Characterization and One- and Two-Electron Redox Chemistry of 1,5-Dicarba-1,5-dideazaisoalloxazines (Flavins)

Rebecca L. Chan and Thomas C. Bruice*

Contribution from the Department of Chemistry, University of California at Santa Barbara, Santa Barbara, California 93106. Received May 15, 1978
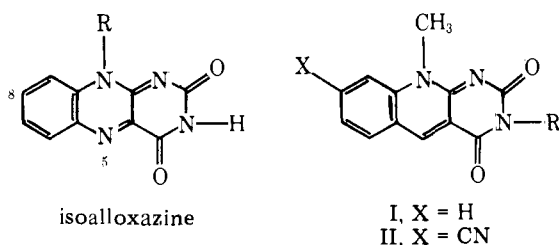
Abstract: The 1,5-carba-1,5-dideazaisoalloxazines IIIa,b,c, and IVa,b,c have been synthesized and their electronic structures, in both oxidized and reduced states, have been determined. The 2e reduction potentials and the pK_a values associated with the ionization of the dihydro forms are compared to the like constants for the 5-carba-5-deazaflavin (I) and to flavins. Reduction of the dideazaisoalloxazine structure with H_2/Pt or $NaBH_4$ (with care) provides the corresponding dihydro species (III-H_2). The protons at C(5) of IIIc-H_2 were shown to be exchangeable with water in the presence of H_2/Pt. Photoreduction (EDTA/$h\nu$) and dithionite reduction were found to be very slow and to reach completion only with great difficulty (the opposite being true with flavins or 5-carba-5-deazaflavins). The oxidation of IIIc-H_2 by $^3O_2$ occurs by way of an autocatalytic mechanism as does the oxidations of I-H_2 and dihydroflavins. These reactions are accelerated by the presence of the oxidized species. Reaction of III and IV with H_2O_2 provides the 5-hydroperoxy adducts. The peroxide adduct of IIIb (i.e., VIII) and also I were isolated and characterized. VIII was shown not to be an intermediate in the $^3O_2$ oxidation of IIIc-H_2. Under anaerobic conditions (H_2O, pH 7-9.5), VIII slowly gives rise to a purple-black, readily oxidizable (O_2, Fe(CN)$_6^-$, nitroxide) product which on isolation and characterization proved to arise via oxygen insertion at the C(1) position (IX). The 1e oxidation of the dihydro-1,5-carba-1,5-dideazaisoalloxazines (IIIc-H_2 and IVc-H_2) as well as the dihydro-5-carba-5-deazaisoalloxazines (I-H_2 and II-H_2) by Fe(CN)$_6^-$ has been studied as has the 1e oxidation of IIIc-H_2 by the nitroxide 4-hydroxy-2,2,6,6-tetramethyl-4-piperidine-1-oxy. The Fe(CN)$_6^-$ oxidations are facile and the rates for the nitroxide oxidation are comparable to that for O_2 oxidation. Second-order rate constants for the oxidation of I$^-$ and thioxane by the 5-hydroperoxides of the various carbadeazaisolloxazines are compared.

Introduction

5-Carba-5-deaza analogues (referred to herein simply as deazaflavins, etc.) of flavin coenzymes were first synthesized by Cheng and co-workers[1] as potential riboflavin antagonists. Various 5-deazaisoalloxazines have subsequently been studied in both enzymatic[2,3] and model systems[3,4] with the view in mind to provide mechanistic insight into flavin-catalyzed reactions. We have previously[5] presented, in detail, the chemical properties of 10-methyl-5-deaza- (I) and 8-cyano-10-

isoalloxazine

I, X = H
II, X = CN

methyl-5-deazaisoalloxazine (II) where R is a methyl or a sulfopropyl group.

