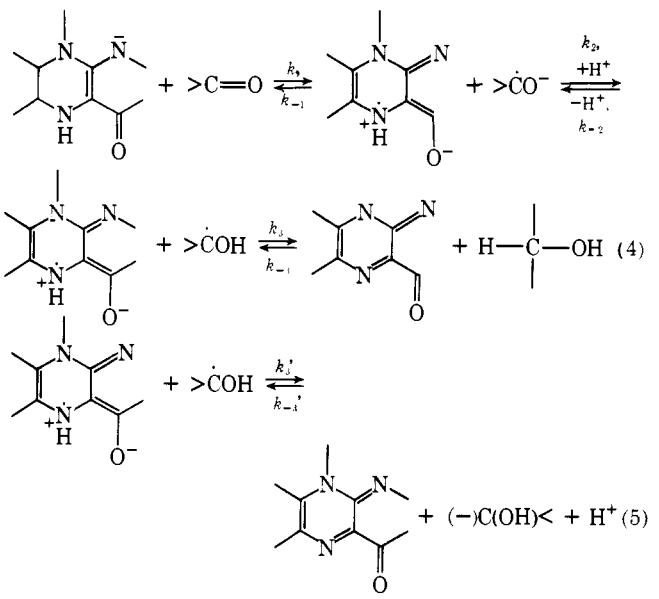


Figure 1. Plots of time dependence for appearance and disappearance of FlMe· on reaction of chloral (0.5 *M*) with FlMeH + FlMe⁻ (1.8×10^{-4} *M*). The points are experimental and the lines computer generated employing the reactions of eq 8 and the constants provided in the manuscript.

lent mechanisms for carbonyl group reduction are not apparent.[6] Direct hydride transfer from the N(5)-H position of FlH_2 or FlH^- cannot be ruled out though the author is unaware of H^- transfer from nitrogen. It has been shown both in model[6a,7] and enzymatic reactions[8] that the elements of H^- are transferred to and from the 5 position of 1,5-dihydro-5-deazaflavine (for example, eq 3). Since the



standard free energies of formation ($\Delta G°$) of flavine radicals from FlH_2 + FlH^- ($\Delta G°$ = 1.69, 3.87, and 5.14 kcal M^{-1} at pH 5, 7, and 9, respectively, as calculated from the data of ref 2e) are not great, the reasonableness of the involvement of radical mechanisms in the flavine >C=O ⇄ HC(OH)< interconversion is greatly dependent upon the $\Delta G°$ values for $>\dot{C}O^-$ and $>\dot{C}OH$ species. Recent considerations have provided the $E°'$ values for the formation of a few of the radical anion and neutral radical species.[9] On the basis that the standard free energies of formation of one or both of the radical pairs, Fl_{rad^+} $>\dot{C}O^-$ and Fl_{rad^+} $>\dot{C}OH$, do not exceed the $\Delta G^‡$ values for the overall reaction (FlH_2 + FlH^-) + >C=O → Fl_{ox} + HCOH, it has been proposed that the mechanism of the reaction is radical in nature.[6c,10] For the purposes of the present study, the mechanism of eq 4 and 5 may be considered (written for the FlH^- species but pertaining to FlH_2 as well). In eq 4, proton transfer may occur in the first step (k_1/k_{-1}) or second (as shown) depending upon the pK_a of the carbonyl compound and the standard free energy of formation of the $>\dot{C}O^-$ species from the carbonyl compound. Equations 4



and 5 differ in that in (4), one-electron transfer is followed by H· transfer [from the (N)5 position] while in eq 5, one-electron + one-electron transfer provides a carbanion. The sequence of eq 4 is proposed to operate in those instances where the carbanion would not be stabilized while the mechanism of eq 5 prevails for resonantly stabilized carbanion products. It should be noted that the sequence of eq 4 amounts to the transfer of the elements of H^- and would not normally be distinguishable from a hydride transfer reaction as noted for the 5-deazaflavine reactions (see eq 3).

Because the species FlMeH and $FlMe^-$ do not possess a hydrogen on the N(5) position, they would not be capable of participating in the mechanism of eq 5. As noted, the radical FlMe· has a greater thermodynamic stability than FlH·. The present study deals primarily with kinetic investigations of the reaction of 1,5-dihydro-3,5-dimethyllumiflavine with carbonyl compounds.

Results

A cursory survey of the reaction of 1,5-dihydro-3,5-dimethyllumiflavine (1.5×10^{-5} *M*; referred to simply as FlMeH and $FlMe^-$) with a series of potential substrates ([S] = 1×10^{-2} *M*) was carried out [30°; μ = 0.5 with KCl; solvent H_2O-CH_3CN or EtOH, 0-10% (v/v)]. The compounds (S) could be placed into one of the three categories of nonreactive (cyclobutanone, pH 7; *N*-methyl-6-chlorouracil, pH 3; thiourea, pH 7), slowly reacting (ethyl pyruvate, pH 7; chloral, pH 7; phenyl glyoxal, pH 2.5; barbituric acid, pH 3), and rapidly reacting (benzoquinone, naphthoquinone, and ninhydrin) on the basis of the appearance of the blue radical of FlMe·. Of the compounds listed, chloral (Ch), benzoquinone (BQ), naphthoquinone (NQ), and ninhydrin (Nin) were chosen for further examination.

Reactions of Chloral with 1,5-Dihydro-3,5-dimethyllumiflavine. Pseudo-first-order conditions of [Ch] ≫ [FlMeH + $FlMe^-$] were employed and the appearance of radical (FlMe·) was monitored at 580 nm. The following observations could be made: (a) the formation of FlMe· followed the first-order rate law accurately when the maximum absorbance was employed as the absorbance at t_∞; (b) the maximum absorbance was found to be dependent on both pH and [Ch]; and (c) after formation, the radical is slowly transformed into 3-methyllumiflavine (eq 6). Examples of the time dependence for radical formation and disappearance are provided in Figure 1. From separate kinetic measurements, the rate of FlMe· → Fl_{ox} was found to be independent of [Ch].

