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The Chemistry of a 1,5-Diblocked Flavin. 1. Interconversion of the Reduced, Radical, and Oxidized Forms of 1,10-Ethano-5-ethyllumiflavin

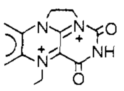
Gert Eberlein and Thomas C. Bruice*

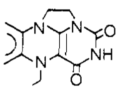
Contribution from the Department of Chemistry, University of California at Santa Barbara, Santa Barbara, California 93106. *Received February 7, 1983*

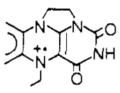
Abstract: The synthesis and conversions of the oxidized, radical, and reduced forms of 1,10-ethano-5-ethyllumiflavin are described. 1,10-Ethano-5-ethyl-1,5-dihydrolumiflavin ($\mathbf{2}_{red}$) is protonated in strong acid at both the N^1- ($pK_a = -6.35$) and N^5-positions ($pK_a = 1.71$) to provide $\mathbf{2}_{red}H_2^{2+}$. The N^5 pK_a and N^3 pK_a (10.43) are comparable to those of other 1,5-dihydroflavins. This is also true for the proton dissociation from N^3 to yield $\mathbf{2}_{red}^-$ ($pK_a = 10.43$). Oxidation by O_2 converts $\mathbf{2}_{red}$ to 1,10-ethano-5-ethyllumiflavin cation radical ($\mathbf{2}_{rad}$). The pK_a for proton dissociation from N^3 to $\mathbf{2}_{rad}$ is 7.92. The spectrum of $\mathbf{2}_{rad}$ establishes that the tautomeric structure of flavin radical cation is identical with that of 1,5-dihydroflavins rather than the previously supposed tautomeric structure with hydroxyl rather than carbonyl at C^4. The disproportionation of $\mathbf{2}_{rad}$, in the H_0 range, is associated with a pseudo pK_a of -7.07 to yield $\mathbf{2}_{red}H_2^{2+}$ and the oxidized species 1,10-ethano-5-ethyllumiflavin dication ($\mathbf{2}_{ox}$) below $H_0 = -7$. The kinetic deuterium isotope effect and product isolation studies on the disappearance of $\mathbf{2}_{rad}$ species in the pH range establish the mechanism to be initiated by a disproportionation and to differ from the mechanism for reaction of N^5-methyllumiflavin radical. These results are discussed. The quinoid dication $\mathbf{2}_{ox}$ may be obtained by oxidation of $\mathbf{2}_{red}H_2^{2+}$ with O_2 in H_2SO_4/H_2O (below $H_0 = -7.07$) or by oxidation of $\mathbf{2}_{red}H^+$ in tetrafluoroboric acid/diethyl ether with Br_2. Corroboration of the proposal that protonation of oxidized flavins occurs preferentially at N^1 rather than N^5 was obtained by a comparison of the pK_a values of various flavins to that of protonated 1,10-ethano-5-ethyllumiflavin. It is possible, for lumiflavin, to calculate the heretofore unknown microscopic pK_a values for protonation of N^1 and N^5 as 0.5 and -3.8, respectively.

Considerable contributions have been made to our knowledge of the mechanism of flavoenzymes by the study of the oxidation and reduction of appropriate substrates by oxidized and 1,5-reduced flavins (respectively) and the reaction of reduced flavins with molecular oxygen.[1] In these investigations N^5-blocked flavins have played a crucial role.[2] The advantages of these analogues arise from the increased redox potential of N^5-blocked flavin and flavin radical and from the stabilization of 4a-covalent intermediates, notably 4a-hydroperoxyflavins.

The present investigation describes the chemical features of the 1,5-diblocked flavins: 1,10-ethano-5-ethyllumiflavin ($\mathbf{2}_{ox}$), 1,5-dihydro-1,10-ethano-5-ethyllumiflavin ($\mathbf{2}_{red}$), and the radical 1,10-ethano-5-ethyllumiflavinylium ($\mathbf{2}_{rad}$). Since $\mathbf{2}_{ox}$ possesses positive charges at the termini of a quinoid structure, it is expected to possess a high free energy content and very positive redox potential. In contrast $\mathbf{2}_{red}$ and $\mathbf{2}_{rad}$ should resemble more closely flavin and N^5-blocked flavin. The value of 1,10-ethano-5-ethyllumiflavins in the elucidation of the mechanism of flavin reactions revolve around the high redox potential for one-electron reduction of $\mathbf{2}_{ox}$ and the stability of the radical species to disproportionation.[3,4] This provides flavin analogues that better mimic the potentials of enzyme-bound flavin cofactors, where stabilization of radical and reduced species is often observed.


2_{ox}


2_{red}


2_{rad}

This paper presents the basic solution chemistry and spectral properties of $\mathbf{2}_{ox}$, $\mathbf{2}_{red}$, and $\mathbf{2}_{rad}$. The following paper[5] presents electrochemical investigations of this (and other flavin) system(s) and the mechanism of the $^3O_2$ oxidation of $\mathbf{2}_{red}$ (and other dihydroflavins).

Experimental Section

Materials. All organic compounds were obtained from Aldrich. Ten percent Pd/C was obtained from Metheson Coleman and Bell. Buffer components were purchased from Mallinckrodt and used without further purification. Water used for kinetic studies was deionized and glass-distilled. Methanol, chloroform, and buffer solutions were purged with N_2 for 30 h/L of liquid. Trifluoroacetic acid was deoxygenated by seven cycles of freeze–pump–thaw employing a degassing vacuum <0.03 torr followed by hydrogenation over Pd/C and distilled in an inert atmosphere glovebox ($[O_2] < 10^{-8}$ M). Sulfuric acid was deoxygenated by pumping at 0.02 torr for 30 h while being agitated in an ultrasonic bath. All deoxygenated solutions and reagents were stored and handled in an inert atmosphere glovebox. Material used for column chromatography was silica gel 60, size 0.063–0.2 mm. For thin-layer chromatography, precoated thin-layer plates, silica gel 60 with fluorescent indicator F_{254}, layer thickness 0.2 mm (Merck Darmstadt), were used. **1,10-Ethanolumiflavinium perchlorate** ($\mathbf{1}_{ox}^+$) was synthesized by the procedures published by Knappe.[6] **1,10-Ethano-5-ethyl-1,5-dihydrolumiflavin** ($\mathbf{2}_{red}$) was obtained from $\mathbf{1}_{ox}^+$ by modification of a previous procedure[2n] for the reductive alkylation of flavins. A 50-mL round-bottom flask was loaded with 1 g (2.72 mmol) of $\mathbf{1}_{ox}^+$, 1 g of 5% Pd/charcoal, 35 mL of 100% ethanol, 0.5 mL of concentrated sulfuric acid, and 5 mL of acetaldehyde (99%). The flask was capped by a secured rubber septum containing two Teflon needles. One needle served as an H_2 inlet and the second as an outlet. The outlet was connected to an Hg pressure gauge. While the reaction mixture was agitated with an ultrasonic bath, it was purged free of O_2 by the free flow of H_2 for about 10 min. The outlet was then placed under 150–200 torr while ultrasonic agitation was continued (50 °C) for 24 h. The reaction was followed by diluting 0.01-mL aliquots with 3 mL of air-saturated sulfate buffer (pH 2.2). After 10 min the spectrum was obtained. The reaction is complete when the ratio of absorbances at 350

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[1] (a) Bruice, T. C. *Prog. Bioorg. Chem.* **1976**, *4*, 1. (b) Hemmerich, P. *Prog. Chem. Org. Nat. Prod.* **1976**, *33*, 29. (c) Bruice, T. C. *Acc. Chem. Res.* **1980**, *13*, 256.

