Changes in the Chemistry of an Isoalloxazine Brought About by Substitution at the 7 and 8 Positions by a Strongly Electronegative Substituent

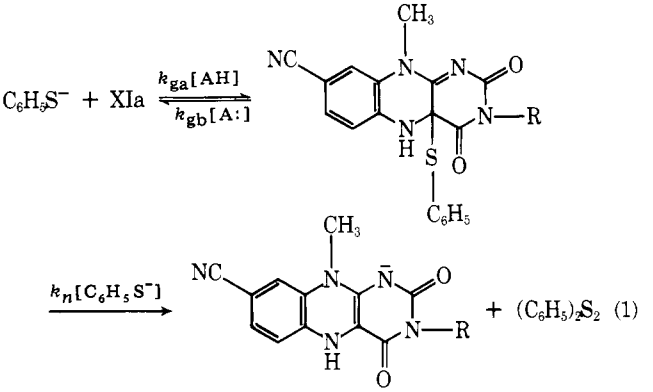
Thomas C. Bruice,* T. W. Chan, Joseph P. Taulane, Ichiro Yokoe, D. Lauriston Elliott, Robert F. Williams, and Michael Novak

Contribution from the Department of Chemistry, University of California, Santa Barbara, California 93106. *Received April 21, 1977*

Abstract: The electron-deficient 7- and 8-cyano-3,10-dimethylisoalloxazines (VII and XIa,b, respectively) are compared to a typical flavin (3-methyllumiflavin) as a means of assessing the mechanistic significance of $C^{8\alpha}$ substitution of flavin in such enzymes as succinic acid oxidase, etc. The spectra, pK_a values of oxidized, radical, and reduced forms, and $E_{1/2}$ values are compared and discussed as are the unusual mechanisms of hydrolysis. The 8-cyano group is shown to provide considerable stabilization to the isoalloxazine radical anion.

Flavin mononucleotide (FMN) was first isolated from old yellow enzyme by Theorell in 1935[1] and in 1936 Kuhn, Rudy, and Weygand[2] established its structure by synthesis. Warburg and Christian first isolated flavin adenine dinucleotide (FAD) from D-amino acid oxidase in 1938.[3] Until 1955, it was assumed that FMN and FAD constituted the sole cofactors for flavoenzymes. The finding by Green, Mii, and Kohout[4] that the flavin cofactor of succinic dehydrogenase could only be released by tryptic digestion and the establishment by Kearney[5] that the flavin moiety was covalently linked to a peptide fragment established that the flavin might exist in modified form allowing its covalent attachment to apoenzyme. It has been established, in the last 5 years, that no less than ten flavoenzymes contain the flavin prosthetic function in covalent linkage to the apoprotein.[6] Further, Singer and colleagues have established that the covalent linkage invariably occurs at $C^{8\alpha}$ through one of four types of chemical structures.[6,7] The 8α substituent groups have been shown to decrease the electron density of the isoalloxazine ring system, and this feature has been suggested to be of importance in the mechanism of reaction of enzymes containing covalently bound 8α-flavins.[8,9]

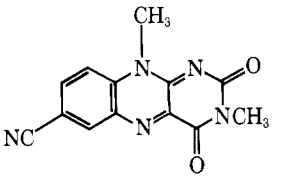
We have previously shown[10] that the 8-cyanoisoalloxazine (XIa) oxidizes thiophenol to phenyl disulfide and nitromethane and nitropropane anions to nitrite ion and aldehydes. Neither thiophenol nor nitroalkane oxidation is seen with flavins as oxidants though D-amino acid oxidase oxidation of nitroalkane anion has been studied by Porter, Voet, and Bright.[11] The reaction of nitroalkane anion with XIa is first order in these species and otherwise independent of pH and buffer concentrations. The reaction of thiophenol with xia was found to be first order in the isoalloxazine and first order in each of thiophenol and thiophenolate and independent of buffer concentration. The mechanism of eq 1 was suggested. General acid



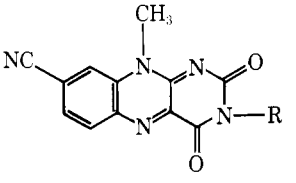
$$C_6H_5S^- + \text{XIa} \underset{k_{gb}[A:]}{\overset{k_{ga}[AH]}{\rightleftharpoons}} \text{adduct} \xrightarrow{k_n[C_6H_5S^-]} \text{XIa} + (C_6H_5)_2S_2 \quad (1)$$

catalyzed protonation of the N^5 position of XIa in concert with nucleophilic attack at C^{4a} is as expected for 4a addition of a nucleophile.[12] An elegant study by Loechler and Hollocher[13] has substantiated the mechanism of eq 1 by showing that a change in rate-limiting step occurs when going from monothiol (k_n(rds)) to a dithiol (k_{ga}[HA](rds)) (where rds stands for the rate-determining step). Additional support for 4a addition of thiol anion involves the establishment of the unimportance of the steric availability of the 9a and 10a[14] as well as the 6 and 8 positions[15] of the isoalloxazine ring.

Electron-attracting groups at the 8 position increase the electrophilicity of the isoalloxazine ring structure and facilitate $2e^-$ transfer to this moiety through formation of intermediate nucleophilic adducts (as in eq 1). However, free-radical processes are also important in the flavin oxidation of organic substrates.[16,17] It is known that aminium radicals are stabilized by placement between electron-withdrawing and electron-donating substituents.[18] It might be anticipated, therefore, that substitution of an electron-withdrawing group at the 8 position of an isoalloxazine will stabilize, to some extent, radicals formed by $1e^-$ addition. We report herein studies of the electron-deficient 7- and 8-cyano-3,10-dimethylisoalloxazines and establish that XIa but not VII supports a particularly stable radical anion.



VII



XIa, R = CH_3
b, R = $(CH_2)_3SO_3^-$

Experimental Section

Apparatus. All kinetic measurements were made on either a Gilford 2000 spectrophotometer or a Cary 16 spectrophotometer at 30 ± 0.1 °C. Absorption spectra were recorded on a Cary 15 or Cary 118C spectrophotometer at 30 °C. pH measurements were made with a Radiometer Model 26 pH meter equipped with a Metrohm EA 125 or Radiometer 2303C combined glass-calomel electrode at 30 °C. Infrared spectra were recorded with a Perkin-Elmer Model 137 sodium chloride spectrophotometer. NMR spectra were recorded on a Varian T-60 spectrophotometer using Me_4Si as an internal standard or as indicated with a Varian XL-100 Fourier transform NMR spectrometer. Mass spectra were recorded with an A.E.I. MS902 mass spectrometer. Melting points and boiling points were uncorrected.

Synthesis of 7-Cyano-3,10-dimethylisoalloxazine (VII). *p*-Trifluoroacetylaminobenzonitrile (I). I was prepared after the procedure of Vogel.[19] *p*-Aminobenzonitrile (Aldrich Chemical Co., 40.75 g, 0.345 mol) was dissolved in a minimum volume of trifluoroacetic acid, and added in portions with swirling to trifluoroacetic anhydride (80 g, 0.381 mol) in an ice bath. After 10 min the reaction mixture was poured over 300 g of crushed ice. The product was collected by suction filtration and recrystallized from ethanol-water, 64.8 g (88% yield): mp 165.5–166.5 °C; IR (KBr) 3210, 2220, 1740, and 1605 cm^{-1}; NMR (acetone-d_6, 60 MHz) δ 7.85 (m, 4 H, $C_{2,3,5,6}$-H), 9.16 (br s, 1 H).

