The Chemistry of an Electron-Deficient 5-Deazaflavin. 8-Cyano-10-methyl-5-deazaisoalloxazine

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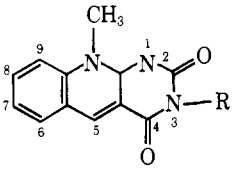
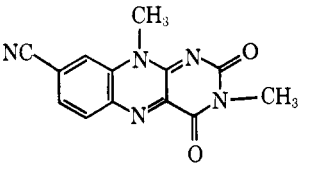
Abstract: The syntheses of 8-cyano-3-R-10-methyl-5-deazaisoalloxazines (III) are described as is a comparison of the reactions of III and 3-R-10-methyl-5-deazaisoalloxazine (I) with a number of nucleophiles. Thiol anions, SO_3^{2-}, HSO_3^-, CN^-, HO^-, and HO_2^- add to the 5 position of III. Though RS^- species provide no detectable reaction with I, the pseudo-first-order rate constants for addition to III exceed 170 s^{-1}. The formation equilibrium constants for β-mercaptoethanol and dithiothreitol were found to be 490 and 1800 M^{-1}, respectively. The second-order rate constants and addition equilibrium constants for SO_3^{2-}, HO^-, and CN^- to III exceed those constants determined with I by 10^1 to 10^2. The reaction of CN^- with III is characterized by a consecutive pseudo-first-order A → B → C process. The intermediate B represents the carbon acid XVII derived by addition of CN^- to the 5 position of III. Hydroxide ion mediated 1,3-prototropic shift converts XVII to 5,8-dicyano-1,10a-dihydro-10-methyl-5-deazaisoalloxazine (XVIII). The carbon acid XVIII possesses a pK_a of 4.5 for dissociation of the 10a proton due to resonance stabilization of the electron pair of the carbanion to both 5- and 8-cyano groups. The 8-cyano-1,5-dihydro-5-hydroperoxy-3,10-dimethyl-5-deazaisoalloxazine (XVI) obtained on reaction of hydrogen peroxide with III has been isolated and characterized. The rather stable hydroperoxide XVI oxidizes I^- to I_3^- with a second-order rate constant 600 times greater than that determined for the oxidation of I^- by *tert*-butyl hydroperoxide (in methanol). The second-order rate constants for oxidation of I^- and thioxane by XVI are only 15 and 7 times smaller than are the corresponding rate constants previously determined with 4a-hydroperoxy-*N*(5)-ethyl-3-methyllumiflavin.

Since the synthesis of 5-deazariboflavin by Cheng and co-workers,[1] 5-deazaflavins have been used extensively as flavin analogues in both model and enzyme reactions. The major purpose of these investigations has been to establish the role of position 5 of the isoalloxazine moiety in flavin-catalyzed redox reactions. Thus, with 3,10-dimethyl-5-deazaisoalloxazine (Ia), Brüstlein and Bruice[2a] demonstrated direct hydrogen transfer from NADH to yield the corresponding 1,5-dihydro-5-deazaisoalloxazine while Shinkai and Bruice[2b] found 1,5-dihydro-3,10-dimethyl-5-deazaisoalloxazine to transfer the elements of hydride directly in carbonyl group reduction. Similar hydrogen transfers have also been observed in a NADH-FMN oxidoreductase system when deaza-FMN is used as the substrate.[3] More recently, apoproteins of a number of flavoenzymes have been reconstituted with the corresponding 5-deaza coenzyme.[4] The enzyme-bound deazaflavins are invariably reduced by the specific enzyme substrates, though at somewhat slower rates. Except for the case of glucose oxidase,[5] reoxidation of the dihydrodeazaflavins by the oxidized substrates has also been observed. Reduction and reoxidation of these enzyme-bound deazaflavins all involve direct hydrogen transfer between substrates and the deaza coenzyme. The transient formation of the semiquinone form of 5-deazariboflavin was observed by Edmondson et al.[6] whereas Hersh et al.[7] recently reported the formation of the free-radical form of 5-deaza FAD bound to D-amino acid oxidase.

In many flavoproteins, an electron-withdrawing group is attached to the α carbon at position 8 of the isoalloxazine moiety.[8] In our investigations on model flavin reactions, we have noted that the electron-deficient 8-cyano-3,10-dimethylisoalloxazine (II) is a better oxidizing agent than normal flavins.[9] Thus, in nonenzymatic systems, 3-methyllumiflavin does not react with nitroalkanes[9] whereas II, like the flavoenzymes glucose oxidase and D-amino acid oxidase,[10] readily reacts with nitroalkanes to give the corresponding aldehydes and the dihydro derivative of II.[9] The comproportionation reaction of reduced and oxidized II to yield two radical species has also been found to be more exothermic than seen with normal flavins.[11] In view of the special behavior of 8-cyanoisoalloxazine (II) relative to normal flavins, it would be of interest to compare the properties of a 5-deazaflavin having a strong electron-withdrawing group at position 8 with those



Ia, R = CH_3
b, R = $(CH_2)_3SO_3K$



II

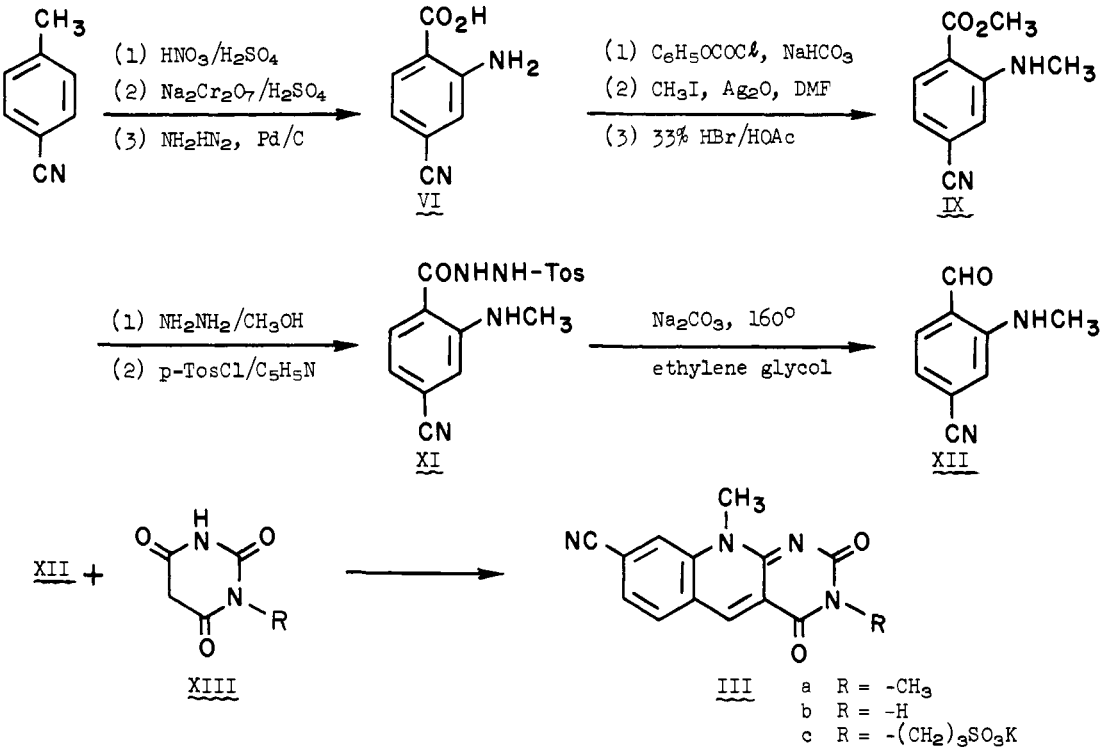
of 3,10-dimethyl-5-deazaisoalloxazine (I). This paper reports the synthesis and chemical properties of 8-cyano-10-methyl-5-deazaisoalloxazines (III). Comparison of this compound with I was made whenever possible.

Experimental Section

Materials. All chemicals were reagent grade and, unless otherwise stated, were used without further purifications. Buffers and stock solutions were prepared in doubly glass-distilled water. The following buffers were used: acetate (pH 4–6), morpholinopropanesulfonic acid (MOPS, pH 6–7.5), phosphate (pH 7–8), borate (pH 8–9.5) and carbonate (pH >9.5). All buffer solutions were 0.1 M in the buffer species unless otherwise mentioned, the ionic strength being adjusted to 1.0 with potassium chloride. Stock solutions of the deazaflavins were prepared either in purified acetonitrile (Ia and IIIa) or in distilled water (Ib and IIIc). Thioxane, nitroethane, and nitropropane were distilled before use. The nitroalkane anions were prepared on the day of use by addition of 1 equiv of potassium hydroxide to cooled suspensions of the nitroalkane in water. Stock solutions of other nucleophiles (potassium sulfite, potassium cyanide, mercaptoethanol, dithiothreitol, and hydrogen peroxide) were prepared within a few days of use. Concentrations of the thiol stock solutions were determined by titration with iodine. Stock solutions of H_2O_2 contained 0.05 M of EDTA disodium salt. The concentration of H_2O_2 was determined by reacting a suitably diluted solution with excess KI in an acidic medium and titrating the triiodide with standard sodium thiosulfate using ammonium molybdate as catalyst.[12]

Apparatus. Melting points were taken in open capillaries on a Thomas-Hoover melting point apparatus or on a Mel-Temp and were uncorrected. All infrared spectra were taken with KBr pellets on a Perkin-Elmer 137 spectrophotometer. Routine NMR spectra were recorded on a Varian T-60 spectrometer. Fourier transform proton magnetic resonance spectra were recorded on a Varian XL100 spectrophotometer using a 4-μs pulse width. Absorption spectra were obtained on a Cary 118 spectrophotometer. Fluorescence spectra were

Scheme I



recorded on a Perkin-Elmer Model 512 fluorescence spectrophotometer. All pH values were measured at 30 ± 0.1 °C with a Radiometer Model M26 pH meter equipped with a Radiometer GK2303C glass-calomel combination electrode. Microanalyses were performed by Chemalytics Inc., Tempe, Ariz., or Elek Microanalytical Laboratories, Torrance, Calif. Spectrophotometric titrations and measurements of redox potentials were performed using the assembly previously described.[13] For redox potential determinations, the glass support cap carries a Metrohm EA 125 U micro glass-calomel combination electrode, an inlet for argon, a platinum electrode, and a microburet filled with a deoxygenated solution of the reducing agent. Side arms on the microburet and on the platinum electrode provide outlets for the argon gas. Reaction rates were determined on a Gilford Model 2000 spectrophotometer, a Cary 118 spectrophotometer, or a Durrum-Gibson Model D-100 stopped-flow instrument.