[2] (a) Kemal, C.; Bruice, T. C. *Proc. Natl. Acad. Sci. U.S.A.* **1976**, *73*, 995. (b) Ibid. *J. Am. Chem. Soc.* **1976**, *98*, 3955. (c) Bruice, T. C.; Taulane, J. B. *Ibid.* **1976**, *98*, 7769. (d) Kemal, C.; Chan, W. T.; Bruice, T. C. *Proc. Natl. Acad. Sci. U.S.A.* **1977**, *74*, 405. (e) Chan, W. T.; Bruice, T. C. *J. Am. Chem. Soc.* **1977**, *99*, 2387. (f) Kemal, C.; Bruice, T. C. *Ibid.* **1977**, *99*, 7064. (g) Kemal, C.; Chan, T. W.; Bruice, T. C. *Ibid.* **1977**, *99*, 7272. (h) Chan, T. W.; Bruice, T. C. *Ibid.* **1977**, *99*, 7287. (i) Kemal, C.; Bruice, T. C. *Ibid.* **1979**, *101*, 1635. (j) Miller, A.; Bruice, T. C. *J. Chem. Soc., Chem. Commun.* **1979**, 896. (k) Bruice, T. C.; Miller, A. *Ibid.* **1980**, 693. (l) Muto, S.; Bruice, T. C. *J. Am. Chem. Soc.* **1980**, *102*, 4472. (m) Iwata, M.; Bruice, T. C.; Cauell, H. L.; Glusker, J. P. *Ibid.* **1980**, *102*, 5036. (n) Ball, S.; Bruice, T. C. *Ibid.* **1980**, *102*, 6498. (o) Muto, S.; Bruice, T. C. *Ibid.* **1980**, *102*, 7559. (p) Shepherd, P. T.; Bruice, T. C. *Ibid.* **1980**, *102*, 7774. (q) Nanni, E. J.; Sawyer, D.; Ball, S. S.; Bruice, T. C. *Ibid.* **1981**, *103*, 2797. (r) Ball, S. S.; Bruice, T. C. *Ibid.* **1981**, *103*, 5494. (s) Wessiak, A.; Bruice, T. C. *Ibid.* **1981**, *103*, 6996. (t) Muto, S.; Bruice, T. C. *Ibid.* **1982**, *104*, 2284.

[3] $\mathbf{2}_{rad}$ has been employed in a recent study of one-electron and two-electron reduction of flavin radical by 1,4-dihydro-*N*-alkylnicotinamides (Powell, M. F.; Bruice, T. C. *J. Am. Chem. Soc.* **1983**, *105*, 1014).

[4] Portions of the work described in this and the following paper have been discussed in a preliminary manner (Eberlein, G.; Bruice, T. C. *J. Am. Chem. Soc.* **1982**, *104*, 1449).

[5] Eberlein, G.; Bruice, T. C. *Ibid.* **1982**, *104*, 1449.

[6] Knappe, W. R. *Chem. Ber.* **1975**, *108*, 2422.

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and 492 nm is ~1.26. At completion of the reductive alkylation the solution was filtered, and the precipitate was washed with 100–150 mL of ethanol in small portions. The filtrate and washings were discarded. The filter cake was next extracted 6 times with 50-mL portions of hot methanol/chloroform (80/20). Each extraction lasted 3–5 min and solvent was removed by filtration while still hot. The combined solvent fractions were refrigerated (−21 °C) for 16 h and the bright yellow crystals of **2**$_{red}$ collected by filtration. After these were washed 3 times with 0.5–1.0-mL aliquots of methanol and 5 mL of diethyl ether, the yield of product was 302 mg. Combination of the mother liquor from the recrystallization and washing followed by concentration to 15 mL provided a second crop of product (138 mg). A third crop of product was obtained by combining the filtrate from the reaction solution with all washings and mother liquors and reducing this by evaporation to a syrup. To the syrup was then added 20 mL of water containing 5 g of sodium acetate and 100 mg of sodium dithionite. The resultant solution was immediately extracted with chloroform, and the chloroform layer was dried over anhydrous magnesium sulfate and evaporated to dryness. Repeated recrystallization of the residue from methanol/chloroform gave 120 mg of **2**$_{red}$ (total yield 560 mg, 57% of theory): mp 240 °C; IR (KBr disk) $\bar{\nu}_{N^3-H}$ 3020, $\bar{\nu}_{C^4=O}$ 1740, $\bar{\nu}_{C^2=O}$ 1680, $\bar{\nu}_{Ar}$ 1510 cm^{-1}; NMR (CF_3COOH, Me_4Si) δ 1.00 (3 H, t, NCH_2CH_3), 2.13 (6 H, s, two aryl-CH_3), 3.60 (2 H, q, NCH_2CH_3), 4.20 (4 H, m, NCH_2CH_2N), 4.77 (1 H, s, NH), 6.72 (1 H, s, aryl-H), 7.19 (1 H, s, aryl-H); MS (200 °C) *m/e* (rel intensity) 298 (60, M^+), 269 (100, $M^+ - CH_2CH_3$), 226 (17, $M^+ - CH_2CH_3$, CONH), 198 (60, $M^+ - CH_2CH_3$, CO–NH–CO), 157 (17, $M^+ - CH_2CH_3$, CO–NH–CO, $CHCH_2N$). Anal. Calcd for $C_{16}H_{18}N_4O_2 \cdot {}^1/_3H_2O$: C, 63.14; H, 6.18; N, 18.41. Found: C, 63.30; H, 6.20; N, 18.19. **1,10-Ethano-5-perdeuterioethyl-1,5-dihydrolumiflavin** was prepared as in the case of **2**$_{red}$ by using ethanol-d_1, acetaldehyde-d_4, sulfuric acid-d_2, and D_2 in place of H_2: MS (200 °C) *m/e* (rel intensity) 303 (51, M^+), 269 (100, $M^+ - CD_2CD_3$), 226 (15, $M^+ - CD_2CD_3$, CONH), 198 [53, $M^+ - CD_2CD_3$, $(CO)_2NH$], 157 [9, $M^+ - CD_2CD_3$, $(CO)_2NH$, $CHCH_2N$].