***p*-Methylaminobenzonitrile (II).** The synthesis of II involved a modification of an established literature procedure.[20] I (25 g, 0.117 mol), methyl iodide (46 g, 0.324 mol), and powdered KOH (~18 g) were added to reagent grade acetone (250 mL). After refluxing for 30 min, the solution was decanted from the solid material and the acetone removed by rotary evaporation. The product was recrystallized from ethanol-water, 12 g (78% yield): mp 84–85.5 °C (lit.[21] mp 85–86 °C).

***p*-Methyltrifluoroacetylaminobenzonitrile (III).** III was prepared after the procedure of Vogel.[19] II (5.43 g, 0.041 mol) was dissolved in trifluoroacetic acid. Trifluoroacetic anhydride (10.5 g, 0.050 mol) was added in portions with swirling to the amine solution in an ice bath. After 15 min the solution was poured over 30 g of ice, the aqueous mixture extracted three times with chloroform, and the combined extracts dried over anhydrous sodium sulfate. The chloroform solution was concentrated by rotary evaporation, and petroleum ether (bp 65–110 °C) was added. The product crystallized as white needles, 8.15 g (87% yield): mp 119–120 °C; IR (KBr) 2215 and 1700 cm^{-1}; NMR ($CDCl_3$, 60 MHz) δ 8.58 (m, 4 H, $C_{2,3,5,6}$-H), 3.22 (s, 3 H).

4-Methylamino-3-nitrobenzonitrile (IV). The nitration of III employed a somewhat different method than that used by Bogert and Wise[22] for the nitration of *p*-acetaminobenzonitrile. III (14.9 g, 0.0655 mol) was added in small portions to a stirred mixture of 150 mL of concentrated nitric acid and 150 mL of concentrated sulfuric acid at 10 °C. After 5 h the reaction mixture was poured over 1 kg of ice, the aqueous mixture extracted five times with chloroform, the chloroform removed by rotary evaporation, and the residue taken up in hot ethanol-water. Potassium hydroxide (5 M, 15 mL, 0.075 mol) was added and IV precipitated from solution. IV was collected by suction filtration and thoroughly washed with water, 9.83 g (85% yield): mp 168–169 °C (lit.[23] mp 169 °C).

4-Methylamino-3-aminobenzonitrile (V). IV (9.55 g, 0.054 mol) was dissolved in hot 95% ethanol (140 mL) under nitrogen. Raney nickel (~2.5 g) and hydrazine hydrate (3.0 g, 0.094 mol) in 95% ethanol (10 mL) were added. After heating for 1.25 h, the solution turned clear. The Raney nickel was removed by filtration, and the solvents were removed by rotary evaporation yielding V, a small sample of which was recrystallized from ethanol: mp 138.5–139.5 °C (lit.[24] mp 141 °C).

7-Cyano-10-methylisoalloxazine (VI). VI was prepared after the method of Kuhn and Weygand.[25] V (8 g, 0.054 mol) from above was dissolved in nitrogen-flushed acetic acid (60 mL) under nitrogen, and added dropwise to a stirred solution of alloxan monohydrate (7.8 g, 0.0485 mol) and boric acid (3.9 g, 0.0631 mol) in hot acetic acid (125 mL), also under nitrogen. The reaction mixture was heated for an additional hour after the diamine addition was completed. The reaction mixture was then concentrated by rotary evaporation and filtered affording a greenish, solid residue and a reddish-brown filtrate. The greenish solid was heated in formic acid, gradually turning yellow, and was collected by suction filtration. The yellow compound was treated twice more with formic acid (25-mL portions), then dissolved in formic acid, and precipitated by the addition of ether. Ether was added to the reaction solution filtrate, precipitating a crude product which was purified in the same manner, total yield 9.23 g (75%): mp 360–362 °C dec; IR (KBr) 3440, 2215, 1715, and 1655 cm^{-1}.

7-Cyano-3,10-dimethylisoalloxazine (VII). VII was prepared after the procedure of Hemmerich et al.[26] VI (2.0 g, 0.00791 mol), methyl iodide (6.84 g, 0.0483 mol), and anhydrous potassium carbonate (11 g, 0.0834 mol) were added to a stirred solution of dimethylformamide (0.8 L). After 2.25 h the reaction solution was filtered, and the dimethylformamide was removed by rotary evaporation. Water (250 mL) was added, and the mixture was extracted six times with chloroform (200-mL portions). The combined chloroform extracts were dried over anhydrous sodium sulfate, the chloroform was removed by rotary evaporation, ether (100 mL) was added, and the product was collected by suction filtration (1.96 g, 93% yield). The crude product was recrystallized from acetic acid, and a sample for analysis was recrystallized three times from dimethylformamide: yellow needles; mp 283–290 °C dec; IR (KBr) 2210, 1720, and 1660 cm^{-1}; NMR (Me_2SO-d_6, 60 MHz) δ 3.37 (s, 3 H, N^3-methyl), 3.66 (s, 3 H, N^{10}-methyl), 7.96–8.56 (m, 3 H). Anal. Calcd for $C_{13}H_9N_5O_2$: C, 58.43; H, 3.37; N, 26.22. Found: C, 58.50; H, 3.49; N, 26.20.

Synthesis of 8-Cyano-3,10-dimethylisoalloxazine (XIa). 3-Methylamino-4-nitrobenzonitrile (VIII). VIII was synthesized after the method of Bower et al.: mp 210 °C (lit. 213–214 °C).

4-Amino-3-methylaminobenzonitrile (IX).[27] VIII (3.05 g, 0.0172 mol), Raney nickel (0.8 g), and hydrazine hydrate (0.95 g, 0.0194 mol) were dissolved in 95% ethanol (150 mL). The mixture was heated on a steam bath under nitrogen. After 3 h, another portion of hydrazine hydrate (0.95 g, 0.0194 mol) was added. After 0.5 h the solution turned clear and was filtered. The solvent was removed by rotary evaporation, and the product was used without further purification.

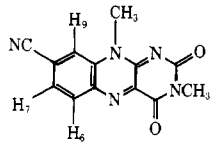
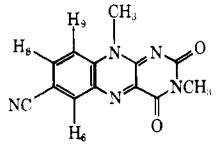
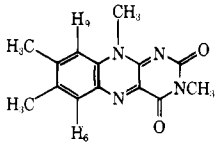
8-Cyano-10-methylisoalloxazine (X). X was prepared after the method of Kuhn and Weygand.[25] IX (0.0172 mol) was dissolved in nitrogen-flushed acetic acid (60 mL) under nitrogen, and added dropwise to a stirred solution of alloxan monohydrate (2.60 g, 0.01625 mol) and boric acid (1.25 g, 0.0202 mol) in hot acetic acid (125 mL), also under nitrogen. The reaction mixture was heated for an additional hour after the diamine addition was completed, and during this time the flow of nitrogen concentrated the solution to a volume of ~50 mL. The solution was cooled and filtered, affording a greenish solid residue. Heating the residue in formic acid (20 mL) gave the desired product as a yellow solid, 3.25 g (72.5%): mp 341–342 °C dec; IR (KBr) 2200, 1720, and 1650 cm^{-1}.