Recently, Rogers and co-workers[6a,b] have synthesized a number of other aza and deaza isosteres of isoalloxazines (i.e., 1-deaza-, 1,5-dideaza-, 3-deaza-, 9-aza-5-deaza-, and 1,3,5-trideazaisoalloxazines). The 1,5-dideazaisoalloxazine was also synthesized independently by Weinstock et al.[6c] Walsh et al.[7,8]

IIIa, R = H; X = H
b, R = CH₃; X = H
c, R = CH₂CH₂CH₂SO₃⁻; X = H

IVa, R = H; X = CN
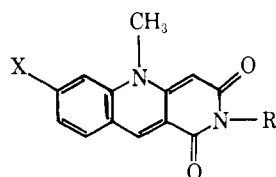b, R = CH₃; X = CN
c, R = CH₂CH₂CH₂SO₃⁻; X = CN

have made a comprehensive survey of the properties of the 1-deazaflavins. The 1-deazaflavins are of interest because unlike the 5-deazaisoalloxazines, their reduced forms are oxidized at rates comparable to those of dihydroflavins.

In view of the generally recognized importance of positions 1 and 5 in the mechanism of flavin-catalyzed reactions, it is of interest to inquire into the properties of 1,5-dideazaisoalloxazines. We present herein some interesting chemical properties we have found with the 1,5-dideazaisoalloxazines III and IV.

Experimental Section

Melting points were uncorrected. Elemental analyses were performed by Elek Microanalytical Laboratories, Torrance, Calif., or Chemalytics Inc., Tempe, Ariz. NMR spectra were taken with KBr pellets on a Perkin-Elmer 137 spectrophotometer. UV and visible spectra were obtained with a Cary 118 spectrophotometer. pH measurements were made with a Radiometer Model M26 pH meter equipped with a Radiometer GK 2402C glass-calomel combination electrode. All kinetics were followed at 30 ± 0.1 °C using a Cary 118 spectrophotometer or a Durrum-Gibson Model D-100 stopped-flow instrument. All buffers were 0.1 M in the buffer species, the ionic strength being adjusted to 1.0 with KCl. *N*-Methylacridinium chloride and 4-hydroxy-2,2,6,6-tetramethyl-4-piperidine-1-oxy were synthesized in our laboratory and were analytically pure. All other chemicals were reagent grade and were used without further purifications unless otherwise mentioned.

Syntheses. 1,5-Dideazaisoalloxazines. The synthesis is outlined in eq 1.