1,5-Ethano-5-ethyllumiflavinium difluoroborate [**2**$_{ox}(BF_4^-)_2$] was obtained by dissolving 10.7 mg of **2**$_{red}$ (36 μmol) in 0.5 mL of 50% tetrafluoroboric acid/diethyl ether complex. Bromine (0.02 mL; 390 μmol) was added and the solution was sonicated for 10 min (exact timing and concentrations are critical to obtain best results). Hydrobromic acid, excess bromine, and tetrafluoroboric acid were removed by washing with 10 mL of dry diethyl ether, and the mixture was sonicated for 30 s. The solution was separated in a centrifuge for 30 s and the ether solution discarded. The remaining syrup was washed with 10 mL of diethyl ether and 3 times with 5 mL of diethyl ether to yield the 1,10-ethano-5-ethyllumiflavinium dication tetrafluoroborate salt as a bright red powder that can be stored at −21 °C indefinitely. A suspension of the salt in the tetrafluoroboric acid/diethyl ether complex is stable at room temperature for several days: UV (concentrated H_2SO_4) 452 nm (ϵ = 23 000), 476 (38 800), 221 (25 000). **1,10-Ethano-5-ethyllumiflavinium radical perchlorate salt** (**2**$_{rad}$) was obtained by oxidizing 21 mg of **2**$_{red}$ (70 mmol) in 5 mL of trifluoroacetic acid with oxygen (125 torr) for ca. 30 h. Solvent was removed from the dark red solution with a stream of dry oxygen and the residue crystallized in good yield by ultrasonic agitation with 1.5 mL of 3 M perchloric acid for about 5 min. The dark red crystals were collected by filtration and washed with small portions of water, methanol, and diethyl ether: mp 245 °C with decomposition; IR (KBr disk) $\bar{\nu}_{C(2)=O}$ 1740, $\bar{\nu}_{C(4)=O}$ 1670, $\bar{\nu}_{aryl}$ 1506, $\bar{\nu}_{ClO_4}$ 1095 cm^{-1}; EPR (toluene, 5% MeOH) 3.257 G; MS *m/e* (rel intensity) 298 (0.6, M^+), 269 (100, $M^+ - CH_2CH_3$). Anal. Calcd for $C_{16}H_{18}ClN_4O_6 \cdot H_2O$: C, 46.22; H, 4.85; N, 13.47; Cl, 8.53%. Found: C, 45.88; H, 4.89; N, 13.37; Cl, 8.41%.

The reduction of 1,10-ethano-5-ethyl flavinylium cation (2$_{rad}$) by 1,5-dihydro-1,10-ethanoflavin (1$_{red}$) was confirmed as follows. A 0.068 mM solution of **1**$_{red}$ was prepared by the photoreduction of 3 mL of an oxygen-free solution of **1**$_{ox}^+$ (0.068 mM) at pH 2.2 (sulfate/bisulfate buffer, μ = 0.1; 9.7 mM in EDTA) with a 250-W tungsten lamp at a distance of 10 cm. The photocatalytic reduction was carried out in a cuvette stoppered with a rubber septum. When the reduction was complete (5 min), the solution was thermostated in the cell chamber of the spectrophotometer for 20 min. Then 0.2 mL of an oxygen-free solution of **2**$_{rad}$ (1.55 mM) in methanol was added through the septum by use of a gas-tight syringe. The UV–vis spectrum was taken immediately after mixing. The red color of the flavin radical disappeared immediately and the concentration of the 1,10-ethanoflavinium cation (**1**$_{ox}^+$) product determined at 370 nm (ϵ 16 900). **The kinetics for the decay of the radical 2$_{rad}$** were studied in the following manner. The appropriate buffer solution (3 mL) was injected into a serum-capped cuvette and the cuvette purged for 2 h with flowing nitrogen. The cuvette was thermostated in the spectrophotometer for 20 min, and there 0.1 mL of a 0.1 mM stock solution of **2**$_{rad}$ was injected through the serum cap with a gas syringe. After the solution was mixed, the disappearance of **2**$_{rad}$ was monitored at 492 nm. On virtual completion of reaction, the concentration of **2**$_{red}$ produced in the course of the reaction was determined by the injection of 0.25 mL of air-saturated acetic acid. This oxidizes (5 min) the product present, **2**$_{red}$, to **2**$_{rad}$ and [**2**$_{rad}$] so formed was determined at 492 nm.

The comproportionation of **2**$_{rad}$ was also studied on a preparative scale at alkaline pH under inert atmosphere. A total of 10 mg of **2**$_{rad}$ (as its trifluoroacetate salt) was added to 1 mL of 1.0 M ammonium hydroxide solution. After the disappearance of the dark red color (~30 s) the yellow green precipitate was collected by filtration and washed with five 1-mL portions of water and 1 mL of methanol. After the mixture was dried, there was obtained 4.77 mg (67%) of **2**$_{red}$. The filtrate from the reaction mixture was acidified with 1 mL of aerated 3 M perchloric acid and its spectrum scanned. The spectral examination revealed the presence of **1**$_{ox}^+$ as well as hydrolytic products of **1**$_{ox}^+$ (see Results).

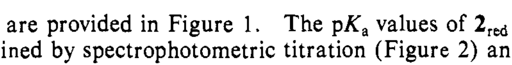
pK_a Determinations with 2$_{red}$ and 2$_{rad}$ were carried out as follows: pK_a values below zero were determined spectrally under anerobic conditions by employing the standard H_0 H_2SO_4/H_2O solutions of Paul and Long.[7] In practice, 0.1 mL of a 4 mM solution of **2**$_{red}$ or **2**$_{rad}$ in H_2SO_4 was added to 3 mL of the appropriate H_2SO_4/H_2O solution, and the absorbance values were determined after mixing (345 nm for **2**$_{red}$, 492 nm for **2**$_{rad}$, 542 nm for 5-ethyl-3-methyllumiflavin cation, 441 nm for 3-methyllumiflavin, and 448 nm for 1,10-ethanolumiflavinium cation). In the pH range, **2**$_{red}$ was titrated by use of a glass electrode and pH meter in a glovebox under an atmosphere of nitrogen. Spectral scans were performed at ~0.3 pH unit intervals and the pK_a was determined from the absorbance change at the appropriate wavelength (see Results). Because of the instability of the neutral (zwitterionic) species of **2**$_{rad}$, the above titration method could not be employed. Titration of **2**$_{rad}$ was carried out by injecting 0.1 mL of a 2.2 mM stock solution (MeOH) of **2**$_{rad}$ into 3 mL of buffer contained in a cuvette. The concentration of **2**$_{rad}$ present in the solution was determined at 492 nm. The ionization of **2**$_{rad}$ was monitored at 553 nm. The calculated ϵ_{553} was then plotted vs. pH and the data were fitted to a standard titration curve for dissociation of a single proton. Since the radical **2**$_{rad}$ is not reactive with $^3O_2$, there was no necessity to carry out the titration under an inert atmosphere. ***Kinetic*** measurements were conducted at 30 ± 0.2 °C in solutions having μ = 1.0 (KCl), and employing as buffers sulfate/bisulfate (pH 1–3), acetate/acetic acid (pH 3.5–5.5), monobasic and dibasic phosphates (pH 6–8), tris(hydroxymethyl)aminomethane hydrochloride (pH 6.5–8.5), cabonate/bicarbonate (pH 9–11), borate/boric acid (pH 8–10), and sodium hydroxide (pH 10.5–12.4).