8-Cyano-3,10-dimethylisoalloxazine (XIa). XIa was prepared after the general procedure of Hemmerich et al.[26] X (3.20 g, 0.0126 mol), anhydrous potassium carbonate (8 g, 0.0579 mol), and CH_3I (6.84 g, 0.0482 mol) were added to a stirred solution of dimethylformamide (1.5 L). After 5 h the solution was filtered, and the dimethylformamide removed by rotary evaporation. Water was added to the wet residue and the crude product was collected by suction filtration, further washed with water and acetone, and twice recrystallized from acetic acid, 2.0 g (59.5%): mp 315–320 °C dec; IR (KBr) 2210, 1710, 1670, 1625, and 1600 cm^{-1}; NMR ($CDCl_3$, 100 MHz) δ 3.55 (s, N^3-methyl), 4.13 (s, N^{10}-methyl), 7.75–8.44 (m, 3 H). Anal. Calcd for $C_{13}H_9N_5O_2$: C, 58.43; H, 3.37; N, 26.22. Found: C, 58.59; H, 3.63; N, 26.14.

A comparison of the NMR spectra (Varian XL-100; Fourier transform) on 1×10^{-4} M solutions of VII and XIa to that of a typical flavin (3-methyllumiflavin) is provided in Table I [positions relative to Me_4Si in $CDCl_3$ or DSS (2,2,3,3-tetradeuterio-3-(trimethylsilyl)-propionic acid sodium salt)] in D_2O. There are substantial solvent effects on the chemical shifts. This observation is in accord with the findings of other workers.[28]

Synthesis of 8-Cyano-10-methyl-3-sulfopropylisoalloxazine, Potassium Salt (XIb). 3-Methyltrifluoroacetylaminobenzonitrile (XII) was prepared according to the general procedures of Morgan and Turner.[29] Anhydrous K_2CO_3 (27.6 g) was added to a solution of 23.6 g (0.2 mol) of 3-aminobenzonitrile (Aldrich) in 350 mL of benzene under a nitrogen atmosphere. While the resultant mixture was cooled in an ice bath, 42.0 g (0.2 mol) of trifluoroacetic anhydride was added in a dropwise fashion with constant stirring over a period of about 1 h. The reaction mixture was then refluxed for 3 h, cooled in an ice bath, and filtered. The filter cake was then thoroughly washed with water to remove inorganic salts. The snow-white product which remained

Table I

	Solvent	Chem-shift position (δ)[a] 6	7	8	9	Coupling constants, Hz J_{6-7}	J_{6-8}	J_{6-9}	J_{7-9}	J_{8-9}	Methyl positions 3	7	8	10
	D_2O	8.51	8.38		8.05	8.5		0.3	1.0		3.46			4.15
	$CDCl_3$	8.44	7.90		7.75	8.3			0.8		3.55			4.13
	D_2O	8.69		8.31	8.13		1.9	0.5		9.0	3.46			4.16
	$CDCl_3$	8.60		8.07	7.71		1.9	0.4		8.9	3.54			4.17
	D_2O	7.94			7.82						3.44	2.48	2.59	4.14
	$CDCl_3$	8.08			7.78						3.54	2.46	2.56	4.12
	CD_3CN[b]	7.91			7.64						3.35	2.43	2.53	4.00

[a] Relative to DDS in D_2O and Me_4Si in $CDCl_3$. [b] Literature values from ref 28.

in the filter funnel was combined with approximately 3 g of material obtained by partial evaporation of the benzene washings, and dried under vacuum to yield 36.1 g (84%) of 3-trifluoroacetylaminobenzonitrile (XIII) (mp 176–179 °C). This material could be recrystallized from 95% ethanol to give crystals with mp 178–180 °C; however, this was not necessary. Unrecrystallized XIII (35.2 g, 0.164 mol), dissolved in 200 mL of freshly distilled dimethoxyethane, was added in a dropwise fashion under nitrogen to a vigorously stirred suspension of 4.3 g (0.180 mol) of NaH (99% Alfa Inorganics) in 50 mL of dimethoxyethane at 0 °C. After addition was complete and hydrogen evolution had ceased, 34.0 g (0.240 mol) of CH_3I was added to the mixture in a dropwise manner. The reaction mixture was then allowed to reach room temperature and stirred for 20 h before it was poured into a mixture of 180 mL of 1 N HCl and 100 g of ice. The mixture was then extracted three times with 200 mL of CH_2Cl_2. The dichloromethane washings were combined, dried ($MgSO_4$), and evaporated to a volume of about 50 mL, which was then applied to a silica gel column and eluted with dichloromethane. Evaporation of the fractions collected immediately after the void volume led to recovery of 35.5 g (95%) of slightly yellow XII: mp 96–98 °C; IR (KBr) 2230 and 1700 cm^{-1}; NMR ($CDCl_3$, Me_4Si) δ 3.40 (s, 3 H), 7.2–8.0 (m, 4 H).

3-Methylaminobenzonitrile (XIV) was obtained by simple alkaline hydrolysis of XII; more exhaustive procedures, such as that of Gassman,[30] led to partial destruction of the product. The amide (XII) (17.6 g, 0.077 mol) was partially dissolved in 300 mL of ethanol, and 300 mL of water containing 12.3 g of NaOH (0.31 mol) was added to the rapidly stirred mixture. After 15 min of stirring the mixture was extracted five times with 200 mL of dichloromethane. The organic extracts were combined, dried ($MgSO_4$), and evaporated to yield a light yellow oil which was distilled (88–89 °C (0.15 mm)) to yield 9.77 g (96%) of XIV: IR 2160 cm^{-1} (C≡N), no carbonyl stretch; NMR ($CDCl_3$, Me_4Si) δ 2.79 (s, 3 H), 4.13 (s, 1 H, NH), 6.6–7.4 (m, 4 H).

8-Cyano-10-methylisoalloxazine N^5-Oxide (XV). 6-(*N*-Methyl-*m*-cyanoanilino)uracil (XVI) was prepared from 6-chlorouracil[31] and XIV by a slight modification of the procedure of Yoneda.[32] The 6-chlorouracil (2.93 g, 0.02 mol) and XIV (5.29 g, 0.04 mol) were heated with vigorous stirring under a nitrogen atmosphere at 215–220 °C for 10 min. The crude product was washed thoroughly with diethyl ether and water and then recrystallized from ethanol to yield 3.45 g of XVI (71% with respect to the 6-chlorouracil): IR (KBr) 2260, 1700, and 1600 cm^{-1}. This material was converted to XV by Yoneda's procedure[32] in 75% yield. After reduction with dithionite[32] 8-cyano-10-methylisoalloxazine (X) was obtained which, after recrystallization from formic acid, was reacted with 1,3-propane sultone by the procedure of Blankenhorn[33] to provide XIb. The UV-vis spectrum of XIb is identical with that of XIa.