Syntheses. The synthetic route to 8-cyano-3,10-dimethyl-5-deazaisoalloxazine (III) is shown in Scheme I.

3-Nitro-4-tolunitrile (IV). To 58 g (0.5 mol) of 4-tolunitrile in a 1-L round-bottomed flask cooled in an ice-salt bath was added, with vigorous stirring, a previously cooled (<5 °C) nitrating mixture of 120 mL of H_2SO_4 and 100 mL of nitric acid at such a rate that the temperature did not rise above 10 °C. When addition was complete, the reaction mixture was stirred at room temperature for another 0.5 h when it turned into a white solid mass. Ice-water (500 mL) was immediately added to the reaction mixture. The product was extracted twice with $CHCl_3$. The $CHCl_3$ solution was dried ($MgSO_4$) and evaporated in vacuo, giving 75 g (94% yield) of 3-nitro-4-tolunitrile. Crystallization of the crude product from $CHCl_3$-petroleum ether (bp 30-60 °C) gave white needles, mp 107 °C (lit.[14] 107 °C).

4-Cyano-2-nitrobenzoic Acid (V). Sixty grams of IV (0.37 mol) was suspended in 1 L of 70% H_2SO_4 at room temperature in a three-necked flask fitted with a mechanical stirrer and a thermometer. Sodium dichromate dihydrate (150 g, 0.5 mol) was added portionwise, with constant stirring, over 2-3 days. The temperature of the reaction mixture was always kept below 30 °C. The dark green reaction mixture was then poured onto 2 L of ice. The white precipitate was collected by filtration, dissolved in 5% aqueous Na_2CO_3 (12 g of the starting material was recovered from the solid that was insoluble in Na_2CO_3 solution), filtered, and reprecipitated by neutralization with dilute HCl. The crude product (44 g, 78%) was recrystallized from ethanol to give white crystals: mp 205.5-206 °C; IR 2500-3000 (COOH), 2250 (CN), 1730 (COOH), 1560 and 1370 cm^{-1} (NO_2); NMR (Me_2SO-d_6) δ 8.17 (d, 1 H, J = 8 Hz), 8.30 (d-d, 1 H, J = 2, 8 Hz), and 8.58 ppm (d, 1 H, J = 2 Hz).

4-Cyanoanthranilic Acid (VI). To 40 g (0.2 mol) of V dissolved in 500 mL of 95% EtOH at 50 °C was added 0.2 g of 5% palladium on charcoal (moistened with EtOH), followed by the slow addition of 25 mL of hydrazine (85% in water) over a period of 1 h. A further 0.2 g of the moistened catalyst was added and the mixture was heated under gentle reflux for 24 h. The reaction mixture was filtered through Celite and evaporated to dryness under reduced pressure. The residue was dissolved in a minimum volume of 5% Na_2CO_3, filtered, and acidified with dilute HCl. The yellow precipitate was collected by filtration and recrystallized from aqueous ethanol. The yield was 30 g (90%) after crystallization: mp 255 °C dec; IR 3250 and 3150 (NH_2), 2500-3000 (COOH), 2230 (CN), and 1675 cm^{-1} (COOH); NMR (Me_2SO-d_6) δ 6.83 (d, 1 H, J = 8 Hz), 7.17 (s, 1 H), and 7.83 ppm (d, 1 H, J = 8 Hz).

Anal. Calcd for $C_8H_6N_2O_2$: C, 59.26; H, 3.73; N, 17.28. Found: C, 59.38; H, 3.93; N, 17.37.

4-Cyano-*N*-benzyloxycarbonylanthranilic Acid (VII). Twenty grams of VI (0.12 mol) was added to a suspension of 84 g (1 mol) of $NaHCO_3$ in 500 mL of water in a 1-L beaker. The mixture was mechanically stirred while 22 g (0.13 mol) of benzyl chloroformate was added in five equal portions over 30 min. Stirring was continued for a further 4 h at room temperature. Then the mixture was neutralized with 1:1 HCl and extracted twice with ether. The ethereal solution was dried over $MgSO_4$ and evaporated to dryness to give 33 g of the crude product. Crystallization from ethyl acetate gave 24 g (65% yield) of white crystals: mp 184.5-185 °C; IR 3300 (NH), 2500-3000 (COOH), 2230 (CN), 1740 ($PhCH_2OC{=}O$), and 1680 cm^{-1} (COOH); NMR ($CDCl_3$) δ 5.25 (s, 2 H, $PhCH_2$), 5.91 (broad, 1 H, NH), 7.40 (m, 6 H, ArH), 8.20 (d, 1 H, J = 8 Hz, ArH), 8.90 (d, 1 H, J = 2 Hz, ArH), and 10.35 ppm (s, 1 H, COOH).

Methyl 4-cyano-*N*-benzyloxycarbonyl-*N*-methylanthranilate (VIII) was prepared by the general method of Olsen.[15] *p*-Cyano-*N*-benzyloxycarbonylanthranilic acid (VII, 23 g, 0.077 mol) was dissolved in 150 mL of anhydrous DMF. Fifty grams (0.22 mol) of Ag_2O and 88 g (0.62 mol) of methyl iodide were added. The mixture was refluxed for 10 h and then filtered. The filtrate was diluted with an equal volume of $CHCl_3$, and washed once with 100 mL of 5% aqueous KCN and five times with water to remove most of the DMF. The chloroform extract was dried ($MgSO_4$) and rotary evaporated to give 22 g of the crude product which was chromatographed on silica gel with 1:1 $CHCl_3$-hexane as eluent to give 18 g (68% yield) of the pure product. Recrystallization from benzene-hexane mixture gave white crystals: mp 109.5-110 °C; IR 2230 (CN), 1720 and 1730 cm^{-1} ($COOCH_3$ and $PhCH_2OCO$); NMR ($CDCl_3$) δ 3.28 (s, 3 H, NCH_3), 3.66 (s,

3 H, $COOCH_3$), 5.20 (s, 2 H, $PhCH_2$), 7.32 (m, 5 H, ArH), 7.5 (d, 1 H, J = 2 Hz, ArH), 7.52 (d-d, 1 H, J = 2, 8 Hz, ArH), and 7.92 (d, 1 H, J = 8 Hz, ArH).

Methyl 4-cyano-*N*-methylanthranilate (IX) was obtained by stirring 17 g (0.052 mol) of VIII in 15 mL of 33% HBr in glacial acetic acid at room temperature for 1 h. Ether was added to precipitate the hydrobromide salt which was filtered and neutralized with aqueous Na_2CO_3. The product was extracted twice with ethyl acetate which after drying ($MgSO_4$) and rotary evaporation gave 9.5 g (95% yield) of the pure product. An analytical sample was prepared by recrystallization from $CHCl_3$-hexane to give light yellow needles: mp 121.5 °C; IR 3320 (NH), 2220 (CN), and 1685 cm^{-1} ($COOCH_3$); NMR ($CDCl_3$) δ 2.90 (s, 3 H, NCH_3), 3.87 (s, 3 H, $COOCH_3$), 6.73 (m, 2 H, ArH), and 7.80 (d, 1 H, J = 8 Hz, ArH).

Anal. Calcd for $C_{10}H_{10}N_2O_2$: C, 63.14; H, 5.30; N, 14.73. Found: C, 63.11; H, 5.38; N, 14.12.

4-Cyano-*N*-methylanthranilic Acid Hydrazide (X). Compound IX (9.0 g, 0.047 mol) was dissolved in 100 mL of 1:1 $CHCl_3$-MeOH and 50 mL of 97% hydrazine was added. The mixture was refluxed for 30 min, cooled, and concentrated to about 30 mL. Upon addition of 300 mL of cold water, the yellow product was precipitated out and was collected by filtration (7.5 g, 84% yield). For analysis, a sample was recrystallized from ethanol (yellow needles): mp 179.5–180 °C; IR 3300 (NH), 2240 (CN), and 1630 cm^{-1} (CO); NMR (TFA) δ 3.43 (s, 3 H, NCH_3) and 8.25 (m, 3 H, ArH).

1-(4-Cyano-*N*-methylanthraniloyl)-2-(*p*-toluenesulfonyl)hydrazine (XI) was prepared from X by the general method of O'Brien et al.[1] in 90% yield. Recrystallization from ethanol gave orange-red crystals: mp 224–225 °C dec; IR 3200, 3300, 3350 (NH), 2230 (CN), 1660 and 1670 cm^{-1} (CO); NMR (TFA) δ 2.47 (s, 3 H= $ArCH_3$), 3.28 (s, 3 H, NCH_3), 7.35 (d, 2 H, J = 8 Hz, ArH), 7.82 (d, 2 H, J = 8 Hz, ArH), and 8.0–8.2 ppm (m, 3 H, ArH).

Anal. Calcd for $C_{16}H_{16}N_4O_3S$: C, 55.80; H, 4.68; N, 16.27. Found: C, 55.60; H, 4.92; N, 16.03.

4-Cyano-*N*-methylanthranilaldehyde (XII) was prepared from XI by McFayden-Stevens' method.[16] To 5 g (0.0145 mol) of XI dissolved in 80 mL of ethylene glycol at 160 °C was added, with vigorous stirring, 5 g of anhydrous Na_2CO_3 over 60 s. The reaction mixture was stirred at the same temperature for a further 30 s whereupon it turned brown. It was then cooled and poured onto 250 mL of ice. The product that precipitated out was extracted into ether. Evaporation of ether after drying (Na_2SO_4) gave 2.2 g of an orange-brown solid which was immediately purified on a column of silica gel (60 g), eluting with chloroform. The pure aldehyde was obtained as a bright yellow solid (1.8 g, 78% yield): IR 3300 (NH), 2220 (CN), and 1670 cm^{-1} (CHO); NMR ($CDCl_3$) δ 2.92 (d, 3 H, J = 5 Hz, NCH_3), 6.8–7.0 (m, 2 H, ArH), 7.50 (d, 1 H, J = 8 Hz, ArH), and 9.83 ppm (s, 1 H, CHO). An analytical sample was prepared by recrystallization from chloroform–hexane to give yellow needles, mp 151–151.5 °C.