Apparatus and Procedures. Spectra were obtained with a Perkin-Elmer 137 IR spectrometer, a Cary 118C or Cary 15 UV–vis spectrometer, a 60-MHz Varian T-60 NMR spectrometer, and a Varian E4 ESR spectrometer. Spectrophotometric titrations used a spectrophotometer titrimetric cell and a pH meter enclosed in an inert atmosphere glovebox. pH measurements were made by using a Radiometer Model 26 pH meter and Sensorex S900 c STD microgel electrode.

Stopped flow measurements used a Durrum stopped-flow spectrophotometer (D110) with a dead time of ~2 ms and a mixing time ~4.6 ms. The spectrophotometer was sealed in a glovebox under nitrogen ($[O_2] \sim 5 \times 10^{-8}$ M). Rate constant calculations were performed on a Hewlett-Packard Model 9825A desk top calculator with a 9864A digitizer and 9867A plotter. Elemental analyses were done by Galbraith Laboratories, Inc., Knoxville, TN.

Results and Discussion

1,10-Ethano-5-ethyl-1,5-dihydrolumiflavin (2$_{red}$) was prepared by reductive alkylation of 1,10-ethanolumiflavinium cation (eq 1). The ultraviolet-visible spectra of **2**$_{red}$ and its acid and base species are provided in Figure 1. The pK_a values of **2**$_{red}$ were determined by spectrophotometric titration (Figure 2) and are summarized in eq 2 (in eq 2 the protonation of **2**$_{red}$H$^+$ is written, without real evidence, at N^1). The pK_{a_2} value for **2**$_{red}$H$^+$ → **2**$_{red}$ is comparable to those for other N^5-protonated 1,5-dihydroflavins. Thus, the corresponding pK_{a_2} values for dissociation of N^5-protonated 5-ethyl-1,5-dihydrolumiflavin[8] and 1,5-dihydrolumiflavin[9] have been reported as 1.94 and 0.8, respectively.



1_{ox}^+ 2_{red} (1)

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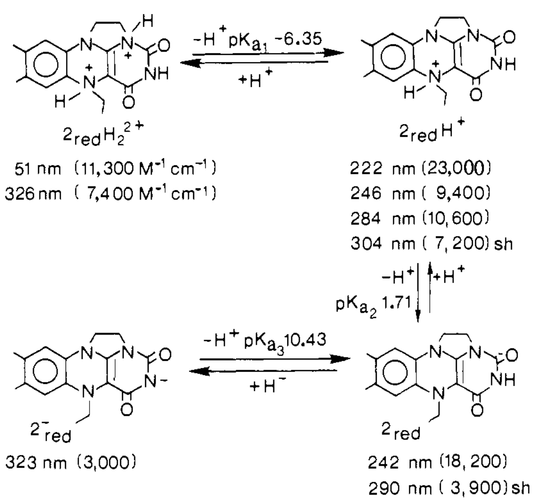
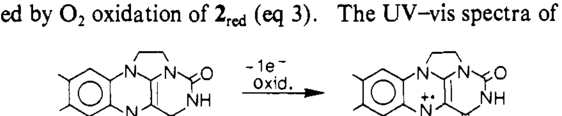
(7) Paul, M. A.; Long, F. A. *Chem. Rev.* **1957**, 57.

(8) Schug, C. Diplomarbeit, University of Konstanz, Konstanz, West Germany, 1978.

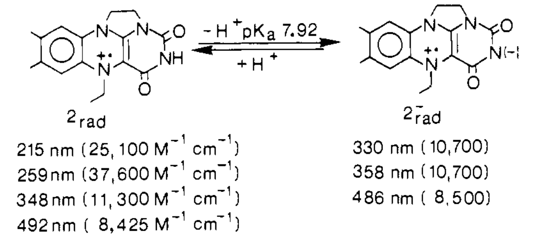


(2)

1,10-Ethano-5-ethyllumiflavinylium cation ($\mathbf{2}_{rad}$) is easily obtained by O_2 oxidation of $\mathbf{2}_{red}$ (eq 3). The UV–vis spectra of $\mathbf{2}_{rad}$ and its anion are shown in Figure 3. The pK_a for acid dissociation of $\mathbf{2}_{rad}$ (eq 4) was determined by spectral titration (Figure 2d).

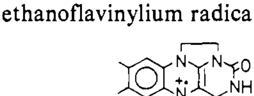


(3)



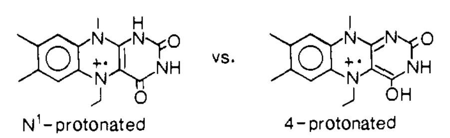
(4)

The spectrum of $\mathbf{2}_{rad}$ (λ_{max} and ϵ values) is almost identical with those of N^5-ethyllumiflavinylium cation ($FlEtH^{+}\cdot$)[8] (Figure 4) and 1,10-ethanoflavinylium radical ($\mathbf{1}_{rad}H^+$) (at pH 0.9, λ_{max} =

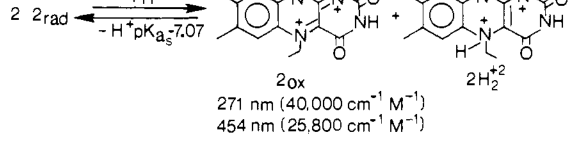


($1_{rad}H^+$)

496 nm, ϵ = ~7800; λ_{max} 355 nm, ϵ = ~13 200) (see Figure 2). The electronic structure of the three must, therefore, be identical. For this reason, the N^5-ethyllumiflavin radical ($FlEtH\cdot$) must be protonated at N^1 and not at the 4-position as previously assigned:[1b]

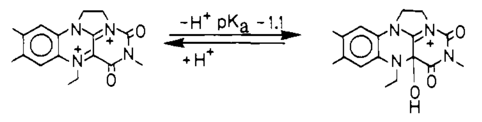


In concentrated sulfuric acid (aerobic), $\mathbf{2}_{rad}$ exhibits a pseudo pK_a at $H_0 = -7.07$ (Figure 2e) that is associated with its disproportionation to $\mathbf{2}_{red}H_2{}^{2+}$ and 1,10-ethano-5-ethyllumiflavin dication ($\mathbf{2}_{ox}$). In the presence of oxygen $\mathbf{2}_{red}H_2{}^{2+}$ is slowly oxidized to