Ethyl α-cyano-β-iminoglutarate (V), mp 54 °C (lit.[9] mp 53 °C), was prepared from ethyl cyanoacetate and converted, following the procedure of Thorpe and co-workers,[9] to glutazine (VI) *m/e* 126, decomposing at 310 °C (lit.[9] mp 300 °C dec). To prepare **1,5-dideazaisoalloxazines,** the appropriate *o*-aminobenzaldehyde (VII), X = H or CN) was prepared as previously described[5] and was stirred with 0.9 equiv of glutazine in 50% aqueous acetic acid. After the reaction mixture was heated on a steam bath for about 1 h, a red, fluffy solid appeared. This was collected by filtration, washed with hot water and acetone, and vacuum dried. IIIa could be recrystallized from DMF: NMR (Me₂SO-d_6) δ 3.46 [s, 3 H, N(10) CH₃], 5.35 [s, 1 H, C(1) H], 7.34-8.01 (m, 4 H, ArH), and 8.57 ppm [s, 1 H, C(5) H]. IVa was
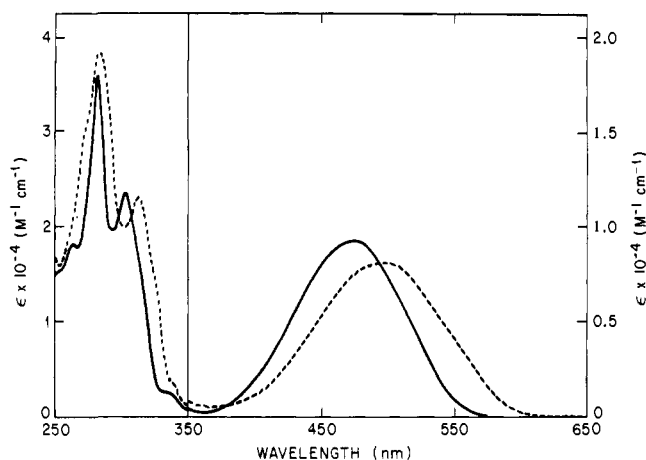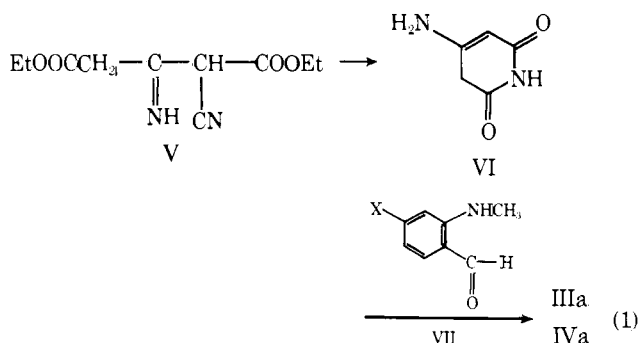
Figure 1. Absorption spectra of 3,10-dimethyl-1,5-dideazaisoalloxazine (III, solid line) and 8-cyano-3,10-dimethyl-1,5-dideazaisoalloxazine (IV, dashed line) at pH 7.0.
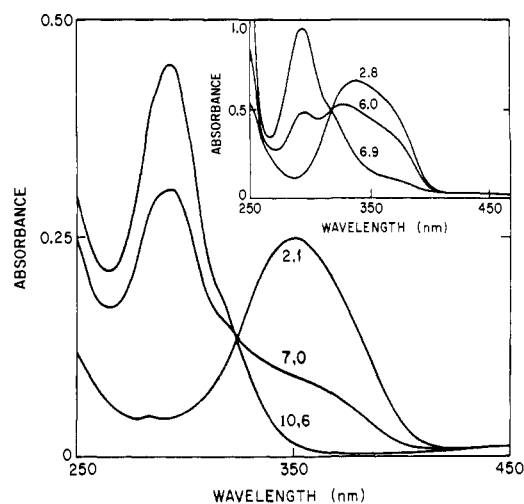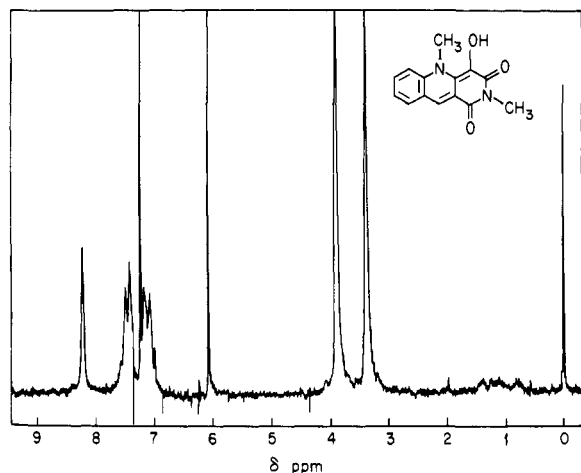

Figure 2. Absorption spectrum of dihydro-10-methyl-3-sulfopropyl-1,5-dideazaisoalloxazine (2×10^{-5} M) and 8-cyanodihydro-10-methyl-3-sulfopropyl-1,5-dideazaisoalloxazine (inset, 4.8×10^{-5} M) at three different pH values. Number on each curve indicates the pH value.

very insoluble in all common solvents. The 3-methyl (IIIb and IVb) and 3-sulfopropyl (IIIc and IVc) derivatives were prepared with methyl iodide or 1,3-propanesultone (carcinogenic) in DMF using NaH as base. IIIb and IVb were recrystallized from EtOH. IIIc and IVc were recrystallized from DMF/water.