Anal. Calcd for $C_9H_8N_2O$: C, 67.48; H, 5.04; N, 17.49. Found: C, 67.63; H, 4.91; N, 17.76.

8-Cyano-3,10-dimethyl-5-deazaisoalloxazine (IIIa). 4-Cyano-*N*-methylanthranilaldehyde (XII, 1.6 g, 0.01 mol) was suspended in 250 mL of water. A solution of 2 g (0.014 mol) of *N*-methylbarbituric acid (XIII, R = CH_3), prepared in 40–60% yield from diethyl malonate and *N*-methylurea,[17] mp 129.5–131.5 °C (lit.[18] 132–133 °C), in 20 mL of water was added and the mixture was stirred in a hot water bath (70–80 °C) for 30 min during which time a bright yellow, fluffy solid was formed. This was collected by filtration and recrystallized from DMF to give 2.0 g (75% yield) of bright yellow fine needles: mp >360 °C; IR 2220 (CN), 1700, 1650, and 1625 cm^{-1} (CO and C=N); NMR (TFA) δ 3.67 [s, 3 H, N(3) CH_3], 4.58 [s, 3 H, N(10) CH_3], 8.17 [d-d, 1 H, J = 1, 8 Hz, C(7) H], 8.60 [d, 1 H, J = 8 Hz, C(6) H], 8.77 [d, 1 H, J = 1 Hz, C(9) H], and 9.83 ppm [s, 1 H, C(5) H]; λ_{max} (pH 7.5, Figure 1), 264 nm (ϵ 4.1 × 10^4 M^{-1} cm^{-1}), 313 (1.34 × 10^4 M^{-1} cm^{-1}), 325 (1.47 × 10^4 M^{-1} cm^{-1}), and 406 (1.11 × 10^4 M^{-1} cm^{-1}).

Anal. Calcd for $C_{14}H_{10}n_4O_2 \cdot (0.3)DMF$: C, 62.10; H, 4.20; N, 20.91. Found: C, 62.19; H, 4.18; N, 20.31.

8-Cyano-10-methyl-5-deazaisoalloxazine (IIIb) was prepared in 80% yield from XII and barbituric acid by the same procedure as that described for IIIa. This compound was very insoluble and was used without recrystallization. NMR (TFA) δ 4.63 [s, 3 H, N(10) CH_3], 8.26 [d, 1 H, J = 8 Hz, C(6) H], 8.63 [d, 1 H, J = 8 Hz, C(7) H], 8.83 [s, 1 H, C(9) H], and 9.82 ppm [s, 1 H, C(5) H].

8-Cyano-10-methyl-3-sulfopropyl-5-deazaisoalloxazine potassium salt (IIIc) was prepared in 86% yield from IIIb and 1,3-propanesultone by the method of Blankenhorn.[19] Recrystallization of the crude product from water–ethanol gave chromatographically (TLC in 1-butanol/acetic acid/water, 2:1:1) pure, fine yellow needles: NMR (D_2O) δ 1.7–2.3, 2.7–3.2, 3.7–4.3 [m, 2 H each, 3 (CH_2)], 4.07 [s, 3 H, N(10) CH_3], 7.78 [s, 1 H, C(6) H], 7.98 [s, 1 H, C(7) H], 8.23 [s, 1 H, C(9) H], and 8.80 ppm [s, 1 H, C(5) H]. This compound has an absorption spectrum identical with that of IIIa (Figure 1, inset). Fluorescence spectrum of this compound (excitation wavelength 390 nm) shows λ_{max} at 459 nm.

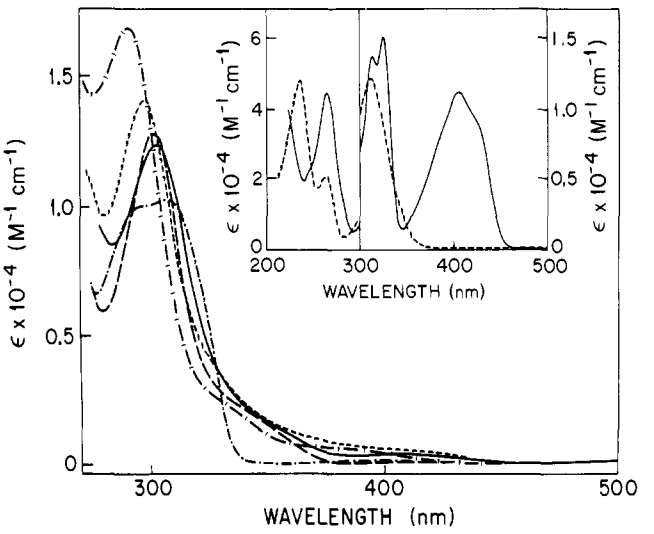


Figure 1. Absorption spectra of derivatives of 5-deazaisoalloxazines I and III: (—) 2-mercaptoethanol adduct to III at pH 9.8, [2-mercaptoethanol] = 0.3 M; (- - - -) sulfite adduct to III at pH 8.6, [K_2SO_3] = 0.001 M; (— | — | —) hydroxide adduct to III in 5 M KOH; (- - - - - -) nitroethane adduct to III at pH 9.3, [nitroethane] = 0.3 M; (- · - · -) cyanide adduct to I at pH 9.2, [KCN] = 0.01 M. All spectra were recorded against reference cuvettes containing equal concentrations of the nucleophiles in the appropriate buffers. The absorption spectrum of dithiothreitol adduct to III is almost identical with that of 2-mercaptoethanol adduct to III and is therefore not shown. Inset shows the absorption spectra of 8-cyano-10-methyl-3-sulfopropyl-5-deazaisoalloxazine (IIIc, solid line) and 8-cyano-1,5-dihydro-3,10-dimethyl-5-deazaisoalloxazine (XIV, broken line) at pH 7.5.

10-Methyl-3-sulfopropyl-5-deazaisoalloxazine potassium salt (Ib) was prepared by published procedures.[19] The UV spectrum of this compound is identical with that of the corresponding 3-methyl derivative, at pH 7, λ_{max} 405 nm (ϵ 9.6 × 10^3 M^{-1} cm^{-1}), 390 (1.11 × 10^4 M^{-1} cm^{-1}), 323 (1.04 × 10^4 M^{-1} cm^{-1}), and 256 (4.03 × 10^4 M^{-1} cm^{-1}). Fluorescence spectrum (excitation at 390 nm) shows λ_{max} at 441 nm.

8-Cyano-1,5-dihydro-3,10-dimethyl-5-deazaisoalloxazine (XIV) was prepared from IIIa under an atmosphere of nitrogen. Typically, 0.26 g (1 mmol) of IIIa was suspended in 15 mL of 0.1 N KOH. Sodium dithionite (0.3 g, 1.7 mol) was added in small portions while the reaction mixture was stirred magnetically. The mixture became reddish-brown at first but gradually turned into a bright yellow homogeneous solution. The product was precipitated out upon acidification with acetic acid. This was collected by filtration, dissolved in dilute NH_4OH, filtered, and reprecipitated with acetic acid. The yield was 70–85%. The dried product usually contains traces (<1%) of the oxidized form (IIIa) and was stable indefinitely under nitrogen. The absorption spectrum is shown in Figure 1. NMR (Me_2SO-d_6) δ 3.28 [s, 3 H, N(3) CH_3], 3.38 [s, 3 H, N(10) CH_3], 3.83 [s, 2 H, C(5) H_2], and 6.1–6.9 ppm (m, 3 H, ArH).

1,5-Dihydro-3,10-dimethyl-5-deazaisoalloxazine (XV) was prepared from Ia in a similar manner as above. The NMR data have been reported elsewhere.[2a] λ_{max} in water, pH 7: 256 nm (ϵ 1.64 × 10^4 M^{-1} cm^{-1}), 277 (1.07 × 10^4 M^{-1} cm^{-1}), and 316 (1.15 × 10^4 M^{-1} cm^{-1}).

8-Cyano-1,5-dihydro-3,10-dimethyl-5-hydroperoxy-5-deazaisoalloxazine (XVI). To a suspension of 130 mg (0.5 mmol) of finely ground 8-cyano-3,10-dimethyl-5-deazaisoalloxazine (IIIa) in 45 mL of dioxane (freshly distilled over sodium metal) was added 15 mL of an

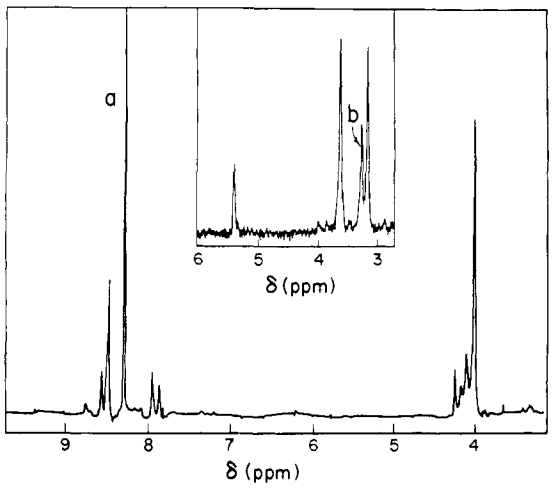


Figure 2. The proton magnetic resonance spectrum of 5,8-dicyano-1,10a-dihydro-10-methyl-3-sulfopropyl-5-deazaisoalloxazine (XVIII in polysol-*d*). This compound was obtained by lyophilizing a solution of IIIc (20 mg) in 50 mL of water containing 0.002 M KCN (pH 9.3) after the mixture has been left overnight at room temperature and acidified to pH 3 with HCl. Peak (a) is due to the solvent. The broad peak between 5.8 and 6.4 ppm disappeared upon addition of a drop of D_2O. Inset is the proton magnetic resonance spectrum of 8-cyano-1,5-dihydro-3,10-dimethyl-5-hydroperoxy-5-deazaisoalloxazine (XVI) in Me_2SO-d_6, (b) is the water peak. The peak at 5.40 ppm remained unchanged upon addition of a drop of DCl.