(5)

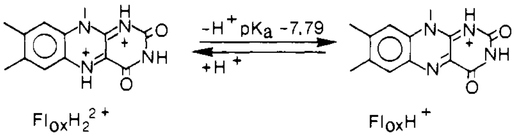
$\mathbf{2}_{ox}$. Formation of $\mathbf{2}_{ox}$ from $\mathbf{2}_{red}H^+$ is easily accomplished preparatively by oxidation of the latter in a diethyl ether solution of tetrafluoroboric acid with bromine. The pK_a for pseudobase formation with $\mathbf{2}_{ox}$ was determined by spectral titration (Figure 2f). Comparison of the heretofore unknown pK_a values for



(6)

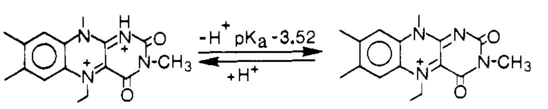
(4a-**2**-OH)

dissociation of $Fl_{ox}H_2{}^{2+} \rightarrow Fl_{ox}H^+$ (eq 7), $Fl_{ox}EtH^{2+} \rightarrow Fl_{ox}Et^+$



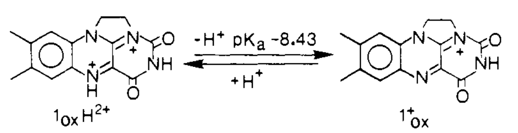
(7)

$Fl_{ox}H_2{}^{2+}$ $Fl_{ox}H^+$



(8)

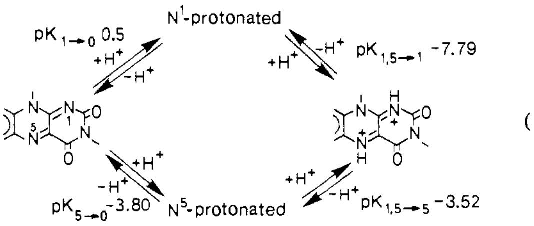
$Fl_{ox}EtH^{2+}$ $Fl_{ox}^{+}Et$



(9)

$1_{ox}H^{2+}$ 1^{+}_{ox}

(eq 8), and $\mathbf{1}_{ox}H^{2+} \rightarrow \mathbf{1}_{ox}{}^+$ (eq 9) (Figure 2g–i) allows corroboration of the initial assignment of the first position of protonation of oxidized flavins being at N^1.[10] The second protonation at N^5 is 5–6 pK_a units lower. Evidence for the identity of the electronic structures of $Fl_{ox}H^{2+}$, $Fl_{ox}EtH^{2+}$, and $\mathbf{1}_{ox}H^{2+}$ with the electron structure of $\mathbf{2}_{ox}$ is provided by comparison of the absorption spectra (Figure 5). From the equilibria of eq 10 [where p$K_{1\rightarrow0}$ is known,[11]



(10)

p$K_{1.5\rightarrow1}$ is from this study (eq 7), and p$K_{1.5\rightarrow5}$ is taken most reasonably as the dissociation of $Fl_{ox}EtH^{2+}$ (eq 8)], there can be calculated the microscopic pK_a (p$K_{5\rightarrow0}$) for the protonation of the

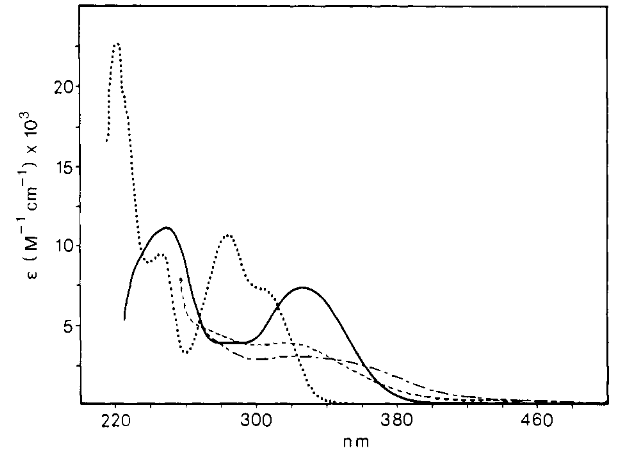


Figure 1. Spectra of the acid/base species of 1,10-ethano-5-ethyl-1,5-dihydrolumiflavin. (···) $\mathbf{2}_{red}H^+$ (1 M HCl); (—) $\mathbf{2}_{red}H_2{}^{2+}$ (H_2SO_4); (- - -) $\mathbf{2}_{red}$ (pH 6.9, phosphate buffer, μ = 0.1); (-·-) $\mathbf{2}_{red}{}^-$ (1 M NH_4OH aqueous). The typical concentrations employed were ~5×10^{-5} M.

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[9] Hemmerich, P.; Veeger, C.; Wood, H. S. C. *Angew. Chem.* **1965**, *77*, 699.

[10] Gawron, O.; Rampol, A.; Johnson, P. *J. Am. Chem. Soc.* **1972**, *94*, 5396, and references cited therein.

[11] Hemmerich, P. *Adv. Chem. Ser.* **1977**, *No. 162*, 312.