Table I. Rate Constants Derived from Eq 7 for the Reaction of 1,5-Dihydro-3,5-dimethyllumiflavine (1.8×10^{-4} *M*) with Chloral (30°, Solvent H_2O, μ = 0.5 with KCl)

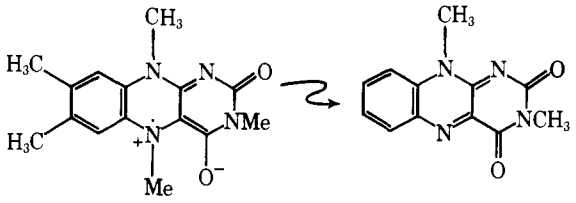
$[Ch]_T$	pH[a]	k_{obsd},[b] min^{-1} $\times 10^3$	% FlMe·max		k_1, M^{-1} min^{-1} $\times 10^4$	k_{-1}, M^{-1} min^{-1} $\times 10$	k_2, M^{-1} min^{-1} $\times 10^4$	k_3, min^{-1} $\times 10^4$
			Spectral	Computed				
0.5	3.98		32.2[c]	30.8	0.18	3.87	7.02	9.5
0.5	4.85		51.5[c]	50.6	0.47	1.83	7.12	10.8
0.5	5.88	2.12	74.3	74.8	2.31	3.57	0.90	8.6
0.3	6.69	4.54	76.7	75.0	8.40	4.88	0.956	8.6
0.4	6.62	5.17	82.0	80.3	8.20	4.61	1.10	8.8
0.5	6.55	5.59	88.4	87.7	8.64	4.49	1.41	7.4
0.5	7.50	6.51	86.6	84.4	9.28	4.92	1.41	9.3
0.5	8.31	7.63	84.7	86.7	10	1.47	0.044	12.5

[a] pH held constant with 0.1 *M* buffer (acetate, phosphate, and borate). [b] Pseudo-first-order rate constant for appearance of OD at 580 nm. [c] Not the maximum value but determined after 6000 min.

Table II. Observed Pseudo-First-Order Rate Constants for the Reaction of 1,5-Dihydro-3,5-dimethyllumiflavine (9×10 *M*) with Ninhydrin [30°; Solvent H_2O–C_2H_5OH, 0.5–4% (v/v); μ = 0.5 with KCl]

pH[a]	[Nin], $M \times 10^3$	k_{obsd}, sec^{-1}
3.04	1	2.30
4.09	1	3.52
5.00	0.5	2.44
5.00	1	3.79, 3.54
5.00	2	7.45
5.00	4	14.7
6.25	1	29
7.01	0.5	33.5
7.01	1	85.2
7.01	2	189
7.62	1	253

[a] pH held constant with 0.05 *M* buffer (acetate and phosphate).



(6)

At any given pH the appearance and disappearance of FlMe· may be represented by the sequence of eq 7

$$FlHMe_T + Ch \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} FlMe\cdot + Ch\cdot$$

$$Ch + Ch\cdot \xrightarrow{k_2} ? \qquad (7)$$

$$FlMe\cdot \xrightarrow{k_3} F_{ox}$$

where $[FlMeH]_T$ represents the concentration of 1,5-dihydro-3,5-dimethyllumiflavine (FlHMe + $FlMe^-$) and Ch·, the radicals formed by one-electron transfer from dihydroflavine species to chloral. Gas-phase chromatography of preparative reactions indicated that 2′,2′,2′-trichloroethanol was not produced in the reaction. Addition of diphenylamine, as an H· donor, to the reaction mixture did not result in the detection of trichloroethanol. It has been reported that the reduction of chloral by 1,5-dihydroflavines does give rise to trichloroethanol.[11]

The rate constants of eq 7 were obtained by fitting the experimental points to curves generated by an analog computer programmed with the differential expressions for $(d[Ch]/dt)$, $(d[FlHMe_T]/dt)$, $(d[Ch\cdot]/dt)$, and $(d[FlMe\cdot]/dt)$. Examples of the fit of eq 7 to the experimental data are provided in Figure 1. The derived constants are presented in Table I. Plots of the logarithms of k_{-1}, k_2, and k_3 vs. pH establish that these constants are independent of pH. Their average values are 0.37 ± 0.11 M^{-1} min^{-1} (k_{-1}), $2.5 \pm 1.5 \times 10^{-4}$ M^{-1} min^{-1} (k_2), and $8.5 \pm 1 \times 10^{-4}$ min^{-1} (k_3). The value of k_1 is clearly pH dependent. In Figure 2 there is plotted log k_1 vs. pH. The points of Figure 2 are experimental and the line is generated from the rate equation

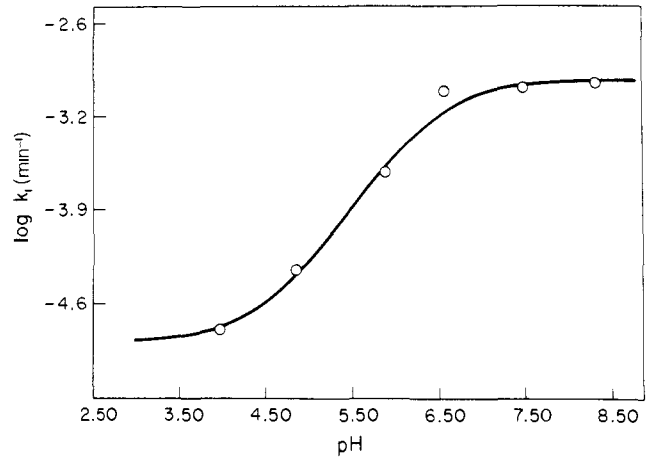


Figure 2. Log k_1 vs. pH profile for reaction of chloral with FlMeH + FlMe· (eq 9).

$$k_1 = k_{FlMeH}\frac{a_H}{K_{app} + a_H} + k_{FlMe^-}\frac{K_a}{K_{app} + a_H} \qquad (8)$$

where K_{app} is the kinetically apparent dissociation constant of the N(1)–H of FlMeH yielding $FlMe^-$, a_H the hydrogen ion activity, and k_{FlMeH} and k_{FlMe^-} the second-order rate constants for electron transfer to chloral from FlMeH and $FlMe^-$, respectively. The values employed to obtain the best fit of eq 8 to the plot of Figure 2 are $k_{FlMeH} = 1.5 \times 10^{-5}$ M^{-1} min^{-1}, $k_{FlMe^-} = 1.05 \times 10^{-3}$ M^{-1} min^{-1}, and $pK_{app} = 6.4$. The pK_a's of *N*(5)-alkyldihydroflavines are reported in the range of 7–8.[12] That pK_{app} is below this value may find explanation in the lack of precise fit of the experimental points to the pH–log k_1 profile (Figure 2) or to some pH dependence of the hydration equilibrium constant of chloral.

The reaction of 3,5-dimethyl-1,5-dihydrolumiflavine with ninhydrin was followed in a stopped-flow spectrophotometer. Reactions were carried out at constant pH values and under the pseudo-first-order conditions of [Nin] ≫ [FlMeH + $FlMe^-$]. The pertinent kinetic data are collected in Table II.