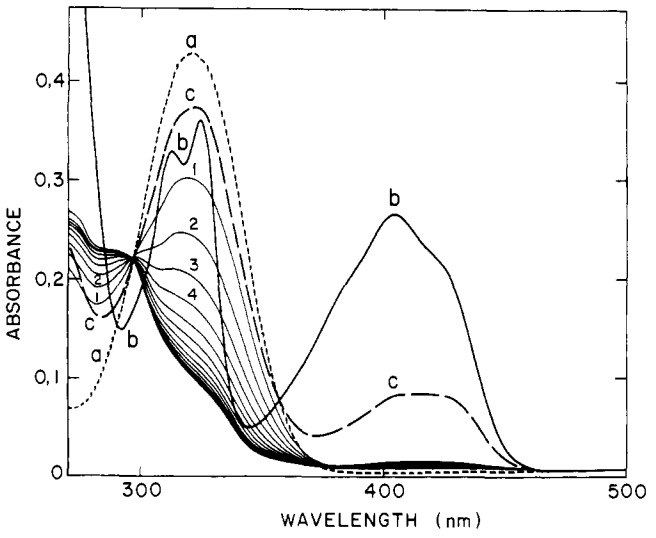


Figure 3. Repetitive scan of the reaction of IIIc with H_2O_2 at pH 9.0. Trace (a) is the spectrum of a solution of 8-cyano-1,5-dihydro-3,10-dimethyl-5-hydroperoxy-5-deazaisoalloxazine in water (2.4×10^{-5} M). Line (b) is the spectrum of an equal concentration of IIIc at pH 9.0. Line (c) was recorded at 2 nm/s 15 s after adding H_2O_2 (0.01 M). Lines 1 onward were scanned at 180-s intervals.

approximately 0.1 M solution of ammonium bicarbonate at pH 9.3. Hydrogen peroxide solution (30%, 0.5 mL) was added. The mixture was stirred vigorously. After 30–45 min, almost all the starting material had gone into solution. The yellow nonfluorescent solution was rapidly filtered and freeze-dried to give a white powder (130 mg, 93% crude yield). This was washed with small amounts of water and ethanol and recrystallized from DMF-ether: mp 269–272 °C dec; IR 3300–3500 (-OH), 2230 (-CN), and 1730 cm^{-1} (-CO); NMR (Figure 2, Me_2SO-d_6) δ 3.20 [s, 3 H, N(3) CH_3], 3.63 [s, 3 H, N(10) CH_3], 5.40 [s, 1 H, C(5) H], 7.72 [d-d, 1 H, J = 1, 8 Hz, C(7) H], 7.95 [d, 1 H, J = 1 Hz, C(9) H], and 8.15 ppm [d, 1 H, J = 8 Hz, C(6) H]. The UV spectrum (Figure 3) shows λ_{max} at 320 nm.

Anal. Calcd for $C_{14}H_{12}N_4O_4$: C, 56.00; H, 4.03; N, 18.66. Found: C, 58.34; H, 3.94; N, 19.09.

Determination of E_m Values. Sodium dithionite solutions cannot be employed as the titrant since dithionite contains traces of sulfite

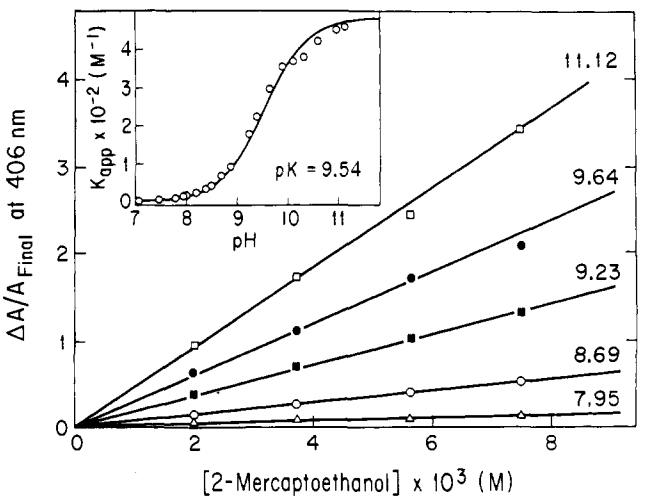


Figure 4. Representative plots of $\Delta A/A_{final}$ at 406 nm vs. concentration of 2-mercaptoethanol at various pH values. Inset is a plot of K_{app} vs. pH for the reaction of 2-mercaptoethanol with III.

that readily forms an adduct with deazaflavins. Instead, sodium borohydride (0.01 M in dilute KOH) was used. The pH meter was calibrated with saturated quinhydrone solutions at two different pH values. The calomel terminal of the combination electrode and the platinum electrode were used for measurement of potentials.

The deazaflavin solution (25 mL, 2×10^{-5} M) in pH 8, 0.025 M Tris buffer contained 1×10^{-7} M of riboflavin as a redox mediator. It was deoxygenated with purified argon gas for 2 h before titration began. At least 15 min was required after addition of each small aliquot of the reducing agent for the potential reading (E_{obsd}) to stabilize. The fraction of deazaflavin reduced was determined from the decrease in the absorption of λ_{max} 406 nm. The E_m values were read off from plots of E_{obsd} vs. % reduction as the value of E_{obsd} at 50% reduction.

Determination of Equilibrium Constants. All equilibrium constants referred to in this paper are association constants. The determination of equilibrium constants for the reaction of III with dithiothreitol is typical. Two milliliters of the buffer in a 1-cm path length quartz cuvette was equilibrated to 30 ± 0.1 °C in the cell compartment of a Cary 118 spectrophotometer. The baseline (390–480 nm) was recorded. A suitable amount of the deazaflavin (III) stock solution was added and the spectrum in the visible region was obtained. An aliquot (<100 μL) of the thiol stock solution was then added. Equilibrium was reached almost instantaneously. The final spectrum was recorded. At pH 10 and above, measurements were made on a Cary 15 spectrophotometer under an atmosphere of nitrogen. At least three measurements were made for each pH value. Typical plots of $\Delta A/A_{final}$ at 406 nm (λ_{max} of III) vs. concentrations of thiols are shown in Figure 4. The slope of each line gives the apparent equilibrium constant, K_{app}, at the particular pH. With hydroxide ion, an aliquot of the deazaflavin stock solution was added to aqueous solutions of KOH at various concentrations (0.01–0.6 M). The decrease in absorbance at λ_{max} 406 nm, relative to the absorbance of a solution of an equal concentration of the deazaflavin in H_2O, was noted immediately and the equilibrium association constant was calculated as described above.

Equilibrium constants for sulfite addition to III were all determined under nitrogen atmosphere. The dimerization equilibrium $2HSO_3^- \rightleftarrows S_2O_5^{2-} + H_2O$ ($K = 7 \times 10^{-2}$ M^{-1})[20] was ignored in the calculations.

The various equilibrium constants for the 3-methyl and 3-sulfopropyl derivatives of I and III were found to be identical. The 3-sulfopropyl derivatives were used in reactions with cyanide, hydroxide, and nitroalkane carbanions because of their greater solubilities. The K_{app} values for cyanide addition to I were determined from the A_∞ values of the respective kinetic runs.

Kinetics. Unless otherwise stated, all kinetic measurements were carried out in aqueous solutions (2% acetonitrile when Ia or IIIa was used) at 30 ± 0.1 °C, μ = 1.0 with KCl. The rate of reaction of the deazaflavins with sulfite and HO^- were determined anaerobically on the stopped-flow instrument. All other rates were measured aerobically on a Gilford or a Cary 118 spectrophotometer. No buffer catalysis was detectable for any of the reactions investigated. Second-

Table I. Values of the Kinetic Apparent pK_a of Thiols and the Equilibrium Constants (K_{eq}) for Thiol Anion Addition to III

	2-Mercaptoethanol	Dithiothreitol
K_{eq}, M^{-1}	490 ± 5	1800 ± 20
pK_a	9.54 ± 0.02[a]	9.62 ± 0.02[b]

[a] Lit.[21] 9.5. [b] A value of 9.72 was obtained by spectrophotometric titration of dithiothreitol at 270 nm.

Table II. Pseudo-First-Order Rate Constants (k_{obsd}) for the Reaction of III with Sulfite[a]

	k_{obsd}, s^{-1} [b]	
[Sulfite], M	pH 5.0	pH 8.6
1.2×10^{-4}	0.135	4.68
2.4×10^{-4}	0.215	8.17
3.6×10^{-4}	0.295	11.7

[a] [III] = 10^{-5} M. [b] These are average values of duplicate runs.

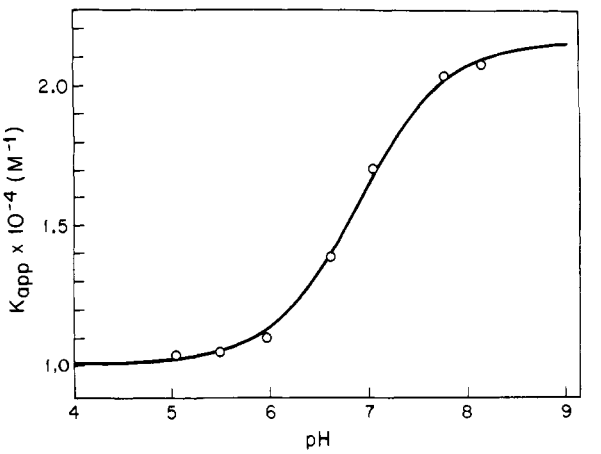


Figure 5. pH dependence of the apparent equilibrium constants of the reaction of 8-cyano-3,10-dimethyl-5-deazaisoalloxazine with sulfite, (○) experimental points. Solid line is the computer fit to eq 3, assuming $K_1 = 1.0 \times 10^4$ M^{-1} and $K_2 = 2.16 \times 10^4$ M^{-1}.

order rate constants were calculated from plots of the pseudo-first-order rate constants (k_{obsd}) vs. concentrations of the nucleophile at a given pH.