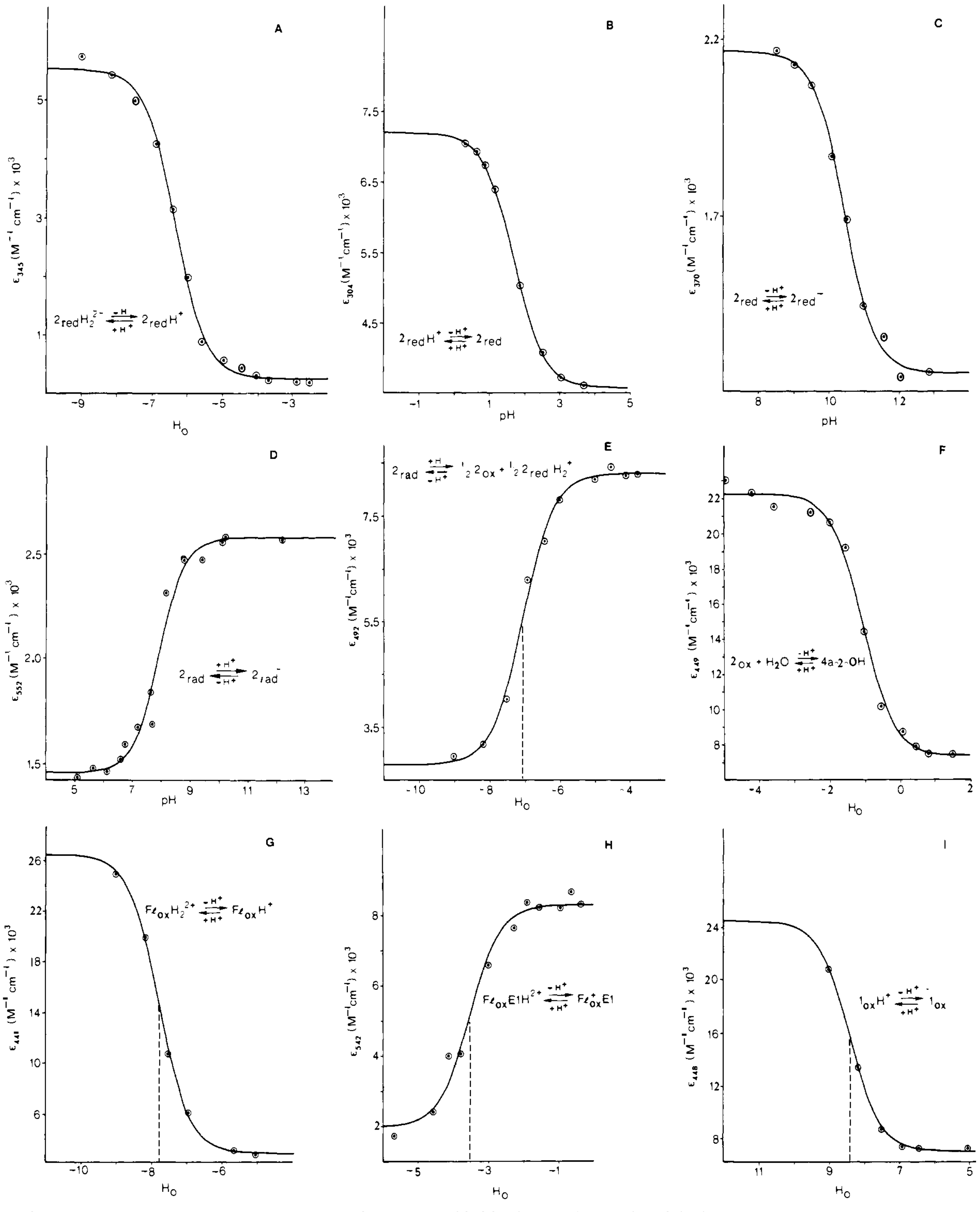

Figure 2. Spectral titration curves. The various pK_a values are provided in the text (see eq 2 and 4–9).

N^5-position of the representative flavin lumiflavin. The microscopic pK_a of N^5 of oxidized flavin has not previously been determined.

Disproportionation and decay of 1,10-ethano-5-ethyllumiflavinylium cation radical ($\mathbf{2}_{rad}$) occurs at pH values above pH 6. The decay of the radical (monitored at 492 nm), under anerobic conditions, was found to be dependent upon the square of its concentration (Figure 6) and to be independent of the buffer concentration required to maintain constant pH (eq 11). Thus,

$$\frac{-d[\mathbf{2}_{rad}]}{dt} = k_2'[\mathbf{2}_{rad}]^2 \qquad (11)$$

in the pH range 6.7–10.1 there was found to be no effect upon rate due to buffer concentration (phosphate, 0.5–0.005 M Tris, 2.0–0.02 M, and carbonate 0.5–0.005 M at μ = 1.0). The pH dependence of k_2 is provided in Figure 7. The experimental points of Figure 7 have been computer fitted by a line generated from eq 12 where a_H is the hydrogen ion activity (glass electrode), k_a = 2.4 × 10^2 M^{-1} s^{-1}, pK_{app} = 8.46, k_{HO} = 2.42 × 10^3 M^{-1} s^{-1}, and K_w is the autoprotolysis constant for water (13.83 at 30 °C, μ = 1.0).[12]

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[12] Robinson, R. A.; Stokes, R. H. "Electrolyte Solutions"; Butterworth: London, 1959; p 544.

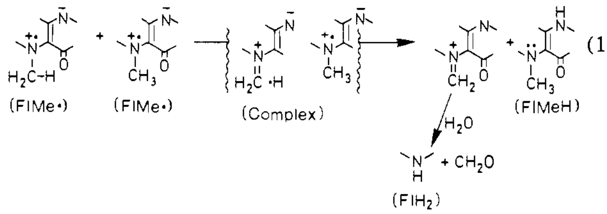
$$k_2' = \underset{A}{\frac{k_a K_{a_1} a_H}{(K_{a_1} + a_H)^2}} + \underset{B}{\frac{k_{HO} K_w}{a_H}} \qquad (12)$$

The pK_{app}, determined kinetically, may be compared to the titrimetric pK_a of 7.92 for acid dissociation of $\mathbf{2}_{rad}$ (eq 4). The bell-shaped portion of the pH-log k_2' profile (*A* of eq 12) represents the product of the mole fraction of undissociated and dissociated $\mathbf{2}_{rad}$. The first step in the reaction would then be disproportionation of the radical species $\mathbf{2}_{rad}$ and $\mathbf{2}_{rad}^-$. The term *B* of eq 12 may then represent the product of [HO$^-$] and [$\mathbf{2}_{rad}^-$]^2 vs.

$$B = k_{HO}\left(\frac{K_a}{K_a + a_H}\right)^2\left(\frac{K_w}{a_H}\right) \qquad (13)$$

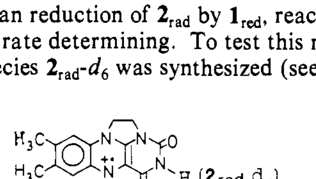
which at higher pH values becomes $k_{HO}K_w/a_H = k_{HO}[HO^-]$. The critical transition state for the reaction at higher pH must contain the kinetic equivalents of two $\mathbf{2}_{rad}^-$ species and an HO$^-$. The first step in the reaction is then, most likely, the disproportionation of two $\mathbf{2}_{rad}^-$ species. In spectral studies, covering the pH range 6.5–12.6, it was found that $\mathbf{2}_{red}$ is formed as a product of the reaction in 75% yield. Thus, after the disappearance of $\mathbf{2}_{rad}$, under anaerobic conditions, acidification with O_2-saturated acetic acid regenerated $\mathbf{2}_{rad}$ in 75% yield. In separate experiments it was shown that mildly acidic solutions of $\mathbf{2}_{red}$, when exposed to air, rapidly generate $\mathbf{2}_{rad}$ in 100% yield based on the [$\mathbf{2}_{red}$] employed.