Plots of [Nin] vs. k_{obsd} at pH 7.01 and 5.00 are linear and provide the pH-dependent apparent second-order rate constants (k_2') of 8.8×10^4 M^{-1} sec^{-1} (pH 7.01) and $3.7 \times$

$$\frac{d[FlMe\cdot]}{dt} = k_2'[Nin_T][FlMeH + FlMe^-] \qquad (9)$$

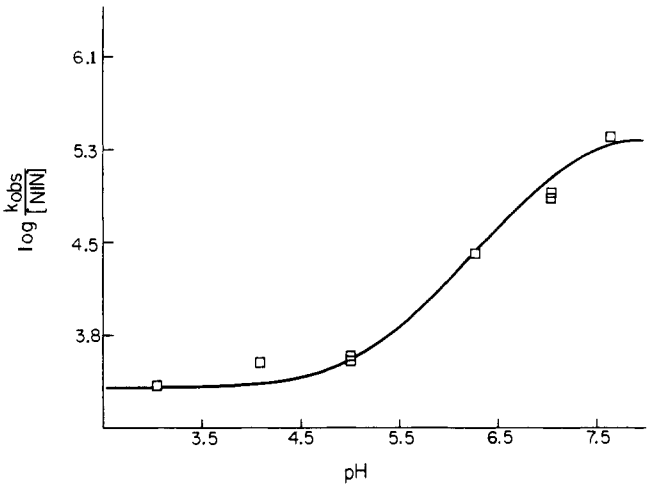
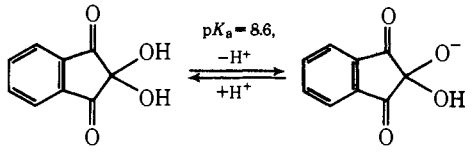


Figure 3. Plot of the log of the apparent second-order rate constant for reaction of ninhydrin with FlMeH + FlMe$^-$ vs. pH. Points are experimental and the line is generated from eq 11.

$10^3\ M^{-1}\ \text{sec}^{-1}$ (pH 5.00). At a value of [Nin] = 1×10^{-3} M a plot of log k_{obsd}/[Nin] vs. pH (Figure 3) reveals that the value of k_{obsd} does not depend only on the mole fraction of FlMeH and FlMe$^-$ ($pK_{app} \sim 7.4$) but increases with increasing pH. The second-order production of FlMe· can be expressed as in eq 10

$$\frac{d[\text{FlMe}\cdot]}{dt} = \left(\frac{k_{\text{FlMeH}}a_H + k_{\text{FlMe}^-}(K_{a_1})}{K_{a_1} + a_H}\right)\left(\frac{a_H}{K_{a_2} + a_H}\right) \times [\text{FlMeH}_T][\text{Nin}_T] \quad (10)$$

where K_{a_1} is the acid dissociation constant of FlMeH, K_{a_2} is the acid dissociation constant of ninhydrin hydrate (eq 11),[13] and k_{FlMeH} is the second-order rate constant for reaction of II with FlMeH ($2 \times 10^3\ M^{-1}\ \text{sec}^{-1}$) and k_{FlMe^-} the second-order rate constant for reaction of II with FlMe$^-$ ($4.25 \times 10^5\ M^{-1}\ \text{sec}^{-1}$).



II III (11)

The formation of FlMe· in a reaction first order in [Nin$_T$] and [FlMeH$_T$] finds rationalization in the mechanisms of eq 12, 13, or 14 where X = ninhydrin.

$$\begin{aligned} \text{FlMeH}_T + X &\longrightarrow \text{FlMe}\cdot + X\cdot \\ X\cdot &\longrightarrow \text{products} \end{aligned} \quad (12)$$

$$\begin{aligned} \text{FlMeH}_T + X &\longrightarrow \text{FlMe}\cdot + X\cdot \\ \text{FlMeH}_T + X\cdot &\longrightarrow \text{FlMe}\cdot + \text{carbinol} \\ \hline 2\text{FlMeH}_T + X &\longrightarrow 2\text{FlMe}\cdot + \text{carbinol} \end{aligned} \quad (13)$$

$$\begin{aligned} \text{FlHMe} + X &\longrightarrow \text{FlMe}_{ox}^+ + \text{carbinol} \\ \text{FlHMe} + \text{FlMe}_{ox}^+ &\longrightarrow 2\text{FlMe}\cdot \\ \hline 2\text{FlHMe} + X &\longrightarrow 2\text{FlMe}\cdot + \text{carbinol} \end{aligned} \quad (14)$$

At pH 4.9 [0.1 M acetate buffer, μ = 0.5, solvent H_2O–EtOH 10% (v/v)] the reaction of ninhydrin with 2.17×10^{-4} M [FlMeH$_T$] provided the following yields of FlMe·.

[ninhydrin]	[radical]
$1 \times 10^{-3} M$	$2.2 \times 10^{-4} M$
$1 \times 10^{-4} M$	$0.6 \times 10^{-4} M$
$0.5 \times 10^{-4} M$	$0.7 \times 10^{-4} M$; $0.6 \times 10^{-4} M$

Table III. Values of Pseudo-First-Order Rate Constants (k_{obsd}) for Appearance of FlMe· in the Reaction of 1,5-Dihydro-3,5-dimethyllumiflavine (9×10^{-5} M) with Naphthoquinone [Solvent H_2O–CH_3CN, 20% (v/v); 30°; μ = 0.5 with KCl]

pH[a]	[NQ] $\times 10^3 M$	k_{obsd}, sec^{-1}
3.17	1	43
4.34	1	50
5.26	1	24
6.23	1	21
7.46	0.5	22
7.46	1	28,23
7.46	1.5	68
7.46	2.0	86
8.56	1	89

[a] pH held constant with 0.05 M buffer (acetate and phosphate).

These results establish that 1 mol of ninhydrin yields 1 mol of FlMe· and, therefore, that a direct one-electron transfer reaction occurs to yield a radical of ninhydrin and FlMe· (eq 12).

Reaction of 1,4-naphthoquinone (NQ) with 1,5-dihydro-3,5-dimethyllumiflavine was followed via stopped-flow as in the case of ninhydrin. The FlMe· radical once formed (580 nm) appears to react only very slowly, if at all, with excess NQ (no change in OD_∞ after 1 hr). The kinetic results are presented in Table III. A plot of the values of k_{obsd} for FlMe· formation at pH 7.46 vs. [NQ] is linear with slope (k_2') of $4.3 \times 10^4\ M^{-1}\ \text{sec}^{-1}$ establishing the reaction to be first order in NQ and first order in [FlMeH$_T$]. At 1×10^{-3} M NQ, k_{obsd} increases by only twofold on change of hydrogen ion activity by $\sim 10^{5.5}$. This may be explained by consideration of eq 15. The rate constant k_c ($\simeq 4 \times 10^4\ M^{-1}\ \text{sec}^{-1}$) pertains to the second-order electron transfers from both FlMeH and FlMe$^-$ to NQ which must be almost of equal facility.

$$\frac{d\text{FlMe}\cdot}{dt} = k_c([\text{FlMeH}] + [\text{FlMe}^-])[\text{NQ}] \quad (15)$$

The reaction of 1,5-dihydro-3,5-dimethyllumiflavine (2.31×10^{-4} M) with varying concentrations of naphthoquinone at pH 5.0 [0.1 M acetate buffer, μ = 0.5, and in H_2O–CH_3CN 27% (v/v)] provided two FlMe· species for each NQ reacted.

[NQ], M	[FlMe·]
1×10^{-3}	2.3×10^{-4}
1×10^{-4}	2.0×10^{-4}
0.5×10^{-4}	1.0×10^{-4}

Since the reactions were determined to be strictly first order in appearance of FlMe· the sequence of either eq 13 or 14 is suggested (X = NQ).