Results

The association equilibrium constants for adduct formation of III with dithiothreitol and 2-mercaptoethanol

$$\text{III} + \text{RS}^- \overset{K_{eq}}{\rightleftharpoons} \text{adduct} \quad (1)$$

$$K_{app} = [\text{adduct}]/[\text{III}][\text{RSH}]_T \quad (2)$$

are appreciable. The pH-dependent apparent equilibrium constant, K_{app}, defined by eq 2 where $[\text{RSH}]_T = [\text{RSH}] + [\text{RS}^-]$ is related to the true equilibrium constant, K_{eq}, by

$$1/K_{app} = a_H/K_aK_{eq} + 1/K_{eq} \quad (3)$$

where K_a is the ionization constant of the thiol. Thus the pH dependence of K_{app} is sigmoidal in nature (Figure 4, inset) and from the slope and intercept of a plot of $1/K_{app}$ vs. a_H (not shown) one obtains the values of K_a and K_{eq}. These values are listed in Table I. The absorption spectra of the thiol adducts of III are almost identical and only that of 2-mercaptoethanol is shown (Figure 1). The pseudo-first-order rate constants for approach to equilibrium (at pH 9.5, [III] = 2×10^{-5} M and [thiol] ≈ 1×10^{-3} M) in thiol adduct formation were too great to be measured by the stopped-flow instrument (mixing time < 4 ms) and thus exceed 170 s^{-1}. The simple 5-deazaflavin (I) does not react with thiols.

The values of K_{app} for the reaction of III with sulfite, when plotted vs. pH, provides a sigmoid plot (Figure 5) which may be fitted by a single acid dissociation curve for a pK_a of 6.90 (pK_a of HSO_3^- 7.21).[22] At pH values above and below the pK_a of HSO_3^-, K_{app} approaches constant values. It is apparent that both HSO_3^- and SO_3^{2-} react with III:

$$HSO_3^- + \text{III} \overset{K_{eq1}}{\rightleftharpoons} \text{adduct} - A$$

$$SO_3^{2-} + \text{III} \overset{K_{eq2}}{\rightleftharpoons} \text{adduct} - B \quad (4)$$

so that

$$K_{app} = K_{eq_1}a_H/(K_a + a_H) + K_{eq_2}K_a/(K_a + a_H) \quad (5)$$

From the plot of Figure 5, the values of K_{eq_1} and K_{eq_2} are 1.01×10^4 and 2.16×10^4 M^{-1}, respectively. The rates of approach to equilibrium were measured anaerobically (406 nm) at pH 8.6 (SO_3^{2-}) and 5.0 (HSO_3^-). The reaction was strictly first order. The pseudo-first-order rate constants (k_{obsd}) are shown in Table II. The rate constants, calculated from the slopes and intercepts of the plots of k_{obsd} vs. sulfite concentrations, are $k_{f_1} = 660$ M^{-1} s^{-1}, $k_{r_1} = 0.065$ s^{-1} (pH 5.0), and $k_{f_2} = 2.9 \times 10^4$ M^{-1} s^{-1}, $k_{r_2} = 1.34$ s^{-1} (pH 8.6). NMR of the sulfite adduct (0.2 M III in D_2O containing 2.3 M K_2SO_3): δ 3.32 [s, 3 H, N(3) CH_3], 3.43 [s, 3 H, N(10) CH_3], 5.27 [s, 1 H, C(5) H], and 7.10-7.33 ppm (m, 3 H, ArH).

The pseudo-first-order rate constants (k_{obsd}) for the reaction of deazaflavins (at 4×10^{-5} M) with cyanide were determined from pH 7 to 11 at fixed cyanide concentrations (1.2×10^{-2} and 1.5×10^{-3} M for I and III, respectively) by following the reaction at 390 (I) and 410 nm (III). The pH dependence of k_{obsd} is again sigmoidal, providing the apparent pK_a of HCN as 9.00 (with I) and 9.05 (with III) (pK_a of HCN at 30 °C in H_2O, μ = 0.15, is 9.17).[23] The formation of an adduct on reaction of I with cyanide is an equilibrium as previously observed.[19,24] At one pH (9.2), k_{obsd} was determined at various concentrations of KCN (0.12-1.2×10^{-2} M). The slope and intercept of a linear plot of k_{obsd} vs. concentrations of KCN give the apparent forward and reverse rate constants as k_f = 0.4 M^{-1} s^{-1} and $k_r = 5.7 \times 10^{-4}$ s^{-1}. The second-order rate constant for addition of cyanide ion to I, calculated from k_f and the pK_a of cyanide obtained above, was $k_{f(CN^-)} = 0.65$ M^{-1} s^{-1} and hence K_{eq} is 1140 M^{-1}. The same value was obtained from the variation of K_{app} (see Experimental Section) with pH. These values may be compared with $k_{f(CN^-)} = 0.2$ M^{-1} s^{-1}, $k_r = 5.0 \times 10^{-4}$ s^{-1}, and $K_{CN^-} = 383$ M^{-1} calculated from the data of Jorns and Hersh[24] (25 °C, pH 8.50) for 5-deaza-FMN.

Unlike deazaflavin I, III reacts with CN^- in an irreversible manner. The repetitive scan of the reaction (Figure 6) reveals an A → B → C pathway. The pseudo-first-order rate constants for the addition of CN^- to III at different pH values are listed in Table III. The second-order rate constant $k_{A\rightarrow B}$ determined from the pK_a of cyanide and from the variation of k_{obsd} with [KCN] at pH 9.1 (see Table III) is 4.6 M^{-1} s^{-1}. The rate constant $k_{B\rightarrow C}$ is independent of $[CN^-]$ but dependent upon pH (Table IV). A plot of log $k_{B\rightarrow C}$ vs. pH (not shown) provides a slope of 0.94 which establishes the reaction to be hydroxide ion catalyzed. The nonreversibility of this reaction made it possible to determine the pK_a values of product C by titration. Spectrophotometric titration (Figure 7) revealed two distinct pK_as: 4.4 ± 0.1 and −0.2 ± 0.05.

Hydroxide ion reacts with I and III in a rapid equilibrium step to form an adduct. This nucleophilic addition is accom-

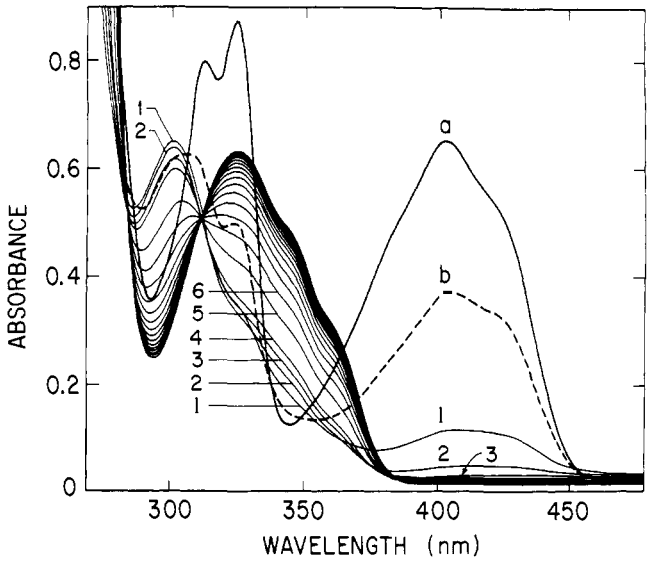
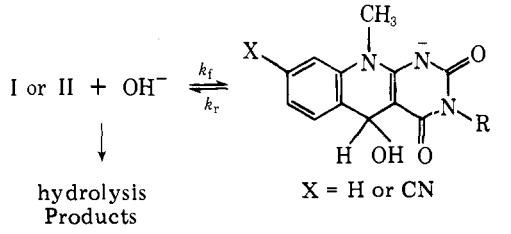


Figure 6. Repetitive scan of the reaction of IIIc with KCN at pH 9.1. Line (a) is the spectrum of IIIc (5.8×10^{-5} M) before adding KCN. Line (b) was recorded at 2 nm/s 30 s after KCN was added (0.004 M). Lines 1-4 were scanned at 150-s intervals, line 5 onward were taken 600 s apart.

Table III. Pseudo-First-Order Rate Constants k_{obsd} for the Addition of CN^- to III at Various pH Values

pH	k_{obsd}, 10^{-3} s^{-1} [a]	pH	k_{obsd}, 10^{-3} s^{-1} [a]
7.43	0.11	9.12	2.41[c]
7.75	0.31	9.10	3.61
8.01	0.46	9.10	5.02[d]
8.13	0.73	9.25	4.30
8.36	1.34	9.80	5.43
8.58	1.81	10.00	5.89
8.78	2.46	10.39	6.57
9.12	1.20[b]	10.99	6.92

[a] All values were determined at [KCN] = 1.5×10^{-3} M (410 nm) unless otherwise mentioned. [b] [KCN] = 0.5×10^{-3} M. [c] [KCN] = 1.0×10^{-3} M. [d] [KCN] = 2.0×10^{-3} M.



(6)

panied by a much slower hydrolysis reaction (eq 6). With excess HO^-, the approach to equilibrium is first order as anticipated. The second-order rate constant, k_f, for III is 120 M^{-1} s^{-1} and K_{eq} is 18.2 M^{-1} ([KOH] = 0.15-0.5 M). Compound I forms an even less stable adduct with HO^-: k_f = 1.13 M^{-1} s^{-1} ([KOH] = 1.0-2.0 M) and $K_{eq} = 0.15 \pm 0.05$ M^{-1}. The UV spectrum of the hydroxide adduct of III (taken immediately after addition of III to 5 M KOH) is shown in Figure 1. No attempt was made to isolate and identify the hydrolysis products.