Compound IIIb: mp 292–293 °C; NMR (CDCl₃) δ 3.39 [s, 3 H, N(3) CH₃], 3.59 [s, 3 H, N(10) CH₃], 5.47 [s, 1 H, C(1) H], 7.24–7.73 (m, 4 H, ArH), and 8.51 ppm [s, 1 H, C(5) H]; UV (Figure 1) λ_{max} (pH 7.0) 474 nm (ϵ 9200), 303 (23 000), 283 (35 000), 263 (17 500), and 252 (15 000). Anal. Calcd for C₁₄H₁₂N₂O₂: C, 69.98; H, 5.04; N, 11.66. Found: C, 70.26; H, 4.88; N, 11.09.

Mp of IVb >360 °C; NMR (CDCl₃) δ 3.39 [s, 3 H, N(3) CH₃], 3.56 [s, 3 H, N(10) CH₃], 5.53 [s, 1 H, C(1) H], 7.38–7.76 (m, 3 H, ArH), and 8.41 ppm [s, 1 H, C(5) H]; UV (Figure 1) λ_{max} (pH 7.0) 498 nm (ϵ 8100), 312 (23 000), and 282 (40 000). Anal. Calcd for C₁₅H₁₁N₃O₂: C, 67.91; H, 4.18; N, 15.84. Found: C, 67.40; H, 4.75; N, 15.01.

NMR of IIIc (D₂O): δ 1.8–2.2, 2.8–3.1, 3.5–3.9 [m, 2 H each, (CH₂)₃], 3.21 [s, 3 H, N(10) CH₃], 5.00 [s, 1 H, C(1) H], 7.1–7.65 (m, 4 H, ArH), and 7.82 ppm [s, 1 H, C(5) H].

NMR of IVc (D₂O): δ 1.9–2.1, 2.9–3.0, 3.8–4.0 [m, 2 H each, (CH₂)₃]. 3.44 [s, 3 H, N(10) CH₃], 5.37 [s, 1 H, C(1) H], 7.50–7.84 (m, 3 H, ArH), and 8.24 ppm [s, 1 H, C(5) H]. The absorption spectra of IIIc and IVc were identical with those of IIIb and IVb.

Dihydro-10-methyl-3-sulfopropyl-1,5-dideazaisoalloxazine (IIIc-H₂). Hydrogen gas (Linde 99.9%) was passed through an aqueous solution of IIIc over platinized asbestos for 2–5 min, when the solution became colorless. The solution of IIIc-H₂ was then filtered through Millipore (0.45 nm) just prior to being used. The concentration of IIIc-H₂ was taken to be that of the starting IIIc, which was determined from the visible spectrum of the solution before reduction. The whole process was carried out under an atmosphere of nitrogen.

To determine the structure of the reduced form, a sample of IIIc was dissolved in D₂O and reduced with gaseous hydrogen over Pt/asbestos for about 10 min. The filtered product solution showed the following NMR spectrum: δ 1.87–2.16, 2.86–3.03, 3.99–4.13 [m, 2 H each, (CH₂)₃], 3.27 [s, 3 H, N(10) CH₃], 3.62 [s, 0.9 H, C(5) H₂] (see Results and Discussion), 6.99–7.29 ppm (m, 4 H, ArH).

The UV spectrum of IIIc-H₂ is shown in Figure 2. IIIc can also be reduced with sodium borohydride by the procedure described below for the reduction of IVc. NMR spectrum of the borohydride reduced IIIc in D₂O (excess borohydride destroyed with D₂SO₄, final pH ≈2): δ 1.9–2.1, 2.8–3.1, 3.7–3.9 [m, 2 H each, (CH₂)₃], 3.10 (s, 3 H, CH₃), 3.50 [s, 2 H, C(5) H₂], and 6.9–7.3 ppm [m, 4 H, ArH]. When the reduction was carried out with NaBD₄, the peak at ≈3.5 ppm integrates to only 0.8 proton.