The reaction of benzoquinone (BQ) with 1,5-dihydro-3,5-dimethyllumiflavine was found to be biphasic, the first reaction reaching infinity at mixing time (4 msec). The flavine product present at completion of the first phase was shown to be FlMe· by manual determination of its spectra at 7 msec after mixing of reactants (Figure 4). The second phase of the reaction was found to involve the first-order conversion of FlMe· to FlMe$_{ox}^+$ [observable at pH 3.65 (545 nm) where FlMe$_{ox}^+$ exists only partially as its pseudo base] which went on in a much slower reaction ($t_{1/2} \simeq 13$ min with 0.05 M acetate buffer) to provide 3-methyllumiflavine.

In Table IV are listed the initial concentration of reactants along with the values of k_{obsd} for reaction of FlMe· with BQ. Consideration of Table IV reveals that k_{obsd} is independent of [FlMe·], [BQ], and pH.

The reaction of 1,5-dihydrolumiflavine-3-acetic acid

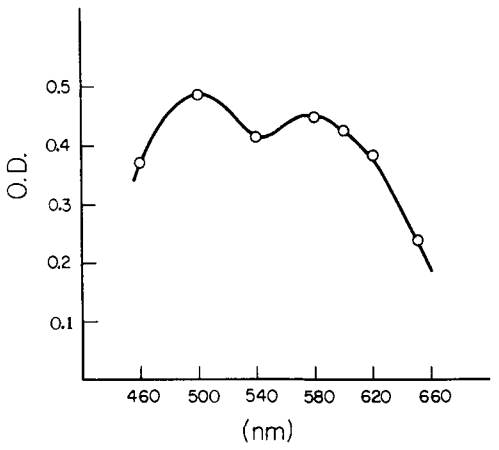


Figure 4. Manual scan of the spectra of a reaction solution of quinone and FlMeH + FlMe· at 7 msec after mixing. Maximal absorbances are those shown by $FlMe\cdot^-$.

Table IV. Pseudo-First-Order Rate Constants for Reaction of Benzoquinone [BQ] with the Radical Generated from 1,5-Dihydro-3,5-dimethyllumiflavine [Solvent $H_2O-C_2H_5OH$, 1–2% (v/v); 30°; μ = 0.5; Buffer 0.05 *M*]

Run[a]	pH	[FlHMe]	[BQ], *M*	k_{obsd}, sec^{-1}
A	5.50	9×10^{-5}	1×10^{-3}	4.2×10^{-1}
A	6.47	9×10^{-5}	1×10^{-3}	3.7×10^{-1}
C	7.01	3.47×10^{-5}	5×10^{-4}	4.0×10^{-1}
C	7.01	3.47×10^{-5}	1×10^{-3}	4.1×10^{-1}
C	7.01	3.47×10^{-5}	2×10^{-3}	3.9×10^{-1}
C	7.01	3.47×10^{-5}	4×10^{-3}	3.9×10^{-1}
C	7.01	6.95×10^{-5}	5×10^{-4}	3.5×10^{-1}
C	7.01	6.95×10^{-5}	1×10^{-3}	4.1×10^{-1}
C	7.01	6.95×10^{-5}	2×10^{-3}	4.4×10^{-1}
C	7.01	6.95×10^{-5}	4×10^{-3}	4.2×10^{-1}
B	7.01	9×10^{-5}	5×10^{-4}	4.5×10^{-1}
B	7.01	9×10^{-5}	1×10^{-3}	5.2×10^{-1}
B	7.01	9×10^{-5}	1.5×10^{-3}	5.2×10^{-1}
B	7.01	9×10^{-5}	2×10^{-3}	5.3×10^{-1}
A	7.62	9×10^{-5}	1×10^{-3}	2.6×10^{-1}
A	8.33	9×10^{-5}	1×10^{-3}	3.0×10^{-1}
A	8.60	9×10^{-5}	1×10^{-3}	2.1×10^{-1}

[a] Indicating reactions carried out with different stock solutions of FlMeH.

(1,5-FlH_2) with Malachite Green (MG) was followed by observing the formation of lumiflavine-3-acetic acid (Fl_{ox}) at 443 nm. The 4,4′-bis(dimethylaminodiphenyl) cation was generated from the corresponding carbinol at pH 5.06. It has been shown previously that the maximum concentration of the carbonium ion species is obtained at this pH.[14] In these experiments, the concentration of FlH_2 was 2.2×10^{-5} *M* and MG was 1.9×10^{-4}–5.8×10^{-4} *M* so that the appearance of Fl_{ox} followed pseudo-first-order kinetics. A plot of k_{obsd} vs. [MG] exhibited a decided upward curvature indicating that the reaction is greater than first order in [MG]. When k_{obsd} is plotted vs. $[MG]^2$, a linear relationship is obtained (Figure 5) from which the value of $k_3' = 2.59 \times 10^5\ M^2\ sec^{-1}$ may be obtained (eq 16).

$$d[Fl_{ox}]/dt = k_3'[FlH_2][MG]^2$$
$$k_{obsd} = k_3'[MG]^2 \qquad (16)$$

Discussion

We have previously proposed that the dihydroflavine (i.e., FlH_2 and FlH^-) reduction of carbonyl compounds occurs through one-electron + 1H· (eq 4) or one-electron + one-electron (eq 5) mechanisms.[6c] Our chief concern in the present study has been to determine if a 1,5-dihydroflavine

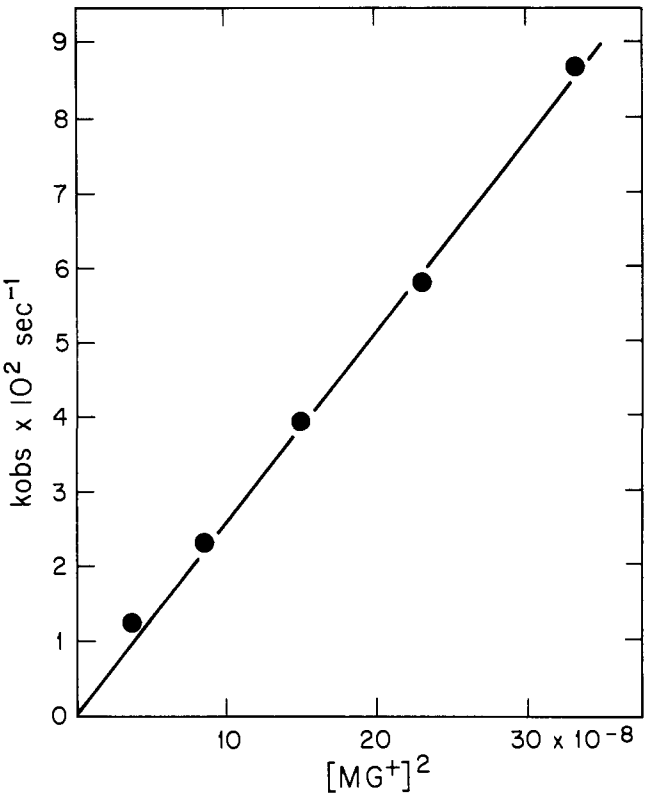
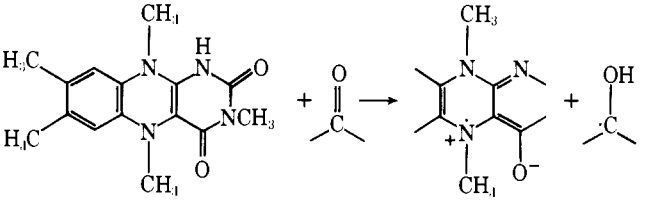


Figure 5. Plot of the pseudo-first-order rate constant for appearance of Fl_{ox} (on reaction of FlH_2 + FlH^- with Malachite Green) vs. the square of the concentration of Malachite Green.