The "disproportionation" of FlCH$_3$·, under anaerobic conditions, has been investigated by Kemal and Bruice.[2f] Between pH 1 and pH 8 the reaction was found to be dependent upon [FlCH$_3$·]^2 and not to be catalyzed by HO$^-$, H_3O^+, or buffer acid and base species. As in the instance of the bimolecular decay of $\mathbf{2}_{rad}$, the reduced flavin (FlMeH) accounted for 75% of the product of the reaction. On the basis that the replacement of FlCH$_3$· by FlCD$_3$· resulted in a kinetic deuterium isotope effect, of (–CH$_3$/–CD$_3$) > 10, and a careful product study, there was suggested the mechanism of Scheme I. The mechanism of reaction (a) of Scheme I was suggested to be that of eq 14.



(14)

Applied to the bimolecular decay of $\mathbf{2}_{rad}$ and $\mathbf{2}_{rad}^-$, the mechanism would be as shown in Scheme II. Reaction (b) of Scheme II was established in separate experiments to be very rapid. Mixing of solutions of $\mathbf{2}_{rad}$ and $\mathbf{1}_{red}$ results in the immediate reduction of $\mathbf{2}_{rad}$. Since the overall reaction, werein $4\mathbf{2}_{rad} \rightarrow 3\mathbf{2}_{red}$, is much slower than reduction of $\mathbf{2}_{rad}$ by $\mathbf{1}_{red}$, reaction (a) would be required to be rate determining. To test this requirement of Scheme II the species $\mathbf{2}_{rad}$-d_6 was synthesized (see Experimental Section).



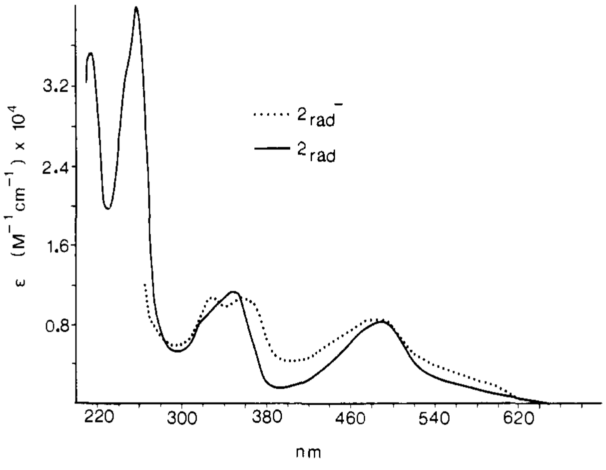


Figure 3. Spectra of the radical species of 1,10-ethano-5-ethyllumiflavin. $\mathbf{2}_{rad}$ at pH 2.10 with H_2SO_4/HSO_4^- buffer (0.5 M) and $\mathbf{2}_{rad}^-$ at pH 11.0 (KOH/H_2O buffer).

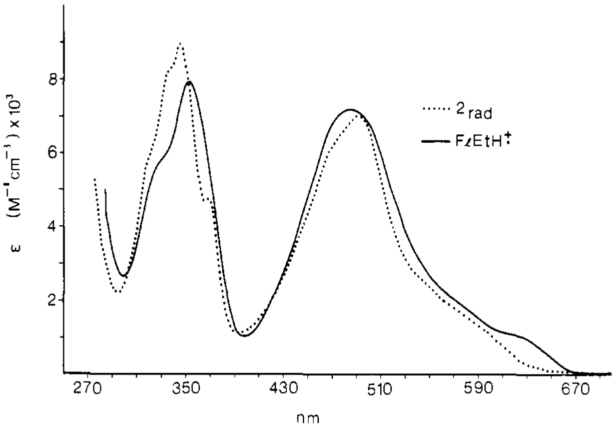


Figure 4. Comparison of the spectra of the radical of 1,10-ethano-5-ethyllumiflavin ($\mathbf{2}_{rad}$ in $CH_3CN/CHCl_3$) to that of the undissociated radical of 5-ethyllumiflavin (FlEtH$^+$ in $CHCl_3$).

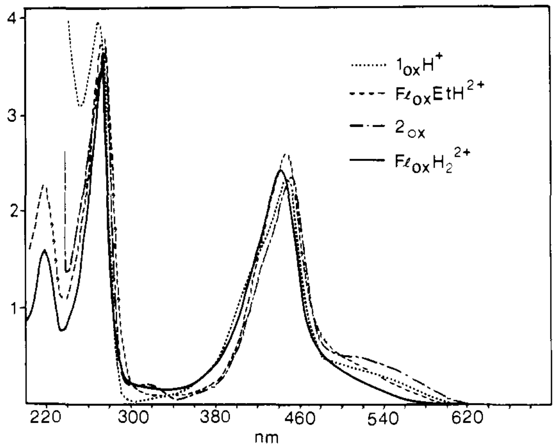
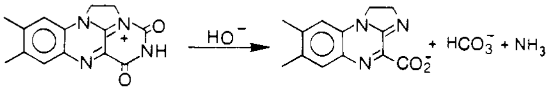


Figure 5. Comparison of the absorption spectra of 1,10-ethano-5-protiolumiflavin ($\mathbf{1}_{ox}H^+$), 5-ethyl-3-methyl-1-protiolumiflavin (Fl$_{ox}$-EtH^{2+}), 1,10-ethano-5-ethyllumiflavin ($\mathbf{2}_{ox}$), and 3-methyl-1,5-diprotiolumiflavin (Fl$_{ox}$H$_2$$^{2+}$). Typical concentrations were 5.5×10^{-5} M in concentrated sulfuric acid.

A comparison of the second-order disproportionation rate constants of $\mathbf{2}_{rad}$ and $\mathbf{2}_{rad}$-d_6 revealed that there is no deuterium kinetic isotope effect [$k(\mathbf{2}_{rad})/k(\mathbf{2}_{rad}\text{-}d_6)$ = 1.09 at pH 6.9 and 0.95 at pH 8.1]. This result establishes that reaction (a) of Scheme II cannot be occurring since electron transfer in the disproportionation reaction is not coupled to proton transfer with rupture of a C–H(D) bond. The disproportionation of $\mathbf{2}_{rad}$ species must differ mechanistically from the disproportionation of FlCH$_3$· (eq 14). The rate-determining step(s) in the disproportionation of $\mathbf{2}_{rad}$ must only involve electron transfer. An additional experimental observation, which is not in accord with Scheme II, is the detection of only traces of $\mathbf{1}_{ox}^+$ (λ_{max} = 390 nm, ϵ = 10 500) as a product. It should be noted that $\mathbf{1}_{ox}^+$ has been shown previously to hydrolyze in aqueous solution (eq 15).[13] However, the pseu-



(15)