The repetitive scan of the reaction of III with hydrogen peroxide at pH 9 is shown in Figure 3. As in the case of CN^- addition, hydrogen peroxide addition also follows an A → B → C pathway. The rate of disappearance of III depends on pH and the peroxide concentration. Table V gives the pseudo-first-order rate constants for the A → B portion of the reaction of H_2O_2 (5.2×10^{-3} M) with III up to pH of about 11. These data represent only a portion of a sigmoid curve. From a linear

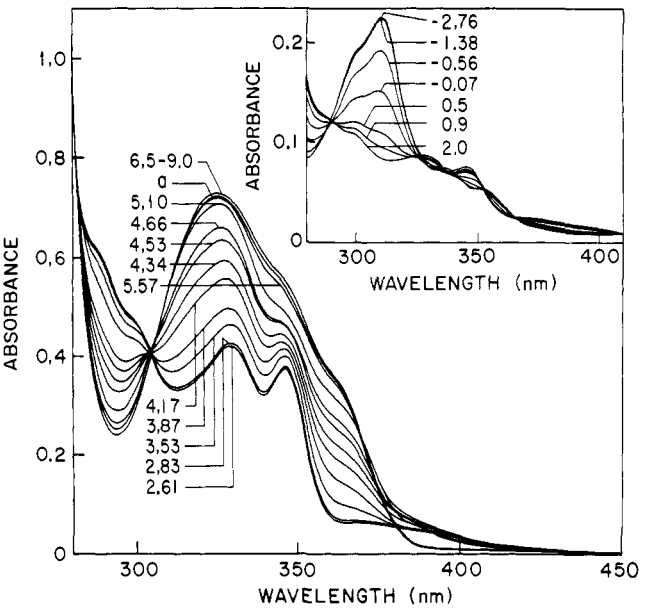


Figure 7. Spectrophotometric titration of 5,8-dicyano-1,10a-dihydro-10-methyl-3-sulfopropyl-5-deazaisoalloxazine (XVIII, ≈2×10^{-5} M) between pH 2.5 and 9 using the assembly previously described.[13] The number on each trace is the pH value. Inset is the titration of XVIII in the H_0 region.[26] A stock solution of XVIII was prepared by adding KCN (to 0.15 M) to a solution of IIIc (2.2×10^{-3} M) at pH 10. Formation of XVIII was complete within 15 min. The spectra were taken after adding 20 μL of this solution of XVIII to 2 mL of suitably diluted solutions of H_2SO_4. The number on each line is the H_0 value. The upper pK_a value was obtained from the sigmoidal plot of A_{325} vs. pH (not shown). The lower pK_a value was determined from a similar plot of A_{310} vs. the corresponding H_0 values.

Table IV. Variation of the First-Order Rate Constant $k_{B\rightarrow C}$ with pH for the Reaction of III with Cyanide[a]

pH	$k_{B\rightarrow C}$, 10^{-3} s^{-1}	pH	$k_{B\rightarrow C}$, 10^{-3} s^{-1}
8.41	0.10	9.82	1.53
8.77	0.21	10.33	5.25
9.10	0.36	10.89	16.8
9.24	0.50		

[a] A solution of the intermediate B (see text) was freshly prepared by adding KCN to a solution of III (3.5×10^{-3} M) at pH 9.0 to a final concentration of 0.1 M. Under this condition the A → B portion of the reaction is complete within 2 min. An aliquot of this solution was then added to the appropriate buffer at 30 °C and the B → C portion of the reaction was followed at 300 nm.

Table V. Pseudo-First-Order Rate Constants (k_{obsd}) for the Reaction of III with Hydrogen Peroxide[a] at Various pH Values

pH	k_{obsd}, 10^{-4} s^{-1}	pH	K_{obsd}, 10^{-4} s^{-1}
7.05	0.2	9.12	83.0
7.44	0.5	9.55	219
7.80	1.2	10.00	377
8.16	2.7	10.49	745
8.63	19.2	10.91	1220
8.82	38.1		

[a] $[H_2O_2] = 5.2 \times 10^{-3}$ M, [III] = 5.8×10^{-5} M, and the reaction mixture contained 4.6×10^{-3} M of EDTA disodium salt.

plot of $1/k_{obsd}$ vs. a_H (eq 7, data for pH 9-11), a very rough estimate for the second-order rate constant for addition of peroxide anion to III is k_2 = 38.5 M^{-1} s^{-1} and the pK_a of H_2O_2 is approximately 10.5 (lit.[25] pK_a of H_2O_2 = 11.6).

$$1/k_{obsd} = a_H(1/k_2K_a[H_2O_2]_T) + 1/k_2[H_2O_2]_T$$
$$[H_2O_2]_T = [H_2O_2] + [^-OOH] \quad (7)$$

Table VI. Dependence of the Apparent Association Constant, K_{app}, for the Reaction of Nitroethane with III on pH[a]

pH	K_{app}, M^{-1}	pH	K_{app}, M^{-1}
6.65	0	8.85	79
7.30	6.5	9.10	89
7.77	18.5	9.5	98
8.02	32.1	9.72	116
8.44	60.5	10.05	114

[a] [III] = 1.7×10^{-5} M, [nitroethane] = $0.7\text{-}2.4 \times 10^{-2}$ M.

The intermediate B has been successfully isolated by carrying out the reaction in 75% aqueous dioxane medium where the B → C step is slow (see Experimental Section). This intermediate is a hydroperoxy adduct. It reacts with potassium iodide to give triiodide, I_3^- (Figure 8). The second-order rate constant for this reaction (0.1 M KI in methanol) is 0.62 M^{-1} s^{-1}. Addition of sodium thiosulfate decolorizes I_3^- and gives the spectrum characteristic of III in methanol (Figure 8). From the known concentration of the hydroperoxy adduct and from the extinction coefficient of I_3^- [ϵ_{349} (2.29 ± 0.03) $\times 10^4$ M^{-1} cm^{-1}],[27] the molecular weight of the hydroperoxy adduct was estimated to be 304 ± 5, which is close to the expected value of 300.3. The hydroperoxy adduct also reacts with thioxane at a slower rate (0.09 M^{-1} s^{-1}, in methanol with thioxane concentration ≈ 0.1 M). Compound I reacts with hydrogen peroxide at a rate too slow to be conveniently investigated. In this instance no attempt was made to isolate the adduct.

The addition of both nitroethane and 2-nitropropane carbanions to III is an equilibrium process. The rapid reversibility is shown by the complete extraction of III into chloroform after it has been decolorized by complexation with the nitroalkane anion. The UV spectrum of the nitroethane adduct to III is shown in Figure 1. The equilibrium constants, K_{app}, for nitroethane addition to III are pH dependent (Table VI). A value of $pK_a = 8.39 \pm 0.05$ (lit.[28] pK_a of nitroethane = 8.6) and K_{eq} = 108 M^{-1} are obtained from a plot of $1/K_{app}$ vs. a_H (eq 3). The association constant of III with 2-nitropropane was determined only at one pH (9.11), K_{app} being 4.1 M^{-1} (cf. K_{app} = 89 M^{-1} for nitroethane at the same pH).

Amines such as DL-alanine, hydrazine, diethyl aminomalonate, and methylamine react with III only at high pH (>10). The reaction was not investigated in detail because of the high pH required. apparently, there is a rapid initial equilibrium followed by a slower reaction. With diethyl aminomalonate, K_{app} for the initial equilibrium is 83.7 M^{-1} at pH 10.2.

Discussion

The equilibrium addition of cyanide and sulfite to 5-deaza-FMN and I has been previously reported.[2a,24] The adducts of these 5-deazaflavins are generally assumed to possess the structure of a 1,5-dihydro-5-deazaflavin with the nucleophile at position 5. Brüstlein and Bruice[2a] provided a proof of the structure of the sulfite adduct of I by way of its NMR spectrum. Addition of nucleophiles to C(5) was found, as expected, to shift the C(5) proton peak from $\delta \approx 3.8$ ppm (in 1,5-dihydro-5-deazaflavins)[2a] to $\delta \approx 5$ ppm in the adduct. We have likewise observed peaks due to the C(5) proton at δ between 5.1 and 5.4 ppm in the NMR spectra of the cyanide adduct of I and the sulfite and hydrogen peroxide adducts of III (Figure 2). However, since these spectra were taken in D_2O, 4a,5 adducts with the nucleophile at C(5) cannot be ruled out solely on the basis of NMR spectra. Additional proof of the structure of these adducts comes from a comparison of their UV spectra with that of 1,5-dihydro-5-deazaflavins. It can be seen (Figures 1 and 3) that the thiol, sulfite, nitroalkane, hydroxide, and hydrogen peroxide adducts to III and the cyanide adduct to I all exhibit maxima in absorbance around 300 nm and thus these spectra resemble those of 1,5-dihydro-5-deazaflavins. The UV spectra of the 1,5-dihydro derivatives of I and III are very similar and only that of the latter is shown in Figure 1. Therefore, the above-mentioned adducts of I and III undoubtedly possess the 1,5-dihydro structures.

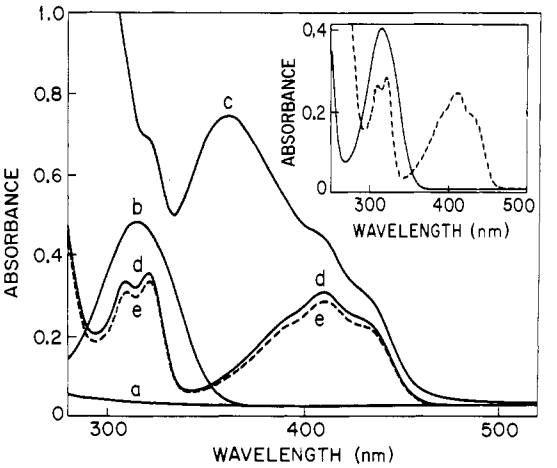


Figure 8. Formation of I_3^- from 8-cyano-1,5-dihydro-3,10-dimethyl-5-hydroperoxy-5-deazaisoalloxazine (XVI) and KI in methanol. (a) is the baseline, (b) is the spectrum of the hydroperoxy adduct (XVI) in methanol (2.6×10^{-5} M), (c) was recorded 30 s after addition of XVI (to a final concentration of 2.6×10^{-5} M) to methanol containing 0.1 M KI, (d) was obtained after adding 20 μL of 0.01 M $Na_2S_2O_3$ to (c), (e) is the spectrum of a solution of IIIc in methanol (2.3×10^{-5} M). The inset illustrates the formation of III from XVI and thioxane: the solid line is the spectrum of XVI in methanol (2.2×10^{-5} M); the dashed line was recorded 10 min after addition of thioxane to a final concentration of 0.1 M.