8-Cyanodihydro-10-methyl-3-sulfopropyl-1,5-dideazaisoalloxazine (IVc-H₂) was prepared as an aqueous solution on the day of use. A solution of a known weight of IVc in pH 7 phosphate buffer was reduced with small amounts of solid NaBH₄ inside a glovebox under nitrogen. The solution turned colorless in 1–2 min when reduction was complete. A small amount of 1 N HCl was added to destroy any excess borohydride. The solution was then diluted to the concentration required with the appropriate buffer. NMR of reduced IVc in D₂O (excess BH₄⁻ destroyed with D₂SO₄, final pH ≈2): δ 1.8–2.1, 2.7–3.0, 3.8–4.0 [m, 2 H each, (CH₂)₃], 3.19 (s, 3 H, CH₃), 3.64 [s, 2 H, C(5) H₂], and 7.2–7.5 ppm (m, 3 H, ArH). The C(5) protons integrate to 0.9 H when reduction was carried out with NaBD₄ in D₂O.

Dihydro-3,10-dimethyl-5-hydroperoxy-1,5-dideazaisoalloxazine (VIII). The preparation of this compound was similar to that for 8-cyano-1,5-dihydro-3,10-dimethyl-5-hydroperoxy-5-deazaisoalloxazine.[5] IIIa (120 mg, 0.5 mmol) was suspended in a mixture of 45 mL of dioxane and 15 mL of 0.1 M NH₄OH in water. Commercial H₂O₂ (30%) was added and the mixture stirred vigorously at room temperature for 4 h when all solid had gone into solution (slightly yellow). The solution was freeze-dried to give 110 mg of a white powder (90% crude yield). This was recrystallized from DMF/ether to give well-defined white crystals: mp 179–180 °C; NMR (Me₂SO-d₆) δ 3.14 [s, 3 H, N(3) CH₃], 3.33 [s, 3 H, N(10) CH₃], 5.13, 5.85 [s, 1 H each, C(5) H and C(1) H], 7.05–7.8 ppm (m, 4 H, ArH). The compound has a complex mass spectrum (see Results and Discussion).

3-10-Dimethyl-1-hydroxy-1,5-dideazaisoalloxazine (IX). Dihydro-3,10-dimethyl-5-hydroperoxy-1,5-dideazaisoalloxazine (VIII), 50 mg, 0.18 mmol) was dissolved in 30 mL of oxygen-free acetonitrile under nitrogen inside a glovebox. This solution was added drop by drop to a flask containing 300 mL of a phosphate buffer solution (0.1 M, pH 8.5). After standing at room temperature for about 10 min, the solution developed a purple color. After about 1 day, dark purple crystals appeared at the bottom of the flask. These were collected by filtration and vacuum dried (yield 40 mg, 82%). The product was purified by chromatography over silica gel inside the glovebox eluting with CHCl₃/EtOAc (3:1) and evaporating off the solvent from the dark purple eluent under vacuum, mp 224–225 °C. Thin layer chromatography (anaerobic, silica gel) of the purified material showed one single spot, R_f 0.34 (CHCl₃/EtOAc, 3:1). The infrared spectrum (KBr pellet) shows a sharp OH peak at 3500 cm⁻¹. NMR (CDCl₃, Figure 3): δ 3.43, 3.94 [s, 3 H each, N(3) CH₃ and N(10) CH₃], 6.03 (s, 1 H, OH), 7.10–7.51 (m, 4 H, ArH), and 8.25 ppm [s, 1 H, C(5)

Figure 3. NMR spectrum of 3,10-dimethyl-1-hydroxy-1,5-dideazaisoalloxazine (0.008 M in $CDCl_3$, anaerobic). The peak at 6.03 ppm disappeared after the solution was shaken with D_2O.

Figure 4. Formation of 3,10-dimethyl-1-hydroxy-1,5-dideazaisoalloxazine (IX) anaerobically from dihydro-3,10-dimethyl-5-hydroperoxy-1,5-dideazaisoalloxazine (VIII), 1.8×10^{-5} M) at pH 8 (phosphate), 30 °C. Lines 1–3 were taken 15 min apart, lines 4–8 30 min apart, and lines 9 onwards were taken at 60-min intervals. Inset is the absorption spectrum of IX at pH 7 (phosphate). Dotted line was obtained after passing oxygen into the solution of IX for a few minutes.