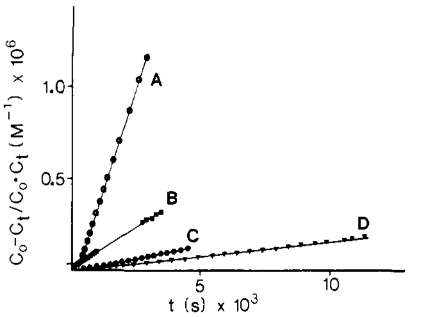
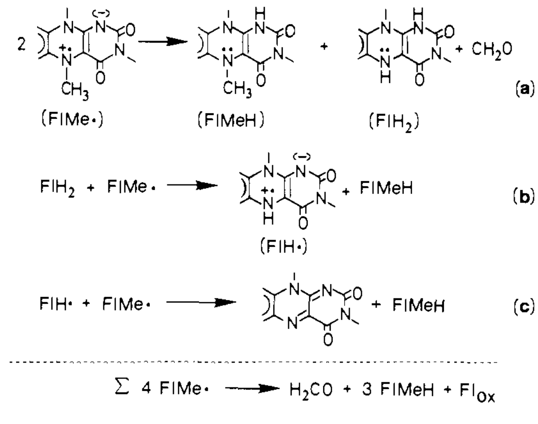
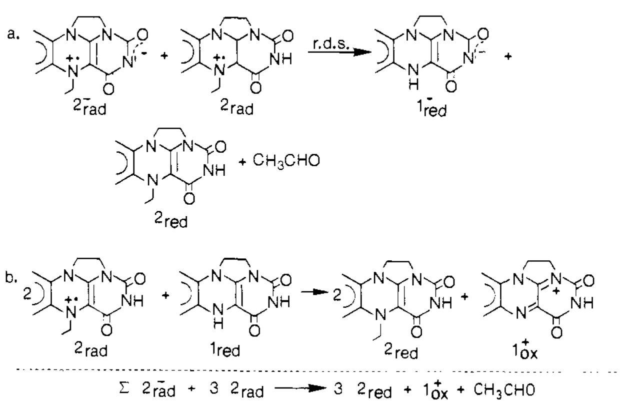


Figure 6. Plot of the change in concentration (at 492 nm) of 1,10-ethano-5-ethyllumiflavinylium cation radical (2_{rad}) with time ($C_0 - C_t$) divided by the product of the concentration of 2_{rad} at time zero (C_0) and at given times (C_t) vs. time. The linearity of the plots establish that the decomposition of 2_{rad} is second order in this species. (A) pH 12.7; (B) pH 8.8; (C) pH 10.9; (D) pH 6.92; at μ = 0.1 in H_2O, 30 °C.

Scheme I



Scheme II. Low pH



do-first-order rate constant for hydrolysis of 1_{ox}^+ at pH 10 was found, in our hands, to be too small ($k_{obsd} = 10^{-5}$ s^{-1}) to account for the inability to detect 1_{ox}^+ as a principal product of disproportionation of 2_{rad} species. The carboxylic acid obtained on hydrolysis of 1_{ox}^+ (eq 15) is itself unstable in base and its reduced form could serve as a two-electron reductant of 2_{rad}. The mechanism of Scheme III is offered as a plausible route to the disproportionation of 2_{rad}. In Scheme III, radical disproportionation provides $2_{ox} + 2_{red}$ in an endergonic reaction. The species 2_{ox} is then trapped by H_2O and HO^- to yield the pseudobase.

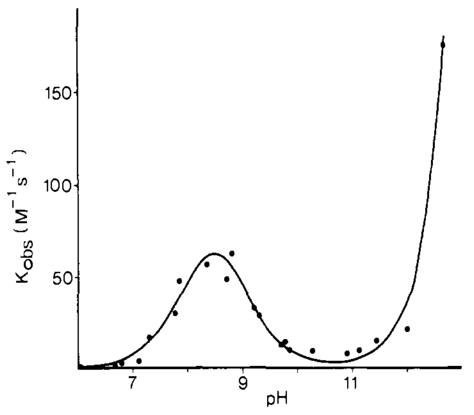
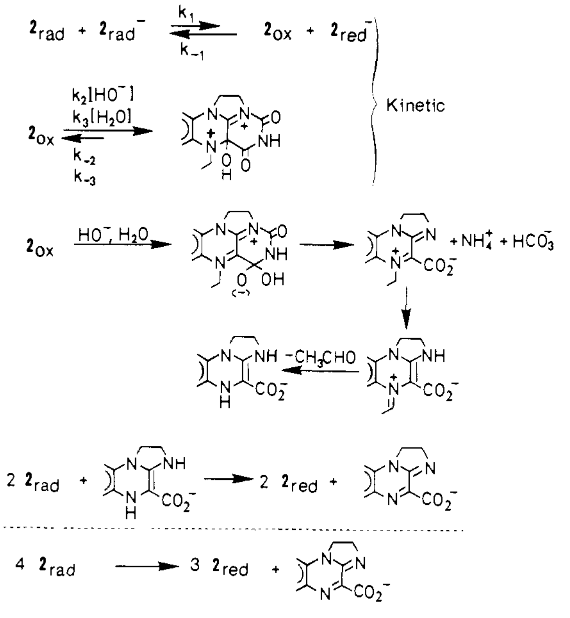


Figure 7. Plot of the apparent second-order rate constants (k_2') for the disproportionation of 2_{rad} vs. pH.

Scheme III



These two reactions constitute the rate-determining steps. Since the pK_a for pseudobase formation from 2_{ox} is −1.1, the formation of pseudobase is exergonic. The rate equation for the kinetic steps of Scheme III (eq 16) is in a form identical with that of the

$$k_{obsd} = \frac{k_1k_3}{k_{-1}}[2_{rad}][2_{rad}^-][H_2O] + \frac{k_1k_2}{k_{-1}}[2_{rad}][2_{rad}^-][HO^-] \quad (16)$$

experimentally determined rate equation for the disproportionation reaction. In competition with reversible pseudobase formation is the irreversible hydrolysis of 2_{ox} etc. The difference in the mechanism for bimolecular decompositions of the radicals FlMe· and 2_{rad} may revolve around the nature of the alkyl groups (Me vs. Et) rather than the flavin moieties employed. The answer to this question is being sought.

Acknowledgment. This work was supported by grants from the National Institutes of Health and the National Science Foundation.

Registry No. 1_{ox}^+, 47043-89-0; $1_{ox}H^{2+}$, 87039-57-4; $1_{rad}H^+$, 87039-58-5; 1_{red}, 25431-13-4; 2_{ox}, 87039-59-6; $2_{ox}(BF_4^-)_2$, 87039-60-9; $2_{rad}ClO_4^-$, 84193-79-3; 2_{rad}^-, 87039-61-0; 2_{red}, 80720-87-2; 2_{red}-d_5, 87039-62-1; $2_{red}H_2^{2+}$, 87039-63-2; $2_{red}H^+$, 87039-64-3; 2_{red}^-, 87039-65-4; $Fl_{ox}H_2^{2+}$, 58243-23-5; $Fl_{ox}EtH^{2+}$, 87039-66-5.

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(13) Wessiak, A. Dissertation, University of Konstanz, Konstanz, West Germany, 1980.