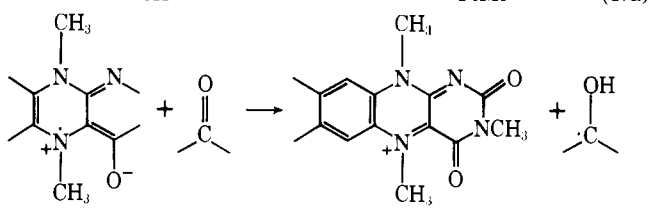
could be shown to undergo one-electron transfer reactions to carbonyl reagents. Because (N)5-methyl substitution greatly stabilizes the flavine radical, we have chosen 1,5-dihydro-3,5-dimethyllumiflavine (i.e., FlMeH and $FlMe^-$) as the potential one-electron donor.

In aqueous solution in the middle pH range the N(5)-flavine radical (i.e., FlMe·) is formed on reaction of (FlMeH + $FlMe^-$) with $CH_2COCO_2C_2H_5$, C_6H_5COCHO, Cl_3CHO, barbituric acid, ninhydrin, benzoquinone, and naphthoquinone. These same substrates are reduced by FlH_2 + FlH^- to yield oxidized flavine (Fl_{ox}) and the corresponding carbinol.[10,12]

The following arguments may be made in support of the one-electron transfer reactions of eq 17. As shown, the reac-



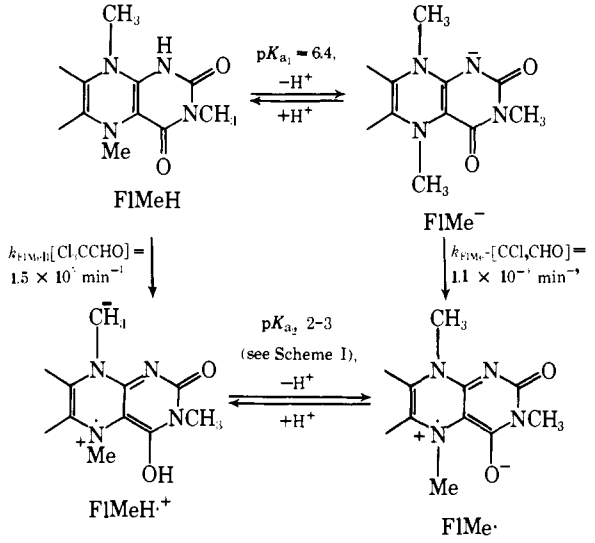
(17a)



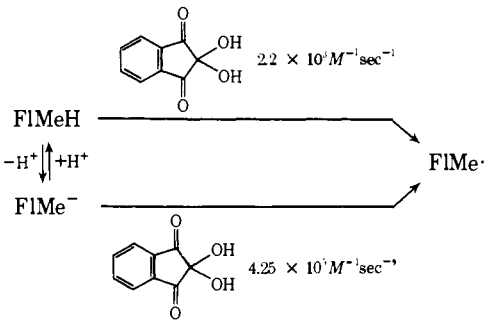
(17b)

tion of chloral with (FlMeH + $FlMe^-$) yields FlMe·. Once formed, the rate of disappearance of FlMe· from solution is independent of chloral concentration. This finding is in agreement with chloral acting as a one-electron acceptor

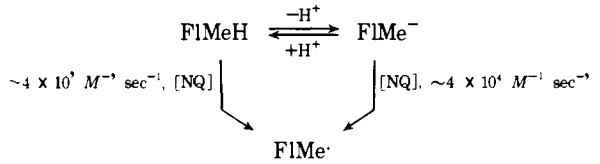
Scheme II. Chloral



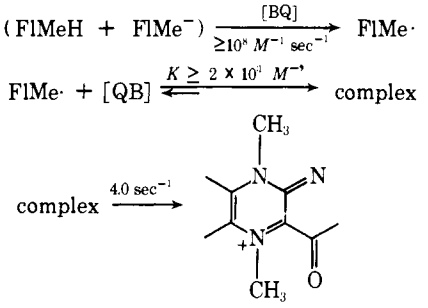
Scheme III. Ninhydrin



Scheme IV. Naphthoquinone



Scheme V. Benzoquinone



from FlMeH and $FlMe^-$ but not from FlMe·. An alternate possibility would be that chloral is reduced by FlMeH in a two-electron process and that the $FlMe_{ox}$ so formed reacts with unreacted FlMeH to yield 2FlMe· (eq 1). However, the two-electron reduction product of chloral is 2′,2′,2′-trichloroethanol and this is not formed on reaction of chloral with FlMeH and $FlMe^-$. Thus, the two-electron reduction mechanism is highly unlikely. It should be noted that 1,5-dihydrolumiflavine-3-acetic acid (FlH_2) has been reported to reduce chloral to trichloroethanol.[11] 1,5-Dihydro-*N*(5)-alkylflavines cannot undergo the H· transfer step of eq 4. In addition, ninhydrin reacts with FlMeH to yield 1 mol of FlMe· species per each mole of ninhydrin. This clearly supports only a one-electron transfer process yielding FlMe· and ninhydrin radicals. The radicals of ninhydrin are known as stable species.[15] Benzoquinone converts FlMeH to FlMe· in a reaction whose second-order rate constant ($\geq 10^8\ M^{-1}\ sec^{-1}$) exceeds that for comproportionation (eq 1). Again a one-electron transfer must be involved.