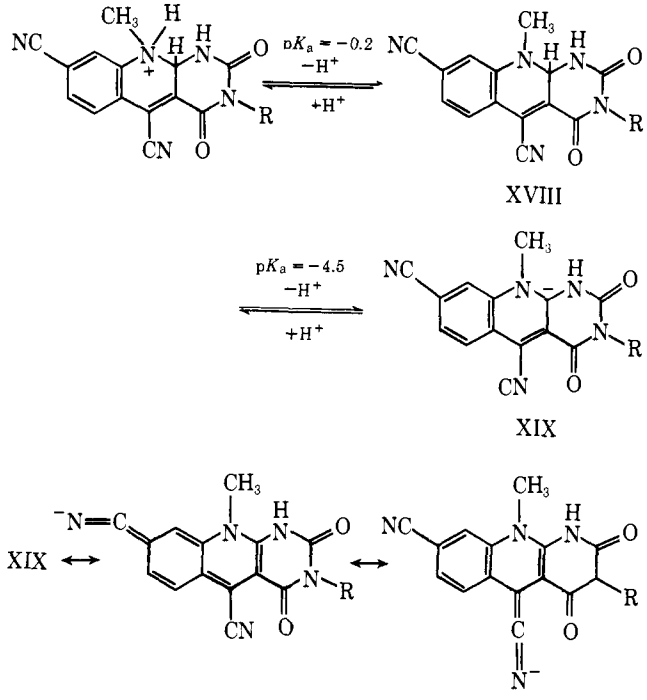
Table VII. Rate Constants and Equilibrium Constants for Nucleophilic Additions to I and III

	I		III	
Nucleophile	k,[a] M^{-1} s^{-1}	K_{eq}, M^{-1}	k[a]	K_{eq}, M^{-1}
$HOCH_2$-CH_2S^-			>170 s^{-1} [b]	490
Dithiothreitol$^-$			>170 s^{-1} [b]	1800
SO_3^{2-}	2.09×10^3	388[c]	2.9×10^4 M^{-1} s^{-1}	2.16×10^4
HSO_3^-			660 M^{-1} s^{-1}	1.01×10^4
CN^-	0.65	1,140	4.6 M^{-1} s^{-1} [d]	
HO^-	1.13	0.15	120 M^{-1} s^{-1}	18.2
HO_2^-			38.5[d]	

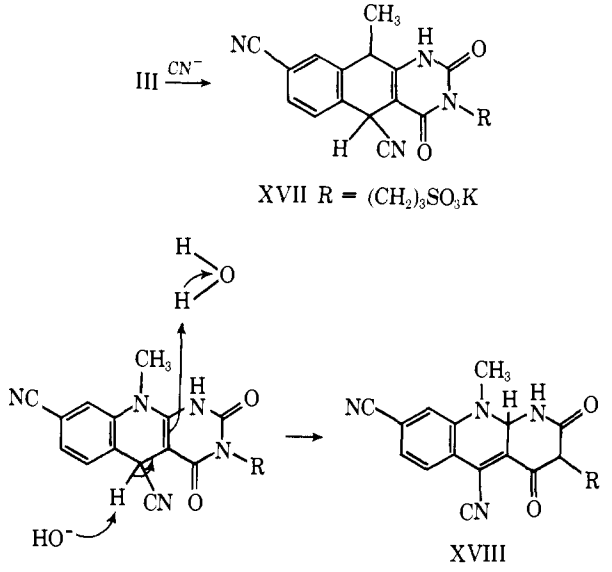
[a] Unless otherwise mentioned, these are forward rate constants, k_f. [b] Pseudo-first-order rate constants determined at [thiol] ≈ 1×10^{-3} M. [c] From ref 2a. [d] These are not equilibrium addition reactions. The numbers are second-order rate constants for addition of the nucleophile (CN^- or HO_2^-) to III.

Table VII summarized the various rate constants and equilibrium constants for nucleophilic additions to I and III. The reaction of cyanide with III follows an A → B → C route. Trace 1 of the repetitive scan (Figure 6) of the reaction largely resembles the spectrum of the intermediate. The λ_{max} (300 nm) and ϵ (≈1.1×10^4 M^{-1} cm^{-1}) imply that this intermediate is a 1,5-dihydro-5-cyano derivative (XVII). The final product has λ_{max} at 325 nm (ϵ 1.1×10^4 M^{-1} cm^{-1}, Figure 6). On the basis of the NMR spectrum (Figure 2) and its two pK_a values this product is identified as 5,8-dicyano-1,10a-dihydro-3,10-dimethyl-5-deazaisoalloxazine (XVIII). The lower pK_a (−0.2, see Results section) of the product is clearly due to protonation at N(10)[29] (Scheme II). The higher pK_a value (≈4.5) could be reasonably assigned to the C(10a) proton in XVIII or to the N(1) proton in a 1,5-dihydro-5-cyano deriv-

Scheme II



Scheme III



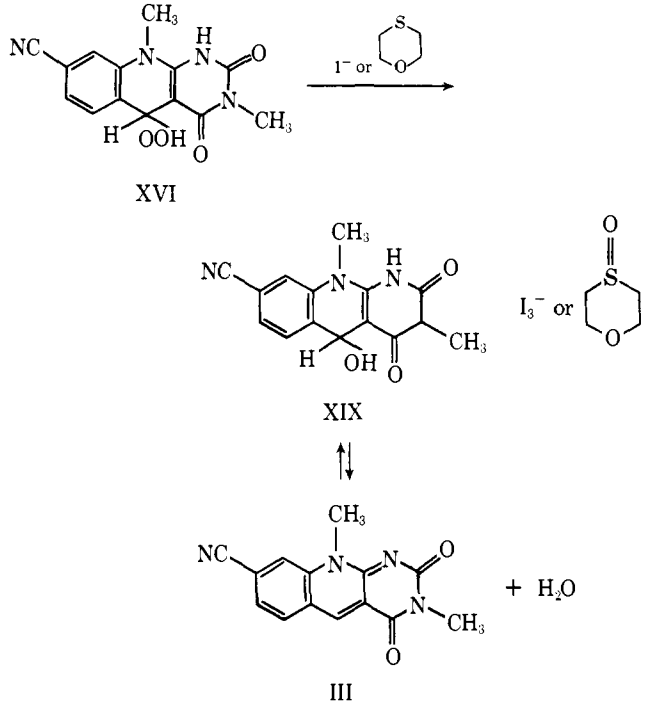
ative (XVII). The lack of peaks between 4.5 and 5.5 ppm in the NMR spectrum of the final product (Figure 2), however, is consistent only with structure XVIII. The C(10a) proton is embedded within the multiplet between 4.0 and 4.3 ppm which integrates to 6 protons [3 methyl, 2 methylene from the N(3) side chain, and one C(10a) proton]. The N(1) proton also appears as a broad peak between 5.6 and 6.4 ppm. It disappeared upon addition of a drop of D_2O as expected. The ionization of the C(10a) proton is expected to be fast enough for titration since it is known that the C(2) protons in malononitriles ionize at rates much faster ($t_{1/2} \approx 30$ s) than other carbon acids,[30]

The formation of XVIII from XVII is hydroxide ion catalyzed (Table IV). The course of cyanide addition to III can be depicted as in Scheme III. The C(5) proton in the intermediate XVII, being under the influence of two strongly electron-withdrawing cyano groups, should be fairly acidic and hence is susceptible to attack by hydroxide ions. Similar attack is not possible for the cyanide adduct to I because the C(5) proton would be much less acidic than that in XVII.

For the equilibrium addition of CN^- to I, we note that the k_r value is close to that of CN^- addition to 5-deaza-FMN[24] (see Results section), but our values of $k_{f(CN^-)}$ and K_{eq} are considerably greater. For 5-deaza-FMN, Jorns and Hersh[24] separated the rate and equilibrium constants into values due to CN^- and HCN. However, their values were not constant at different pH and the validity of their separating these constants seems doubtful. In our case, we observed that both k_{obsd} and K_{app} approach zero as the pH approaches 7 or below. There is no evidence that the protonated form of the nucleophile, i.e., HCN, adds to I. This is similar to the equilibrium addition of thiols to III but different from the addition of sulfite to III where both HSO_3^- and SO_3^{2-} add.

The reaction of hydrogen peroxide with III is also not a simple equilibrium process. It, too, follows an A → B → C pathway (Figure 3). The final product could not be isolated. The intermediate hydroperoxy adduct (XVI), prepared in a separate reaction, is of particular interest. The UV spectrum of this adduct (XVI, trace a in Figure 3) is compared with that of the intermediate (trace c in Figure 3) and their identity is unquestionable. This compound oxidizes thioxane and reacts with potassium iodide to give I_3^- (Figure 8), properties characteristic of peroxides. In Figure 8, trace c, obtained after adding XVI to KI, is in fact a superposition of the spectrum of I_3^- and that of III in methanol. The formation of III from XVI upon reaction with KI or thioxane can be rationalized by Scheme IV. As we have already seen, in aqueous solutions at

Scheme IV



high pH, III is in rapid equilibrium with XIX and this equilibrium is very much in favor of III. Therefore, at low concentrations of HO^-, III would be the only observable deazaflavin product in Scheme IV. It is interesting to note that the second-order rate constants for the reaction of XVI with KI (0.62 M^{-1} s^{-1}) and with thioxane (0.09 M^{-1} s^{-1}) in methanol are only 15 and 7 times smaller, respectively, than the corresponding second-order rate constants for the reaction of 4a-hydroperoxy-*N*(5)-ethyl-3-methyllumiflavin with KI and thioxane,[31] and the reaction of KI with XVI is over 600 times faster than that with *tert*-butyl hydroperoxide. However, unlike 4a-hydroperoxy-*N*(5)-ethyl-3-methyllumiflavin, the hydroperoxy adduct XVI does not oxidize aldehydes such as

formaldehyde and pyridine-4-carboxaldehyde (dioxane solvent).