pK_a Determinations (30 °C, μ = 1 with KCl in H_2O, Argon Atmosphere). Dihydro-8-cyano-10-methyl-3-sulfopropylisoalloxazine, Potassium Salt. Typically, 100 μL of a 1.33×10^{-3} M stock solution of the isoalloxazine was added to 3.0 mL of the appropriate buffer (0.1 M) in the lower section of a Thunberg cuvette. Into the upper chamber of the cuvette was placed 20 μL of a freshly prepared solution of dithiothreitol (9.1×10^{-2} M). Both chambers were deoxygenated with a stream of vanadous-scrubbed and prehumidified argon for 1.5 h. The Thunberg cuvette was then sealed and thermally equilibrated when the contents were mixed and the reduction of isoalloxazine was followed to completion by repetitive scanning of the spectra (reduction on mixing above pH 7.5) using a Cary 118C spectrophotometer (isosbestic points at 262, 289, 327, 340, 397, and 505 at pH 4.49). Above pH 7.5 dithiothreitol and its oxidation product exhibit a minor absorbance in the UV (see Figure 4). The spectra at 8 to 10 half-lives for reduction were determined by subtraction of the thiol spectrum which was determined in separate experiments. On oxygenation, 98 to 100% of oxidized isoalloxazine was recovered. pH values were determined prior to and at the completion of the reaction and found to have remained constant. The absorbance changes at 335, 350, and 390 nm were fit to theoretical titration curves for a monobasic acid of pK_a = 5.9 (inset Figure 4). The pK_a of the neutral radical of 8-cyano-3,10-dimethylisoalloxazine was determined by half-reduction with C_6H_5SH and by half-photoreduction with EDTA employing the same general procedure as outlined above.

Kinetics. All kinetic measurements were carried out at 30 ± 0.1 °C at μ = 1 (with KCl). The anaerobic kinetic experiments were performed in Thunberg cuvettes under vanadous-scrubbed argon atmosphere. The rate of disappearance of 7- and 8-cyano-3,10-dimethylisoalloxazines in various reaction mixtures was followed by measuring the variation in absorbance at 426 and 442 nm, respectively.

Product Analysis. Hydrolysis of Compounds VII and XIa. 8-Cyano-3,10-dimethylisoalloxazine (100 mg) was dissolved in 50 mL of CH_3CN. To this solution was added 50 mL of carbonate buffer (0.5 M; pH 11.30) and 10 mL of MeOH. This mixture was stirred for 2.5 h at room temperature. After acidification with 1 N HCl, the reaction mixture was extracted three times with 15-mL portions of $CHCl_3$. The $CHCl_3$ layer was washed with water and dried over Na_2SO_4. After evaporating $CHCl_3$, the residue was purified by silica gel column chromatography. The fraction eluted by 2% MeOH–$CHCl_3$ was recrystallized from acetone to give spirohydantoin XVIb, mp 300–302 °C dec, 62 mg. By a similar procedure, compound XVIa was obtained from VII. XVIa, mp 290–291 °C (acetone), yield is 95%. Spectral characteristics of compound XVIb are as follows: NMR (Me_2SO-d_6, 60 MHz) δ 2.93 (s, 3 H), 3.40 (s, 3 H), 6.83 (s, 1 H), 7.40 (s, 1 H), 7.53 (s, 1 H), 8.23 (s, 1 H), and 9.23 (s, 1 H); UV (MeOH) λ_{max} 302 (ϵ 1×10^4), 232 (ϵ 3×10^4), and 223 nm (ϵ 2.7×10^4); IR (KBr) 3450 (NH), 2230 (C≡N), 1790, 1730, and 1655 cm^{-1}; *m/e* 283 (M^+). Anal. Calcd for $C_{13}H_{11}N_5O_3$: C, 54.73; H, 3.89; N, 24.55. Found: C, 54.86; H, 4.05; N, 24.51.

Compound XVIa exhibited the following spectra: NMR (60 MHz) δ 2.90 (s, 3 H), 3.36 (s, 3 H), 7.09 (s, 1 H), 7.24 (s, 2 H), 7.85 (s, 1 H),

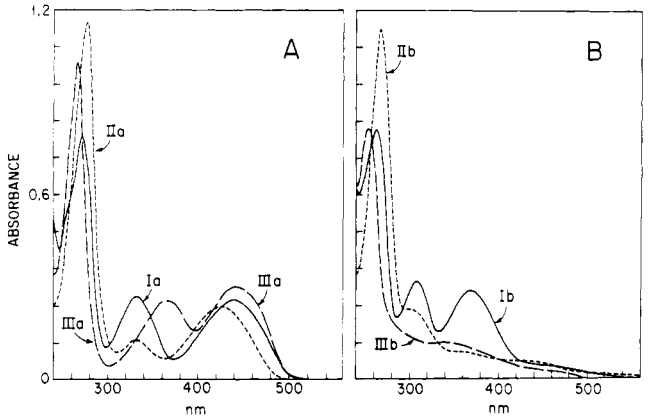


Figure 1. Plots of absorbance vs. wavelengths for oxidized (A) and reduced (B) isoalloxazines. Plots Ia,b pertain to: 8-cyano-3,10-dimethylisoalloxazine; IIa,b, 7-cyano-3,10-dimethylisoalloxazine; and IIIa,b, 3-methyllumiflavin (H_2O, pH 7.46, 0.10 M Tes buffer). Isoalloxazine concentrations, 2.52×10^{-5} M.

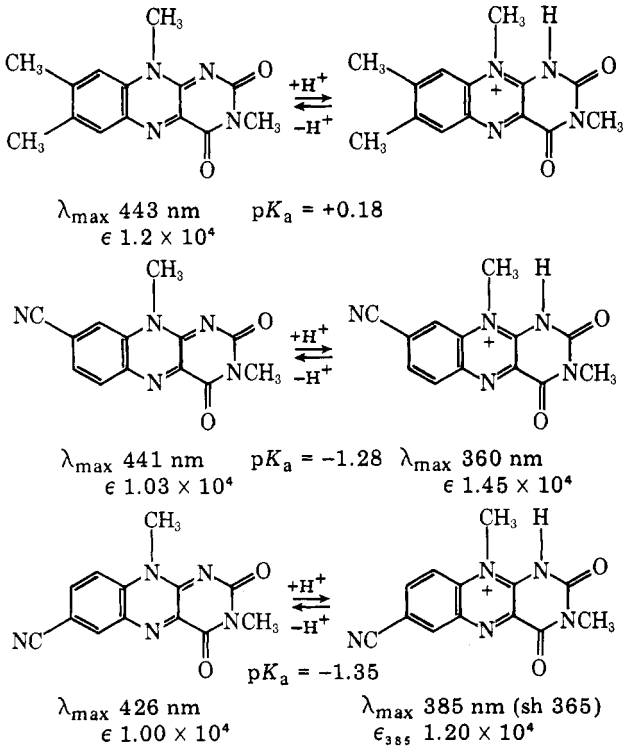
and 9.20 (s, 1 H); UV (MeOH) 322 (ϵ 1.1×10^4), 265 (ϵ 1.84×10^4), and 234 nm (ϵ 7.84×10^4); IR (KBr) 3450 (NH), 2230 (C≡N), 1780, 1730, and 1640 cm^{-1}; *m/e* 285 (M^+). Anal. Calcd for $C_{13}H_{11}N_5O_3$: C, 54.73; H, 3.89; N, 24.55. Found: C, 54.86; H, 4.05; N, 24.51.