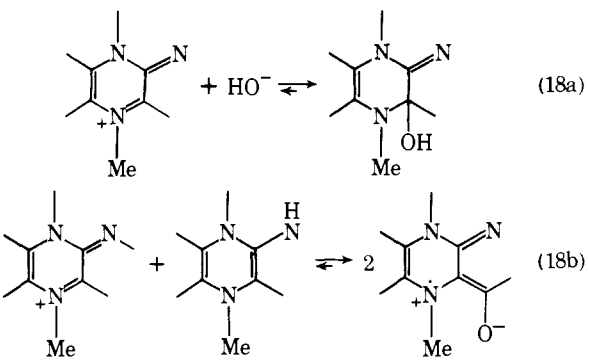
The kinetic studies described in the Results section are in accord with the following pathways for one-electron transfer (Schemes II-V). The ratio of the rate constants for (FlH_2 + FlH^-) reduction of chloral[10] and for reaction of (FlMeH + $FlMe^-$) with chloral are

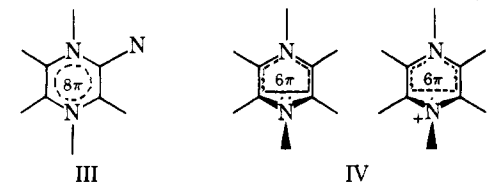
$$k_{FlH_2}/k_{FlMeH} = 4.7 \times 10^2$$

$$k_{FlH^-}/k_{FlMe^-} = 3.23 \times 10^2$$

Therefore, one-electron transfer from 1,5-dihydro-*N*(5)-methylflavine species to chloral occurs at rates which are over 10^2 slower than that for reduction of chloral by 1,5-dihydroflavine. For both we propose initial one-electron transfer. If the first step in reduction by (FlH_2 + FlH^-) is formation of FlH· (eq 4), then it might be anticipated that the rate constants for (FlMeH + $FlMe^-$) would exceed those for (FlH_2 + FlH^-) because the stability of product FlMe· exceeds that of FlH·. This result requires comment.

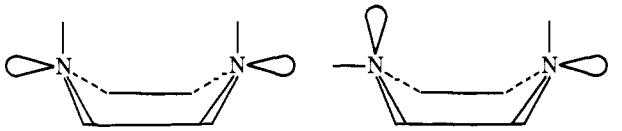
In comparing the comproportionation reaction for 1,5-dihydroflavine (eq 1) with that for the *N*(5)-methyl analog, it is obvious that the equilibrium in the direction of radical formation is greatly favored on *N*(5)-methyl substitution. Although the comproportionation reaction for *N*(5)-flavines includes the very unfavorable pseudo-base equilibrium (eq 18)[12] the radical species is still highly favored. The reason for N(5)-methylation bringing about a great stabilization of the flavine radical, relative to reduced flavine, has not previously been considered. The enhancement of radical stability accompanying N(5)-alkylation may be due to an increase in free energy content of (FlMeH + $FlMe^-$) as compared to (FlH_2 + FlH^-) and/or to a decreased free energy content of FlMe· as compared to FlH·. A search for an explanation based on the acid-base properties of FlMeH ⇄ $FlMe^-$ + H^+ and FlH_2 ⇄ FlH^- + H^+ is not rewarding since the two systems are quite similar in this regard. Conceptually, the central 1,4-dihydropyrazine system of the 1,5-dihydroflavine molecule is capable of existing in a destabilized planar antiaromatic state (III). In addition, both the dihydroflavine and radical states can be conceived as existing in stabilized half-chair homoaromatic conformations (IV). The enhanced stability of FlMe· as compared to FlH· would find explanation if it were assumed that (FlMeH + $FlMe^-$) possessed greater antiaromatic character than (FlH_2 + FlH^-) and FlMe· was more homoaromatic than FlH·. Structure III should be favored by 1,5-axi-





III IV

al,axial repulsion and IV by peri interaction of the methyl substituent with the substituents in the 4 and 6 positions. The possession of an 8π available electron system in the 1,4-dihydropyrazine moiety of the dihydroflavine ring does confer upon the latter *potential* homo- and antiaromaticity.[16] Streitwieser,[17] employing molecular orbital calculations, has predicted the thermodynamic destabilization brought about by placing the last two electrons of 1,4-dihydropyrazine in antibonding orbitals. However, the degree of antiaromaticity must depend critically upon the conformation of the dihydropyrazine ring which controls the ability of the nitrogen lone pairs to interact as part of a π system. It has been suggested that the degree of antiaromaticity in 1,4-dialkyldihydropyrazines may be controlled by the substituents on the pyrazine ring.[18] It has also been suggested[19] that forced flattening of the 1,5-dihydroflavine ring structure at an enzyme active site should bring about a destabilization due to the increase in antiaromaticity. The degree of planarity required to obtain a significant thermodynamic destabilization due to antiaromatic character is so critical that in our view this possible contributing factor may be dismissed. The steric constraint upon the system is not so great to dismiss a homoaromatic contribution but the energies involved are probably not great. Of greater concern should be the degree of lone-pair lone-pair splitting of the electrons on the N(5) and N(10) nitrogen of dihydroflavine.[20] This through-space and σ-bond interaction should result in a destabilization of the dihydropyrazine moiety of the dihydroflavine which is relieved on radical formation. Though the causitive effects are different, both antiaromaticity and lone-pair lone-pair splitting favor radical formation. A suitable model for lone-pair lone-pair splitting triethylenediamine which exhibits a reversible one-electron oxidation to yield a relatively long-lived (seconds) radical cation.[21] Hoffmann's[20b] extended Hückel calculations dealing with the lone-pair lone-pair splitting between the energies of the nitrogen lone-pair orbitals have been experimentally verified by Heilbronner and Muszhat.[22] Accompanying aminium cation (i.e., $-{}^{+}\dot{N}<$) formation there is a considerable flattening of the nitrogen.[23] This flattening is seen, to some degree, even in the case of the cation radicals of caged ethylenediamines and may be expected for the N(5) position of the dihydroflavine radical. Lone-pair lone-pair splitting (and radical formation) is favored, for ethylenediamines, when the electron pairs reside in eq,eq orbitals as compared to ax,eq orbitals. It is known[24] that the dihydroflavine conformation resembles a butterfly so that the 1,4-dihydropyrazine moiety of the flavine may be in the conformations of V and VI, respectively. Due to peri interaction with the



V VI

4(CO) and 6(H), the $N(5)$-methyl group of (FlMeH + $FlMe^-$) should be axial[24] and the conformation of V favored. The preferred conformation of FlH_2 + FlH^- would be that of VI since the peri interactions are minimal. The result is that FlMeH + $FlMe^-$ is of greater free energy content than is FlH_2 + FlH^- due to nonbonded electron pair interactions while FlMe· is of less free energy content than FlH· as a result of the greater planarity of the $N(5)$-aminium cation. The latter feature should be enhanced by 5,10-ax,ax repulsion. The lessened reactivity of (FlMeH + $FlMe^-$) with chloral may be due to steric hindrance of approach of the oxidant due to $N(5)$-alkyl substitution (VIII).