H]. UV (pH 2–9, Figure 4): λ_{max} 535 nm (ϵ 7200), 322 (17 700), 289 (29 500). Anal. Calcd for $C_{14}H_{12}N_2O_3$: C, 65.61; H, 4.72; N, 10.93. Found: C, 65.83; H, 5.03; N, 11.94.

Determination of Redox Potential. The redox potentials of the dideazaisoalloxazines III and IV were determined at pH 8 in Tris buffer by equilibration with 1,5-dihydro-10-methyl-3-sulfopropyl-5-deazaisoalloxazine ($1-H_2$) obtained by catalytic reduction (H_2/Pt) of an aqueous solution of 1. The procedure is similar to that developed by Stankovich and Massey.[10] An aliquot of the reduced 5-deazaflavin was added to a Thunberg cuvette containing the buffer under an atmosphere of nitrogen. Solutions of IIIc or IVc were placed in the side arm. The Thunberg was sealed and the contents mixed. Equilibrium was reached in about 45 min. The equilibrium concentrations of IIIc or IVc were determined from the final spectra (ϵ_{473} 9.23 × 10³ M^{-1} cm^{-1} for III, ϵ_{500} 8.11 × 10³ M^{-1} cm^{-1} for IV). From the known initial concentrations of each reactant (all at about 9×10^{-5} M), the equilibrium concentration of each component can be calculated and hence the redox potential of III or IV determined from the equation
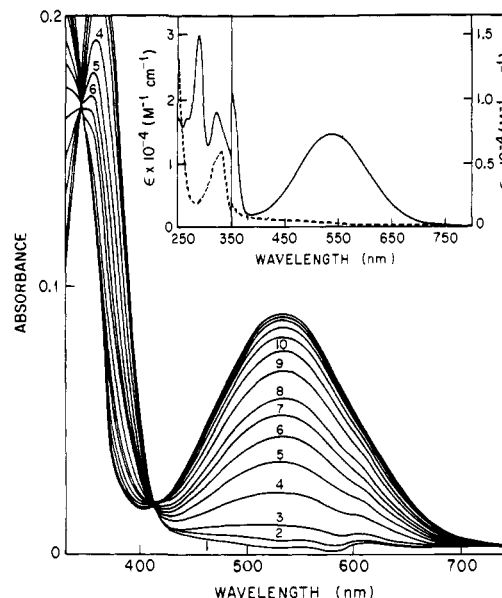
$$E°_{III} = E°_I + \frac{0.059}{n} \log \frac{[I]_{eq}}{[1-H_2]_{eq}} \frac{[III-H_2]_{eq}}{[III]_{eq}} \quad (2)$$

Kinetics. Reaction of reduced deazaisoalloxazines with potassium ferricyanide and *N*-methylacridinium chloride was followed anaerobically on the stopped-flow instrument. Other reactions were followed on the Cary 118 spectrophotometer with Thunberg cuvettes except for the reoxidation by oxygen of reduced dideazaisoalloxazine (III-H_2), which was followed in oxygen-saturated buffers in normal 3-mL, 1-cm path length quartz cuvettes sealed with rubber septums. Freshly prepared solutions of III-H_2 were injected into the cuvette with a syringe needle.
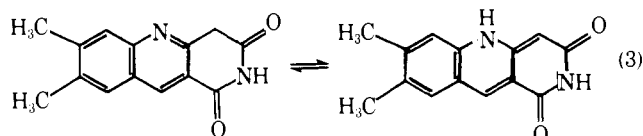
Results and Discussion

We found that the most recently reported procedure of Rydon et al.[11] for the preparation of glutazine (VI), an intermediate in the synthesis of 1,5-dideazaisoalloxazines, was entirely unsatisfactory. Instead, the older method of preparation of VI by Thorpe and co-workers[9] gave an excellent yield of the compound. With glutazine and a slight excess of a pure sample of the appropriate *o*-aminobenzaldehyde (VII), we were able to obtain the dideazaisoalloxazines (IIIa and IVa) in 70–92% yield.