Results and Discussion

The introduction of the strongly electron-withdrawing cyano substituent group into the 7 and 8 positions of the 3,10-dimethylisoalloxazine ring system has a profound influence upon both the physical and chemical properties of the isoalloxazine moiety. Comparison of the 7- and 8-cyano-3,10-dimethylisoalloxazines (VII and XI, respectively) points up several marked differences.

Oxidized State. In Chart I there is presented a comparison

Chart I



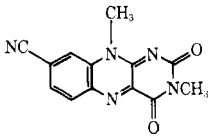
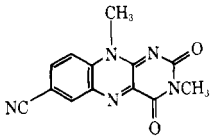
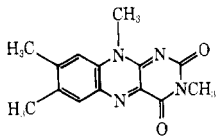
of the pK_a and visible–near-ultraviolet spectra (Figure 1A) of VII and XIa to those for 3-methyllumiflavin (XVII).[34] As anticipated, cyano substitution results in a decrease in basicity.

The substitution of an electron-withdrawing group in the 7 position of isoalloxazine also seems to have a large influence upon the UV–vis spectrum (Figure 1). A comparison of the spectrum (H_2O solvent) of 3-methyllumiflavin to that of 7-cyano-3,10-dimethylisoalloxazine reveals that the λ_{max} for the latter is shifted by 14 nm to shorter wavelength (λ_{max} 426 nm). Furthermore, the secondary peak at 330 nm (shifted from 360 nm in flavins) is of less intensity when compared to other flavins ($\epsilon_{330}/\epsilon_{426} = 0.5$). The λ_{max} of the 8-cyano derivative is only slightly shifted (λ_{max} 441 nm) and the position of the 330-nm absorbance is as seen with VII. The ratio $\epsilon_{330}/\epsilon_{441}$ is 1.0 and that is usually about 0.7 to 0.8 ($\epsilon_{360}/\epsilon_{443}$) as illustrated by the 3-methyllumiflavin spectra (λ_{max} 443 nm) of Figure 1.

The half-wave potentials ($E_{1/2}$) for the overall $2e^-$ reduction of the 7- and 8-cyano analogues, when compared to that of a simple flavin, establish, as anticipated, that electron withdrawal by the cyano substituents increases, thermodynamically, the oxidizing ability of the isoalloxazine ring system. The increase of $E_{1/2}$ to a more positive potential by 200 mV (Table II) may be compared to a like increase of 30–40 mV for the $2e^-$ redox potential on 8α substitution of riboflavin.[8]

Kinetic data for the hydrolysis of 7- and 8-cyano-3,5-dimethylisoaloxazines are provided in Tables III and IV. All reactions were found to be pseudo-first-order at constant pH and examination of the tables reveals that the hydrolytic reactions are not buffer catalyzed. A repetitive spectral scan of the hydrolysis of XIa at pH 9.15 is shown in Figure 2. The spectra of the products of hydrolysis of both VII and XIa (Figure 2) resemble that of the spirohydantoin XX obtained[35]

Table II. $E_{1/2}$ vs. SCE for a Series of Isoalloxazines[a]

Isoalloxazine	$E_{1/2}$, V
	−0.317
	−0.353
	−0.538

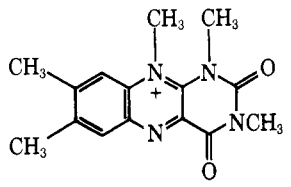
[a] Differential pulse polarography at pH 9.0 (0.1 M borate, 30 °C, μ = 1 with KCl).

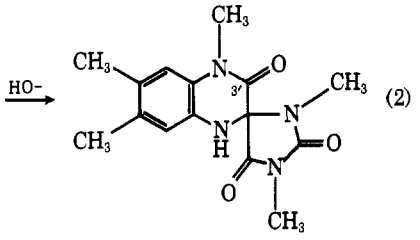
Table III. Hydrolysis of 7-Cyano-3,10-dimethylisoalloxazine in Carbonate Buffer ($[B_T] = [HCO_3^-] + [CO_3^-]$, 30 °C, H_2O, μ = 1.0 with KCl)

pH	$[B_T]$, M^{-1}	k_{obsd}, min^{-1}	k_{HO^-}, M^{-1} min^{-1}
9.90	1	9.97×10^{-4}	
	0.7	1.01×10^{-3}	
	0.5	1.04×10^{-3}	
	0.25	1.04×10^{-3}	$(8.64 \pm 0.16) \times 10$
9.55	1	4.08×10^{-3}	
	0.7	4.25×10^{-3}	
	0.5	4.40×10^{-3}	
	0.25	4.55×10^{-3}	$(8.25 \pm 0.46) \times 10$
9.10	1	2.07×10^{-3}	
	0.7	1.78×10^{-3}	
	0.5	1.96×10^{-3}	
	0.25	1.96×10^{-3}	$(1.04 \pm 0.09) \times 10$

Table IV. Hydrolysis of 8-Cyano-3,10-dimethylisoalloxazine in Carbonate Buffer ($[B_T] = [HCO_3^-] + [CO_3^-] + [CO_3^-]$, 30 °C, H_2O, μ = 1.0 with KCl)

pH	$[B_T]$, M^{-1}	k_{obsd}, min^{-1}	k_{OH^-}, M^{-1} min^{-1}
10.25	1.0	1.67×10^{-2}	
	0.7	1.65×10^{-2}	
	0.5	1.64×10^{-2}	
	0.25	1.63×10^{-2}	$(6.26 \pm 0.07) \times 10$
10.00	1.0	6.93×10^{-3}	
	0.7	6.97×10^{-3}	
	0.5	7.06×10^{-3}	
	0.25	7.19×10^{-3}	$(4.76 \pm 0.1) \times 10$
9.55	1	2.78×10^{-3}	
	0.7	2.88×10^{-3}	
	0.5	3.00×10^{-3}	
	0.25	3.10×10^{-3}	$(5.62 \pm 0.3) \times 10$
9.10	1	1.04×10^{-3}	
	0.7	1.01×10^{-3}	
	0.5	9.49×10^{-4}	
	0.25	8.07×10^{-4}	$(5.10 \pm 0.47) \times 10$

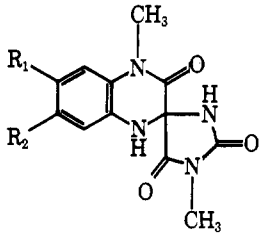




(2)

XX

on hydrolysis of the 1,3-dimethylflavinium cation (eq 2). From this observation as well as IR, NMR, and mass spectra (experimental), the hydrolytic products are assigned structures XVIa,b. The hydrolysis of 3-methylflavins generally occurs



XVIa, R_1 = H; R_2 = CN
b, R_1 = CN; R_2 = H

via nucleophilic addition of HO^- to the 4-carbonyl and 10a position to provide products in which the uracil ring is opened[36] (eq 3). Thus, the hydrolyses of VII and XI are unusual. The intermediate species in the hydrolysis of the 1-alkylflavinium ion of eq 2 and reaction B of eq 3 undoubtedly possess the structures of XVIII and XIX, respectively. Formation of the spirohydantoin XX from XVIII accompanies conversion of the hydroxyl function to the carbonyl group at the 3′ position. The formation of spirohydantoins (XVIa,b) on hydrolysis of VII and XIa must be related to the increase in electronegativity of the N^5 nitrogen of the tetrahedral intermediate (XIX) which facilitates formation of structure XVI. In this regard, repetitive spectral scans during the hydrolysis of VII and XIa (Figure