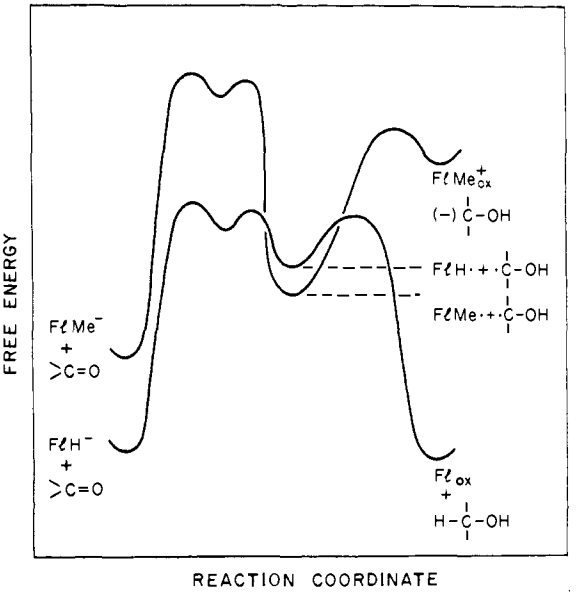
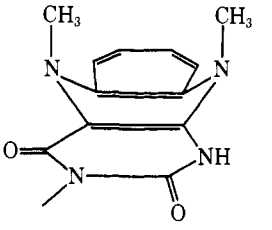


Figure 6. Reaction coordinate diagrams for one-electron transfer from FlH^- and $FlMe^-$ to a carbonyl group.



VII

With this line of reasoning, the rate of electron transfer from (FlMeH + $FlMe^-$) relative to (FlH_2 + FlH^-) is decreased but FlMe· is more stable than FlH·. In the case of dihydroflavine reduction of Ch the intermediate state ⁞ FlH· >ĊOH ⁞ may yield Fl_{ox} + HC(OH)< by H· transfer. This is not possible in the case of ⁞ FlMe· >ĊOH ⁞ and since the species (-)C(OH)< probably exceeds in free energy content that of >ĊOH, it is not formed by one-electron transfer but decomposes by nonproductive path(s). These arguments are summarized in the reaction coordinate diagrams of Figure 6.

If one compares the rate constants associated with one-electron transfer to chloral and to ninhydrin, it is seen that for both cases $FlMe^-$ is $\sim 10^2$ more reactive than FlMeH. However, the second-order rate constants for reaction of 1,4-naphthoquinone with $FlMe^-$ and FlMeH are comparable and about equal to that for reaction of ninhydrin with $FlMe^-$. The leveling effect with NQ may be due to preequilibrium complex formation which occurs preferably with the slower reacting FlMeH species.

Gibian and Rynd[25] found that the reduction of benzoquinone by 1,5-dihydroriboflavine occurred at a rate exceeding that of the mixing time of their stopped-flow spectrometer. From their determined mixing time, they were able to conclude that any second-order rate process would be associated with a rate constant greater than $3.3 \times 10^7\ M^{-1}\ \text{sec}^{-1}$. Our value of $k \geq 10^8\ M^{-1}\ \text{sec}^{-1}$ has been estimated in the same manner. Nothing should be made of the differences in these constants since neither can be determined directly. With (FlMeH + $FlMe^-$) we find the initial product to be FlMe· which in a second step with quinone yields $FlMe_{ox}^+$. With (FlH_2 + FlH^-) Gibian and Rynd report no observable radical formation in the production of Fl_{ox}. They point out that a very reasonable mechanism would involve elec-

tron transfer within an initially formed donor-acceptor complex. It is known that hydroquinones and oxidized flavines form stable 1:1 complexes in acidic solution.[26] In the reaction of Fl_{ox} with dihydropyridines, there is a linear relationship of log k vs. the log of the equilibrium constant for complexing of Fl_{ox} by tryptophan and β-resorcylic acid. This finding led to the proposal of preequilibrium face to face complexing.[27] This suggestion has been supported by the finding of saturation kinetics in the reduction of lumiflavine by NADH.[28] The difference in the kinetics of reaction of 1,5-dihydroflavine and 1,5-dihydro-N(5)-methylflavine with benzoquinone must reside in the existence of ⟦FlH·BQ·⟧ at steady state whereas in the former instance FlMe· is formed as a distinct intermediate. On the basis of the preceding discussion, this finding is not at all unexpected.

The inability to detect a dependence of the rate constant for reaction of FlMe· with benzoquinone on the concentration of either reactant can be explained (Scheme V) with the assumption of the equilibrium formation of an intermediate if it is assumed that the product of the equilibrium constant for the formation of intermediate and [benzoquinone] > 1. This requires the equilibrium constant for complexing of FlMe· and benzoquinone $\geq 2 \times 10^3$ M. Complexing constants of this order of magnitude are known for Fl_{ox}[29] and other systems[30] but exceed by 10^1–10^2 those generally seen in aqueous solution.[31]

The reduction of 5,10-methylenetetrahydrofolate (XI) to 5-methyltetrahydrofolate (X) by 5,10-methylenetetrahydrofolate reductase involves FADH as reducing agent.[32,33] As shown by the borohydride reduction of IX → X the former exists to some extent as the iminium cation.[34] The stable carbocation Malachite Green (MG) is reduced by N-benzyldihydronicotinamide via hydrogen transfer.[35] Because of our interest in one-electron transfer reactions from dihydroflavines and the flavine mediated enzymatic and borohydride reduction of IX, it was of interest to determine if 1,5-dihydroflavine would transfer one electron or H^- to a carbocation in a model system. The reduction of MG by 1,5-dihydrolumiflavine-3-acetic acid was found to be first order in [FlH_2] and second order in [MG] with an apparent third-order rate constant (k_3 of eq 16) equal to 2.6×10^5 M^2 sec^{-1}. This finding is in keeping with a radical mechanism (eq 19) in which the rate of one-electron transfer from

$$\begin{aligned} FlH_2 + -\overset{|}{\underset{|}{C}}{}^{+} &\rightleftharpoons FlH\cdot + -\overset{|}{\underset{|}{C}}\cdot + H^+ \\ FlH\cdot + -\overset{|}{\underset{|}{C}}{}^{+} &\rightleftharpoons Fl_{ox} + -\overset{|}{\underset{|}{C}}\cdot + H^+ \\ 2\text{—}\overset{|}{\underset{|}{C}}\cdot &\longrightarrow \text{product} \end{aligned} \qquad (19)$$

FlH· to $>C^+$- exceeds that for H· transfer (eq 20). At-

$$FlH_2 + -C^+ \rightleftharpoons FlH_2\cdot^+ + -C\cdot \longrightarrow Fl_{ox} + -CH + H^+ \qquad (20)$$

tempts to examine the reduction of X by FlH_2 were not successful due to the latter's hydrolytic instability. These results establish that one-electron transfer from an unmodified dihydroflavine to a carbon compound can be facile. One-electron reduction of resonance-stabilized carbonium ions is a well-established phenomenon (for recent examples, see ref 36).