Compound III reacts with nitroalkane carbanions. The reaction is not cleanly first order probably because of the nitroalkane ⇄ nitroalkane carbanion equilibrium. Upon standing, other products are formed. The smaller K_{eq} for 2-nitropropane compared with that for nitroethane reflects the importance of steric effects in such addition reactions. Contrary to the observation of Walsh et al.,[32] we found that nitroethane carbanion does not react with the normal 5-deazaflavin I in the pH range of 4–9.

We have demonstrated in this investigation that 8-cyano-5-deazaflavin (III) is much more susceptible to nucleophilic attack (at position 5) than the normal 5-deazaflavin I and its derivatives. Thus, nitroalkane carbanions and thiols react with III but not with I. The equilibrium constant and rate for the reaction of sulfite with I (K_{app} = 388 M^{-1} at pH 6.93, k_f = 2.09 × 10^3 M^{-1} s^{-1} at pH 6.89)[2a] are an order of magnitude smaller than the corresponding values for III (K_{app} = 1.71 × 10^4 M^{-1} at pH 7.03, k_{fSO_3} = 2.9 × 10^4 M^{-1} s^{-1} at pH 8.6).

At pH 8, the E_m value for III (see Experimental Section) was determined to be −390 ± 10 mV. This value is not too different from that reported for I (−380 mV at pH 8.0).[33] *Thus the presence of the cyano group at position 8 is reflected only in its enhanced reactivity with nucleophiles but not in its reduction potential!* Also, the 1,5-dihydro derivative of III, like that of I[6] (compounds XIV and XV), is oxidized only slowly ($t_{1/2}$ several hours) by O_2 alone but much faster ($t_{1/2}$ 10–30 min) in the presence of normal flavins. In the presence of oxidized 5-deazaflavin, the oxidation of XIV is also accelerated, though the effect is less pronounced. Thus, a strongly electron-withdrawing group in the 8 position does not greatly affect the rate of oxidation of 1,5-dihydro-5-deazaflavin. Unlike 8-cyano-3,10-dimethylisoalloxazine (II),[9] but like deazaflavin I, compound III does not form any stable free radicals.

Sander and Jencks[34] have provided the linear free energy equation (8) to correlate the equilibrium constants for nucleophilic additions to carbonyl compounds.

$$\log K_{eq} = \Delta\gamma + A \qquad (8)$$

The γ constant was originally defined as the logarithm of the ratio of K_{eq} for the addition of a nucleophile to pyridine-4-carboxaldehyde to the equilibrium constant for addition of methylamine to the same substrate. The Δ and A values are constants for a given substrate. The relationship of eq 8 has recently been shown to be applicable in nucleophilic addition reactions to quinoxaline.[35] The values of Δ have been found to be close to unity for nucleophilic additions to carbonyl compounds and to quinoxaline. Pitman[35] has shown that a unity value for Δ implies that no steric interaction exists between the nucleophile moiety and other parts of the adduct and that the electronic interactions between the nucleophile moiety and the remainder of the adduct are similar for a given substrate and for pyridine-4-carboxaldehyde.

In spite of our limited data, we obtained reasonably linear plots of the logarithm of the equilibrium constants, K_{eq}, for nucleophilic addition reactions to I and III vs. γ (Figure 9). Our values of Δ are close to 0.5 (0.53 and 0.46 for I and III, respectively). From the definition of γ and the relation of $\Delta G°$ and K_{eq}, eq 9 can be easily derived

$$\log K_{eq} = \gamma + \frac{G°_{add_0} - G°_{add}}{2.3RT} + C \qquad (9)$$

where $G°_{add_0}$ is the standard free energy of the adduct between a given nucleophile and pyridine-4-carboxaldehyde, $G°_{add}$ is that of the adduct between the same nucleophile and the 5-deazaflavin, and C is a constant for a given substrate. The fact

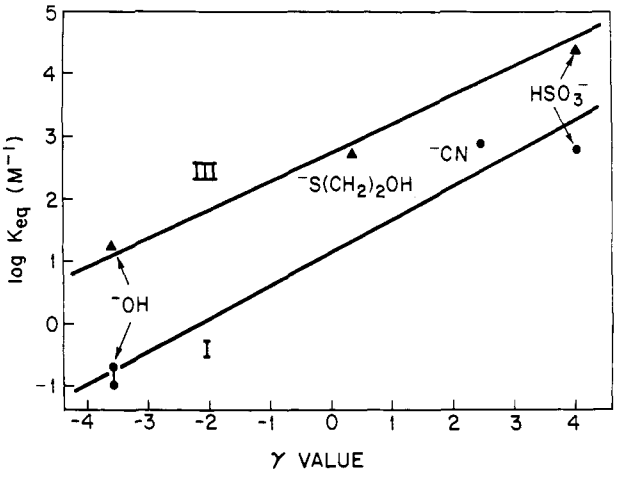


Figure 9. Plot of the logarithm of K_{eq} for the nucleophilic addition reactions to I and III vs. the γ values of the nucleophiles.

that $\Delta < 1$ for nucleophilic additions to the deazaflavins I and III implies that these additions are less sensitive to the affinity of the nucleophilic reagents. It also implies that there may be steric interaction between the nucleophilic group and other parts on the adduct and that electronic interactions between the nucleophile moiety and the remainder of the adduct molecule are not the same for the 5-deazaflavins as those for quinazoline and aldehyde adducts.

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Biological Analogues. On the Nature of the Binding Sites of Copper-Containing Proteins

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Abstract: An extended series of ligands and their copper(II) complexes have been prepared as spectroscopic models for determining the geometries and ligand coordinations of copper proteins. The electronic properties of thioether, imidazole, amide anion, amine, phenolate anion, and thiolate anion coordination to copper(II) have been determined. In addition, the electronic spectra of some of these ligands in square-planar, square-pyramidal, and tetrahedral geometries about copper(II) have been obtained by appropriate ligand design. On the basis of these results and an analysis of the protein spectra, structures for the copper coordination and geometry in (blue) type I copper, copper in galactose oxidase, type III copper, and copper in oxyhemocyanin are proposed.

Copper, in its various roles in biological systems, displays differing spectroscopic and chemical properties presumably because of the differing ligand environments and coordination numbers. Included among this variety of centers are "blue" or type I copper, "nonblue" or type II copper, ESR "nondetectable" or type III copper, and the copper present in hemocyanin.[1,2]

Type I copper occurs in the blue electron-carrying proteins stellacyanin, plastocyanin, and azurin where it is the only type of copper present. It also occurs, accompanied with types II and III, in the blue oxidases, laccase, ceruloplasmin, and ascorbate oxidase. By itself, blue copper is spectroscopically spectacular; it is characterized by an exceedingly intense electronic absorption at around 600 nm ($\epsilon \sim 5000$) as well as a succession of weaker bands which start at ~450 nm and carry on to the near-infrared; a total of 9 bands are seen in the visible and near-infrared regions for plastocyanin.[3]

The geometry and ligand environment of the type I site have not been determined directly, but on the basis of spectroscopic evidence, nearly all the conceivable permutations have been proposed.[3-8] Resonance Raman studies have been interpreted both in terms of distorted tetrahedral[4] and trigonal-bipyramidal[5] coordination geometries. Cobalt(II) reconstituted azurin and plastocyanin show electronic spectra typical of distorted tetrahedral coordination,[6] and more recent, additional data, in the near-infrared region, provide persuasive evidence for tetrahedrally surrounded copper(II) in plastocyanin and stellacyanin.[3]

The presence of RS^-, from cysteine, in the coordination sphere of blue copper has been proposed on the basis of binding by *p*-mercuribenzoate,[1,2] x-ray photoelectron spectroscopy,[7] electronic and circular dichroism spectra,[3] resonance Raman data,[4,5] and inferred from the Co(II) reconstituted materials. Ligands proposed, other than RS^-, include imidazole,[3,4] deprotonated amide,[3] and unspecified oxygen donors.[5] The coordination of thioether sulfur (from methionine) instead of RS^- has also been claimed;[8] this is unlikely for various reasons, not least of which is that no methionine has been found in stellacyanin.[9] Amino acid compositions of plastocyanin,[10] azurin,[11] ceruloplasmin,[12] ascorbate oxidase,[13] and laccase[14] indicate sufficient cysteine, methionine, histidine, and tyrosine to account for any of the proposed ligand environments.

Type II or nonblue copper is usually found in combination with types I and III but it occurs alone in galactose oxidase.[15] There has been little speculation as to the geometry and ligand environment of the copper in this enzyme although RS^- (cysteine) coordination is suggested by chemical studies on the enzyme and the apoenzyme.[16] Amino acid analyses disagree on the number of cysteines present in galactose oxidase[16,17] but considerable amounts of methionine and histidine are present.

So far, type III copper has not been found alone; it occurs along with types I and II in the blue oxidases, where selective titration studies with reducing agents show that the 330-nm ($\epsilon \sim 3000$) band of these proteins is associated with the type III copper and that two electrons are required to reduce this site. Type III copper is believed to consist of magnetically coupled pairs of copper ions.[19]

Superficially, the spectra and constitution of the copper in oxyhemocyanin appear to be remarkably similar to that of type III copper.[1,2,20,21] The coppers occur as spin-coupled copper(II) pairs which bind a peroxide ion.[22] The electronic absorption spectrum of oxyhemocyanin consists of two major visible bands, one at 345 nm ($\epsilon \sim 9000$ per Cu), the other at 570 nm ($\epsilon \sim 500$ per Cu).[23] Amino acid analyses of a variety of hemocyanins[20,24] indicate a large amount of histidine and of methionine per copper pair to be present as well as cysteine, although the number involved in disulfide bridges was not determined. However, it is significant that hemocyanin can be reconstituted after the RS^- groups have been blocked in the apoenzyme.[20,21] Thus, RS^- coordination appears to be excluded and the most widely assumed coordination ligand is imidazole.

This paper describes an attempt at determining the struc-