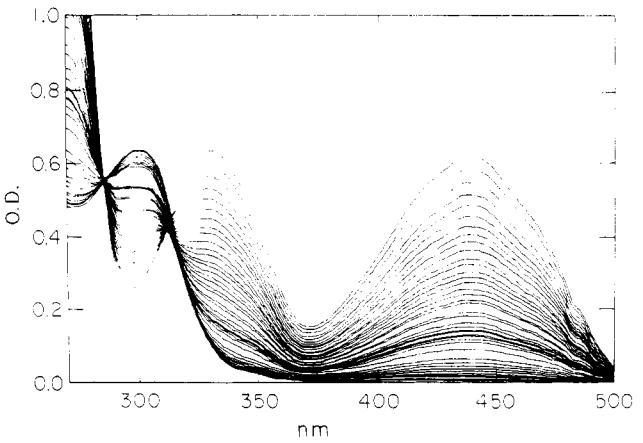
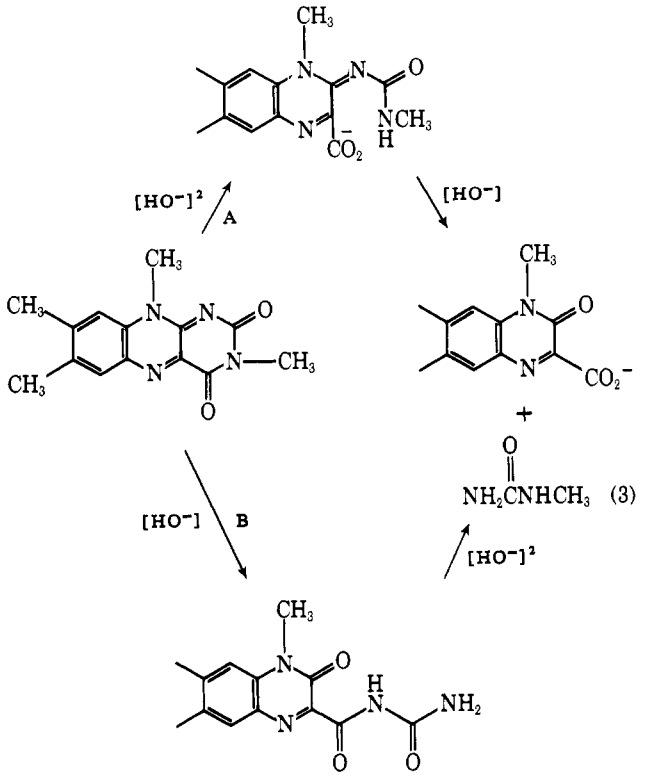
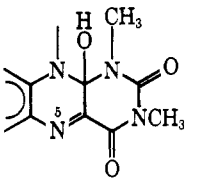


Figure 2. Repetitive spectral scan of the time course for the alkaline hydrolysis of 8-cyano-3,10-dimethylisoalloxazine (6.25×10^{-5} M, pH 9.15, with 0.5 M carbonate buffer, 30 °C, solvent H_2O).

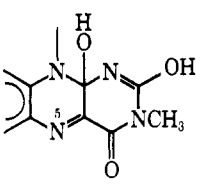


(3)

2) provide no evidence for the accumulation of 10a- or 4a-hydroxyl adducts.



XVIII



XIX

Structure of the Dihydro Forms of VII and XIa,b. In either basic or acidic aqueous solutions, reduction of the 7-cyanoisoalloxazine (VII) yields a product possessing a spectrum that is very similar to that which has become recognized as characteristic of 1,5-dihydroflavins. Thus, the spectra of reduced VII are comparable to that for reduced 3-methyllumiflavin (Figure 1B). In contrast, the spectrum of reduced 8-cyanoisoalloxazine (XI) is quite different. As shown in Figure

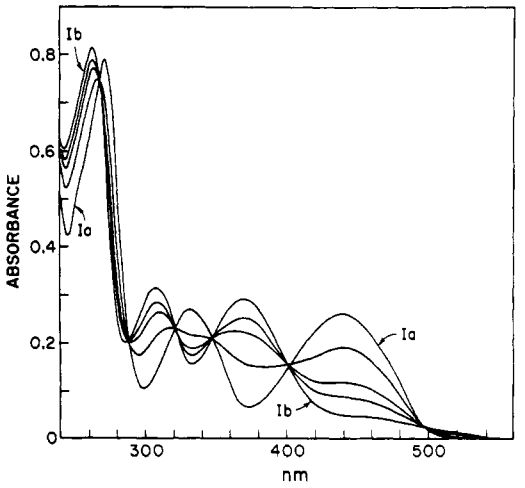


Figure 3. Repetitive spectral scans taken in the course of the reduction of 8-cyano-3,10-dimethylisoalloxazine (2.52×10^{-5} M) employing dithiothreitol as the reducing agent (pH 7.4, 0.1 M Tes buffer, H_2O, 30 °C). Curves Ia and Ib represent the spectra of oxidized and reduced isoalloxazine, respectively.

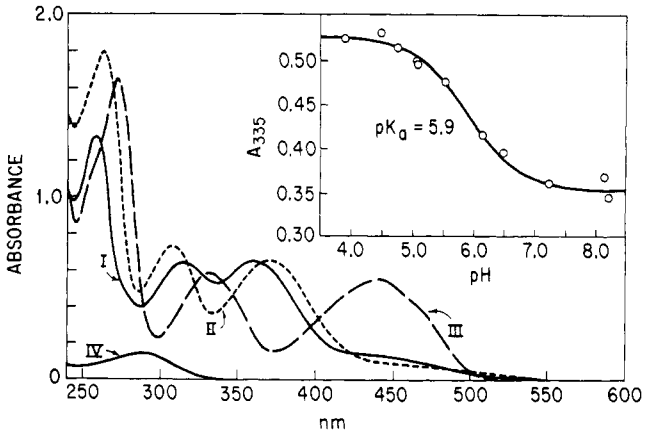


Figure 4. Curves I and II represent the spectra of reduced 8-cyano-10-methyl-3-sulfopropylisoalloxazine (5.49×10^{-5} M) at pH values of 4.49 and 8.15. Curve III is the spectrum of the oxidized isoalloxazine. Curve IV represents the differential spectra obtained by subtracting the absorbance vs. nanometer plot of the product obtained upon reduction of the isoalloxazine and reoxidation with O_2, all at pH 8.15. This differential absorbance increases upon cyclic reduction and oxidation and represents decomposition products. Cyclic reduction and oxidation are quantitative at pH 7 and below. The inset represents the pH dependence of the absorbance of reduced isoalloxazine. The points are experimental and the line represents a theoretical titration curve for a single proton ionization.