Structures of compounds III and IV are evident from their NMR spectra. In this respect, it is interesting to note that for 1,5-dideazalumichrome, Ashton et al.[6a] reported that the proton(s) at the C(1) position gives two peaks in the NMR spectrum and that these are exchangeable. Thus, in Me_2SO, 1,5-dideazalumichrome exists as two tautomers in the ratio of 0.15:0.85 (eq 3). For the dideazaisoalloxazines III and IV, distinct vinyl protons at C(1) and C(5) are observed in their


NMR spectra and these are not exchangeable with solvent. Thus, only the 1,5-dideazaisoalloxazine structure exists for both compounds III and IV. In general (see Table I) the C(1) vinyl proton appears at δ between 5 and 5.5 ppm whereas the C(5) vinyl proton appears at much lower field ($\delta > 8$ ppm).

Chemical reduction of the dideazaisoalloxazines was examined employing catalytic hydrogenation, photoreduction (EDTA/$h\nu$), and sodium borohydride or sodium dithionite. Hydrogenation with platinized asbestos as catalyst proved satisfactory for the reduction of IIIc but cannot be used for IV because of the presence of the cyano group. Photoreduction of III and IV is very slow, reaching completion only with difficulty. Not surprising is the finding that the 8-CN group of IV greatly enhances its rate of photoreduction over that for III. Thus, with a 100-fold excess of EDTA, about 50% of IVc was photoreduced in 5 h whereas less than 5% IIIc was reduced under identical conditions. Reduction of the dideazaisoalloxazines with sodium dithionite was sluggish. A large excess of dithionite had to be used and even so the reduction was incomplete. In comparison, the dithionite reduction of 5-deazaflavins or of normal isoalloxazines occurs readily.

Unlike 1-deazariboflavin, which has been reported[7] to give O_2-stable reduced products with sodium borohydride, we found that under carefully controlled conditions (pH 7 during reduction and addition of acid to destroy the excess borohydride when reduction was complete), reduction of IIIc or IVc by $NaBH_4$ not only proceeded rapidly, but the reoxidation of the resultant product by air was near quantitative. If, however, an unbuffered solution of IIIc or IVc was reduced by solid $NaBH_4$ and the resulting solution (pH ≈9) exposed to air, less than 50% reoxidation was observed. Reduction of IIIc by either H_2/Pt or $NaBH_4$ gave the same reduced product as evidenced from the identical UV spectra and from the same pK_a value (6.8) determined from spectrophotometric titration of the

reduced form. The pK_a of the 8-CN derivative (IVc) reduced by borohydride was determined to be 6.3. This difference in pK_a is not unexpected on account of the presence of the strongly electron-withdrawing cyano group in IV.

As for the structure of reduced III (III-H$_2$) and IV (IV-H$_2$), we propose, on the basis of their NMR spectra, that both compounds exist essentially as the 1,5-dihydro form, as is the case for dihydroisoalloxazines and dihydro-5-deazaisoalloxazines. Thus, in the NMR spectra of the borohydride-reduced IIIc and IVc (D$_2$O, pH $\approx$2), the vinyl protons at $\approx$5 ([C(1) H] and $\approx$8 ppm [C(5) H], which are characteristic of oxidized 1,5-dideazaisoalloxazines, have disappeared, showing that reduction has occurred at these positions. The two C(5) methylene protons of III-H$_2$ and IV-H$_2$ appear at $\approx$3.5 ppm. The C(1) methylene protons do not appear in the NMR spectra. This implies a facile enol–keto tautomerism as shown in eq 4. The pK_a values of 6.8 and 6.3 for III-H$_2$ and IV-H$_2$,