Experimental Section

Materials. **1,4-Benzoquinone** was recrystallized from water (charcoal) and sublimed: mp 114–115° (lit.[37] 115.7). **1,4-Naphthoquinone** was recrystallized from petroleum ether and sublimed: mp 121° (lit.[37] 124°). **Ninhydrin** was of analytical grade (Calbiochem). **4,4′-Bis(dimethylaminodiphenyl)carbinol** was synthesized according to the literature,[13] mp 103° (lit. 103–104°). **Lumiflavine-3-acetic acid** was a sample from a previous study.[6a] **3,5-Dimethyl-1,5-dihydrolumiflavine** was prepared as described by Ghisla et al.:[38] mp 245° (lit. 250°); λ_{max} (6 N HCl) 305, 285 nm. **3,5-Dimethyllumiflavine perchlorate** was prepared in the manner of the same authors: λ_{max} (pH 3.3) 545, 432 nm; λ_{max} (pH 2) 548, 430 nm, λ_{max} (pH 7) 354, 310 (sh), 262 nm. The zwitterionic **3,5-dimethyllumiflavine radical** was prepared after the method of Müller et al.:[39] black-blue crystals; λ_{max} (pH 7, anaerobic) 580, 502 nm.

Apparatus. Kinetic measurements were carried out in H_2O at 30°, μ = 0.5 (KCl), by either of two procedures: (a) in Thunberg cuvettes under argon (scrubbed of traces of O_2 by passing through a vanadous trap)[40] on a Gilford Model 2000 spectrophotometer equipped with four thermospacers through which water at 30° was circulated; or (b) in a Durrum-Gibson stopped-flow spectrophotometer which was contained in a nitrogen box and thermostated at 30°. All solutions were made up in doubly distilled water and were deoxygenated by bubbling argon.

Kinetics. A typical example of **the reaction of 3,5-dimethyl-1,5-dihydroflavine with chloral** follows. Into the upper compartment of a Thunberg cuvette was placed 0.5 ml of an appropriate aqueous solution of chloral (μ = 0.5) and into the lower portion of the cell there was placed 4.25 ml of a 0.1 M aqueous buffer solution. Both solutions were degassed by bubbling argon for 30 min. The cuvette was then sealed and transferred to an anaerobic box and opened, and 0.25 ml of the 3,5-dimethyl-1,5-dihydrolumiflavine stock solution was added (the aqueous stock solution was prepared and stored in the anaerobic box under N_2). The cuvette was again sealed and transferred from the anaerobic box to the spectrophotometer. After temperature equilibration (15 min) the reaction was initiated by mixing the contents of the Thunberg and the progress of the reaction monitored at 580 nm. The following buffers at 0.1 M were employed to maintain pH: CH_3CO_2K + CH_3COOH for pH 3.5–5.5; K_2HPO_4 + KH_2PO_4 for pH 5.5–7.5; boric acid and KOH above pH 7.5.

Reactions with Benzoquinone, Naphthoquinone, and Ninhydrin. Stock solutions (prepared daily) 0.1 M in substrate were made up using argon degassed solvent in an N_2 box (ethanol was the solvent for benzoquinone and ninhydrin, and acetonitrile was distilled under N_2 and stored over molecular sieves for naphthoquinone). Stock solutions of 1,5-dihydro-3,5-dimethyllumiflavine were made up in aqueous 0.005 N KOH (μ = 0.5 with KCl) for benzoquinone and ninhydrin experiments and in H_2O–CH_3CN (20% v/v) solvent 0.005 N in KOH (μ = 0.5 with KCl) for naphthoquinone experiments. The dihydroflavine solutions and the appropriate buffer were degassed for 2 hr by bubbling argon. The storage syringes of the stopped-flow spectrophotometer (sealed in a N_2 atmosphere) were then charged with the dihydroflavine solution and with the buffer solution (10 ml) to which 0.2 ml of the stock solution of substrate had been added. The kinetic determinations were then initiated.

Reaction of Malachite Green with lumiflavine-3-acetic acid was carried out in water–acetonitrile solution (5:3, v/v) at μ = 0.3 and at 30°. A typical kinetic experiment was carried out in the following manner. Into one compartment of a Thunberg cuvette there was placed 0.05 ml of a 2.2×10^{-3} M solution of the flavine and 0.45 ml of a 0.1 M acetate buffer containing 10^{-3} M in EDTA. Into the second compartment there was added 4.5 ml of 0.1 M acetate buffer containing the desired concentration of MG. Both compartments of the Thunberg cuvette were degassed by bubbling vanadous ion scrubbed argon prehumidified with acetonitrile–H_2O (5:3, v/v) for 30 min. The cuvette was then closed and the flavine in the upper compartment photoreduced by irradiation with a 100-W lamp bulb for 20 min. After equilibration at 30° for 15 min, the reaction was initiated by mixing the contents of the upper and lower compartment of the Thunberg cuvette and the course of the reaction followed at 443 nm (λ_{max} of oxidized flavine).

Comproportionation Experiments Were Carried Out as Follows. Into one of two side arms of a Thunberg cuvette was placed a crystal of 1,5-dimethyllumiflavinium perchlorate and into the second side arm there was added 0.125 ml of the stock solution of 1,5-dihydro-3,5-dimethyllumiflavine. Into the bottom of the cuvette was placed 4.875 ml of buffer (0.1 M phosphate, μ = 0.5). These oper-

ations were carried out in a N_2 atmosphere box. The two solutions were degassed with argon and the Thunberg was closed and removed from the box. The buffer solution was employed to dissolve the crystal of flavinium perchlorate when the total contents of the cuvette were mixed and the absorbance of the radical was monitored at 580 nm.

Analyses for 2',2',2'-trichloroethanol in the reaction of 1,5-dihydro-3,5-dimethyllumiflavine with chloral were carried out in the following manner. (a) Under an N_2 atmosphere (N_2 box) 20 ml of an H_2O-CH_3CN solution [50% (v/v), 0.1 *M* in phosphate buffer (pH 7.0), μ = 0.5 with KCl] was degassed by bubbling argon for 6 hr when 15 mg (5×10^{-5} mol) of 3,5-dimethyl-1,5-dihydrolumiflavine and chloral hydrate (0.5 g, 3×10^{-3} mol) was added. The reaction mixture was kept in the N_2 box for 3 days when it had turned deep blue (FlMe·). To the reaction mixture was added 10 ml of H_2O and the solution extracted five times with 5-ml aliquots of CH_2Cl_2. The CH_2Cl_2 layers were combined and dried over anhydrous Na_2SO_4 and the solvent was removed by distillation (42°). The remaining volume, 0.5 ml, was subject to GLC analysis (columns 20% Apiezon L on Chromosorb W, 5 ft, 90°). (b) The exact procedure as in (a) was followed but employing 5 ml of CH_3CN as reaction media. (c) The procedure of (a) was employed using an EtOH-H_2O 20% (v/v) reaction media containing 85.5 mg (5×10^{-4} mol) of diphenylamine as an H· donor.

In no case was trichloroethanol detected. Procedures a, b, and c were repeated but using authentic 2',2',2'-trichloroethanol in place of chloral hydrate. In each case the trichloroethanol could be recovered as indicated by GLC analysis.

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