3, reduced XIa,b exhibits a well-defined λ_{max} at 370 nm not seen for other dihydroflavins. The reduction of XIa,b to the fully reduced state was accomplished with all the normal agents employed for this purpose (photoreduction in the presence of EDTA, $Na_2S_2O_4$, Zn and hydrochloric acid, BH_4^-, dithiothreitol, etc.) with the exception of hydrogenation using Pd on asbestos. Not surprisingly, the cyano group on the aromatic ring is reduced by the latter agent. Regardless of the reducing agent employed, the dihydroflavin obtained exhibits the same characteristic spectrum with a λ_{max} at 370 nm (ϵ 1.37×10^4 M^{-1} cm^{-1}). Below neutrality, reduction does not result in the appearance of any detectable intermediate. Repetitive spectral scans of the course of reduction by dithiothreitol (pH 7.4) are presented in Figure 3. The reaction is seen to be accompanied by isosbestic points at 497, 404, 347, 321, 288, and 268 nm. On completion of reduction XIa was re-formed in 98% yield on admittance of oxygen. Absorbance at 370 nm has been shown to be a useful diagnostic tool for the identification of

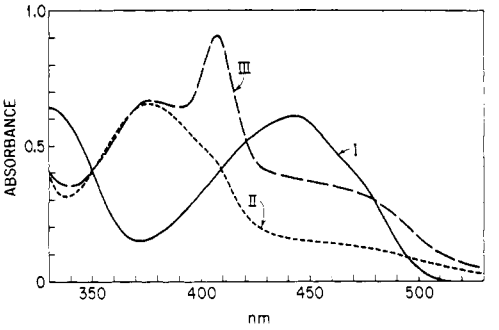
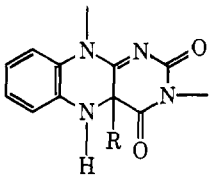


Figure 5. Plots of the absorbance spectra of oxidized (I), reduced (II), and half-reduced (III) 8-cyano-3,10-dimethylisoalloxazine at a concentration of 5.8×10^{-5} M and at pH 10.37. The typical anion radical absorbance at OD_{406} (optical density) may be seen in plot III.

substituted flavins possessing the 4a,5-dihydro structure (XXI).[37] Thus, spectrally the reduction of the 8-cyanoisoal-



XXI

loxazines (XIa,b) appears to be accompanied by the formation of a 4a,5-dihydroisoalloxazine.[37] On the other hand, 1,5-dihydroisoalloxazines possess three maximum absorbances, the first two (λ_{max_1} 350–400 nm and λ_{max_2} ~280 nm) appear as shoulders and λ_{max_3} (~250) as a distinct peak.[39] The 8-cyano substituent may simply favor the electronic transition responsible for λ_{max_1} which would provide for the rather unique spectrum of XIa.

Differentiation between 1,5-dihydro- and 4a,5-dihydroflavins may be made on the basis of the acidity of the N^1 position in the case of the 1,5 isomer (eq 4). Because of the insolubility of XIa at low pH, spectral determinations of the pK_a of dihydro-XIa could not be carried out. For this purpose XIb was employed. Reduction of XIb was easily carried out under argon with dithiothreitol (Experimental Section). The spectra of dihydro-XIb at pH values of 4.49 and 8.15 are presented in Figure 4. Examination of Figure 4 establishes spectral changes to exist on an increase in pH [pH 4.49: λ_{max} 370 (1.37×10^4 M^{-1} cm^{-1}), 345 (1.36×10^4 M^{-1} cm^{-1}), and 259 nm (2.8×10^4 M^{-1} cm^{-1}); pH 8.15: λ_{max} 370 (1.38×10^4 M^{-1} cm^{-1}), 308 (1.5×10^4 M^{-1} cm^{-1}), and 264 nm (3.7×10^4 M^{-1} cm^{-1})]. The inset to Figure 4 displays the change in A_{335} as a function of pH. The data points have been fitted to a single proton titration curve for an acid of $pK_a = 5.9$ (the same pK_a value is obtained when A_{390} and A_{350} are plotted vs. pH). Taking into account the electron-withdrawing ability of the 8-cyano group, the pK_a of dihydro-XIb may be compared to that of the 1,5-dihydroflavins (6.7).[16] Therefore, it appears that reduction of 8-cyanoisoalloxazines provides a 1,5-dihydro derivative.

The Radical Anion of 8-Cyano-3,10-dimethylisoalloxazine. Above neutrality the reduction of XIa,b by any number of reagents (NADH, dithionite, EDTA and light, thiols, etc.) results in the transient appearance of an absorbance at 406 nm (Figure 5). No intermediate is seen on reduction of VII. The transient absorbance at 406 nm is also seen in the oxidation of reduced XIa,b (by CH_2O, O_2, etc.). A plot of the absorbance at 406 nm vs. time invariably increases to a maximum at 50% reduction and then decreases. A plot of percent reduction (A_{443}) with thiophenol and nitroethane vs. A_{406} is presented in Figure 6.

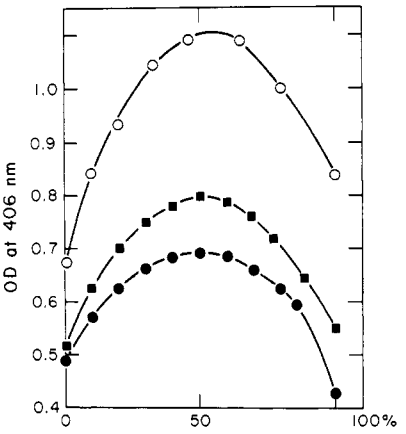


Figure 6. Plots of A_{406} vs. percent reduction of 3,10-dimethyl-8-cyanoisoalloxazine (5×10^{-5} M): $C_2H_5NO_2$ (2×10^{-2} M) as reductant at pH 10.15 (○); C_6H_5SH (1×10^{-3} M) as reductant at pH 9.90 (●) and at pH 10.15 (■).

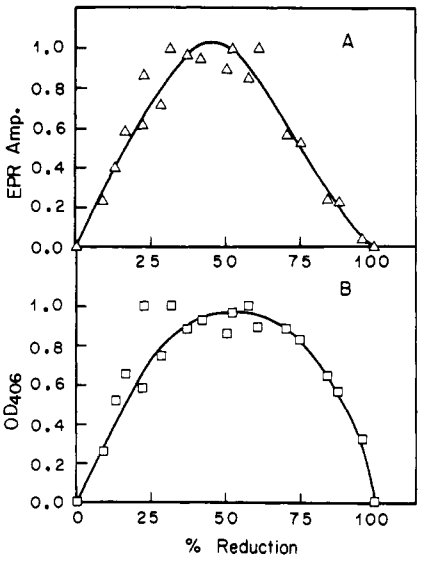


Figure 7. (A) Amplitude of the EPR signal obtained by freezing solutions (pH 9.7 carbonate buffer) at −35 °C vs. percent photocatalytic (with EDTA) reduction of 3,10-dimethyl-8-cyanoisoalloxazine. (B) Absorbance of the same solutions at 406 nm plotted vs. percent reduction.

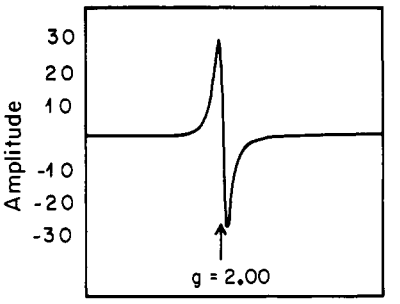


Figure 8. The EPR signal obtained at −35 °C from a half-reduced (52.7%) and frozen (−35 °C) solution (pH 9.7, 0.10 M carbonate buffer) of 8-cyano-3,10-dimethylisoalloxazine at 5.86×10^{-5} M.

These results establish that the species absorbing at 406 nm is formed by reaction of oxidized and reduced 8-cyano-3,10-dimethylisoalloxazine. This, in turn, suggests that the absorbing species is an 8-cyano-3,10-dimethylisoalloxazine radical formed by comproportionation. Electron paramagnetic resonance spectra were obtained at −35 °C in frozen buffered solution. Neither anaerobic (argon) solutions of the oxidized or reduced flavin exhibited detectable electron paramagnetic resonance (EPR) signals. However, when the photoreduction (EDTA) of the oxidized species was carried out and the reaction monitored by both EPR and UV-vis spectroscopy the well-defined EPR signals which were obtained increased to 50% reduction and then decreased as reduction continued (Figure 7). Inspection of Figures 6 and 7 reveals that the appearance and disappearance of both 406-nm absorbance and EPR signal parallel one another. Figure 8 shows the typical EPR signal obtained at −35 °C (in this instance at 52.7% reduction). The value of $g = 2.0021$ is in reasonable agreement with g values determined for other flavin radicals.[40]

In the comproportionation of oxidized and reduced XIa,b the equilibria of eq 4, 5, and 6 are in effect:

$$\mathrm{FlH_2} \underset{+H^+}{\overset{-H^+}{\rightleftharpoons}} \mathrm{FlH^-} \overset{K_{a1}}{} \tag{4}$$

$$\mathrm{FlH^-} + \mathrm{Fl_{ox}} \overset{K_e}{\rightleftharpoons} \mathrm{FlH\cdot} + \mathrm{Fl^-\cdot} \tag{5}$$

$$\mathrm{FlH\cdot} \underset{+H^+}{\overset{-H^+}{\rightleftharpoons}} \mathrm{Fl^-\cdot} \overset{K_{a2}}{} \tag{6}$$

At pH $\gg$ pK_a all dihydroflavin is effectively in the form of FlH^- so that eq 4 may be ignored. If the reduction of XIa is followed to 50% completion where the concentration of radical is maximized and $[FlH^-] = [Fl_{ox}] = [\alpha]$ then the material balance of eq 7 pertains:

$$[\mathrm{Fl}]_{total} = 2\alpha + [\mathrm{FlH\cdot}] + [\mathrm{Fl^-\cdot}] \tag{7}$$

and

$$\mathrm{Fl}_{total} = \frac{2[\mathrm{Fl^-\cdot}]a_H^{1/2}}{(K_{a_2}K_e)^{1/2}} + \frac{[\mathrm{Fl^-\cdot}]a_H}{K_{a_2}} + [\mathrm{Fl^-\cdot}] \tag{8}$$

Assuming $1/K_e^{0.5} \ll 1.0$ the concentration of $[Fl^-\cdot]$ is provided by eq 9:

$$[\mathrm{Fl^-\cdot}] = [\mathrm{Fl}]_{total}\left(\frac{K_{a_2}}{K_{a_2} + a_H}\right) \tag{9}$$

It follows that the titration of a solution composed initially of 50% oxidized and 50% reduced XIa should provide the pK_a of FlH· The 8-cyanoisoalloxazine XIa (5.5×10^{-5} M) was 50% reduced (by thiol or EDTA and light), as shown by the maximum appearance of A_{406}, and an aliquot of an appropriate buffer tipped in and the absorbance at 406 nm determined. Separate experiments were carried out with buffers of known pH and at the end of each experiment the pH was determined by means of a glass electrode. Plots of A_{406} vs. pH employing thiophenol and A_{307} employing photoreduction with EDTA are shown in Figure 9. The points of Figure 9 are experimental and the curves fitted for pK_a values of 10.25 and 10.3, respectively.

The 8-cyano substituent of XIa imparts a great stabilizing influence upon the isoalloxazine radical. Thus, at the concentrations of Fl_{ox} and FlH^- employed it is not possible to detect radical formation with 7-cyano-3,10-dimethylisoalloxazine (VII) or 3-methyllumiflavin. The positions of the λ_{max} values (370, 406, and 480 nm)[41] for the species formed with a pK_a of 10.3 unquestionably identify this as an isoalloxazine radical anion. Here a dilemma arises. The 8-cyano group decreases the pK_a of the 1,5-dihydroisoalloxazine from the usual value of ca. 6.7 to 5.9 but at the same time increases the pK_a of the neutral radical from ~8.4[42] to 10.3. The stabilizing influence of the 8-CN group upon the radical anion is reflected in a decrease in the acidity of the N^5 position of the neutral radical. At present we can offer no assured rationale for this phenomenon. Spin densities of the "normal" neutral flavin

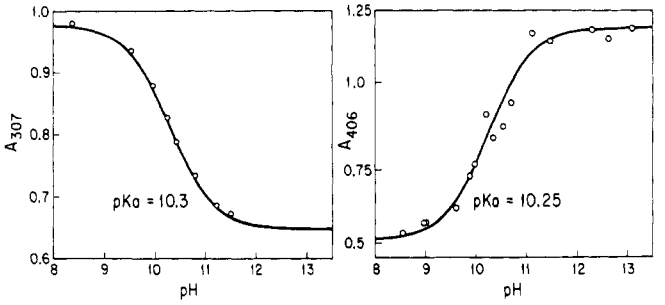
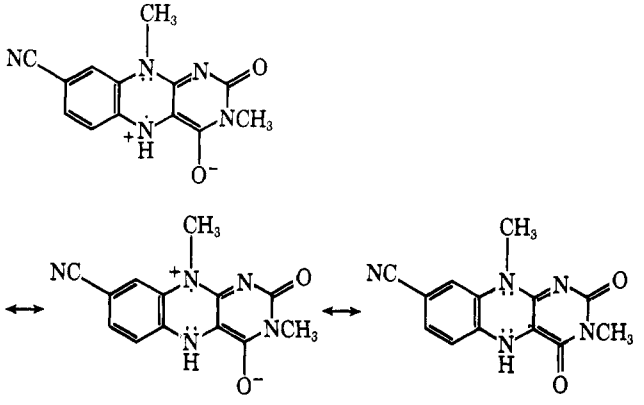


Figure 9. Plots of the absorbances at two wavelengths of half-reduced 8-cyano-3,10-dimethylisoalloxazine vs. the adjusted pH (concentrations of XIa total are 2.5×10^{-5} M for the measurements at A_{307} and 5×10^{-5} M for those at A_{406}).

radical and the flavin radical anion indicate that electron localization in the vicinity of N^5 is about twice as great as at N^{10} and that electron localization at C^6 and C^8 is but slightly less than at N^{10}.[43] (If the 8-cyano substituent were to decrease the spin density at N^5 in favor of N^{10} or C^{4a} by conjugative effect, then the partial positive charge at N^5 would be decreased and the pK_a of the neutral flavin radical increased.)



In summary, a strongly electron-withdrawing substituent at the 8 position of the isoalloxazine ring not only enhances the rates of substrate oxidation when a nucleophilic addition of substrate is required but also stabilizes the isoalloxazine radical and, therefore, should enhance free-radical oxidation processes.

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