(4)
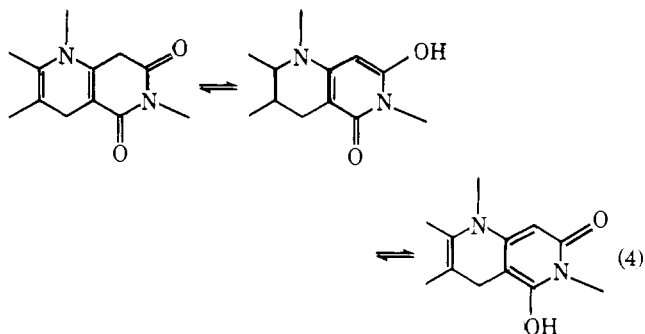
respectively, are associated with the formation of the enolate ion (eq 5). Walsh and co-workers[7] proposed similar structures

(5)
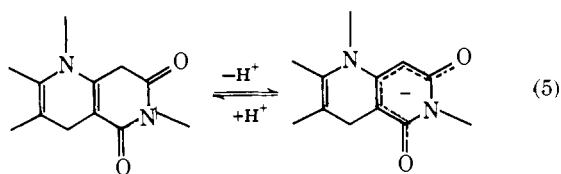
for dihydro-1-deazariboflavin that would account for a pK_a value of 5.6. Other possible dihydro derivatives of the dideazaisoalloxazines, such as the 1,10a-dihydro or 4a,5-dihydro forms, are ruled out because they cannot account for the observed pK_a values.

Apparently the C(5) hydrogens of IIIc-H$_2$ are exchangeable with the protons of water in the presence of hydrogen gas and platinum catalyst. The C(5) methylene protons of IIIc-H$_2$, prepared by hydrogenation in D$_2$O over Pt," appear (δ 3.57 ppm at pH 7) at essentially the same position (δ 3.55 ppm at pH 2) when IIIc-H$_2$ is obtained by borohydride reduction of IIIc. However, for the catalytically hydrogenated product, the peak corresponding to the C(5) protons integrates to less than two protons (see Experimental Section). When IIIc-H$_2$ is allowed to remain in D$_2$O and the passage of hydrogen continued for a few more minutes in the presence of Pt, the integrated intensity of the C(5) protons continues to decrease. When H$_2$O was used as the solvent for catalytic hydrogenation, it was observed that the duration of passage of H$_2$ gas through the solution of IIIc over platinized asbestos (up to 15 min) did not affect the UV spectrum of the reduced product and that over 90% reoxidation to III was observed at all times. Therefore, it appears that extensive hydrogenation of the isoalloxazine ring structure does not occur when hydrogen is passed through the solution for less than 15 min. The same phenomenon was observed when the 5-deazaflavin I was reduced by H$_2$/Pt in D$_2$O.

We found that when the 5-deazaflavins I and II were reduced by NaBH$_4$ only 50% re-formation of I and II occurred

upon O$_2$ reoxidation even when reduction was carefully controlled. Extensive reduction of the carbonyl groups must have occurred, as previously reported (but to less extent) in the borohydride reduction of normal flavins[12] and 1-deazariboflavin.[7]

Reaction with Hydrogen Peroxide. We have previously reported the reaction of the 5-deazaisoalloxazine II with hydrogen peroxide.[5] Likewise, compounds III and IV react with H$_2$O$_2$ in an A → B → C process, where B is the hydroperoxy adduct and C is some unidentified hydrolysis and/or oxidation product(s). The B → C process is several times slower than is the hydroperoxide formation. By using a reaction medium that inhibits the B → C process (see Experimental Section), we could isolate the intermediate hydroperoxy adducts. For compound IIIb, a pure hydroperoxy adduct was prepared and its NMR spectrum was found to resemble that of a 4a,5 derivative. The characteristic C(5) vinyl proton at about 8 ppm is lost whereas two protons appear in the δ 5–6 ppm region. One of these (δ 5.83 ppm) is attributable to the C(1) vinyl proton and the other (δ 5.11 ppm) to the C(5) proton (VIII). The C(4a) proton is not visible in the NMR spectrum. It is either embedded in the HOD peak ($\delta \approx$3.3 ppm) or, more likely, the compound exists as tautomers b or c (eq 6). For the hydro-

(6)
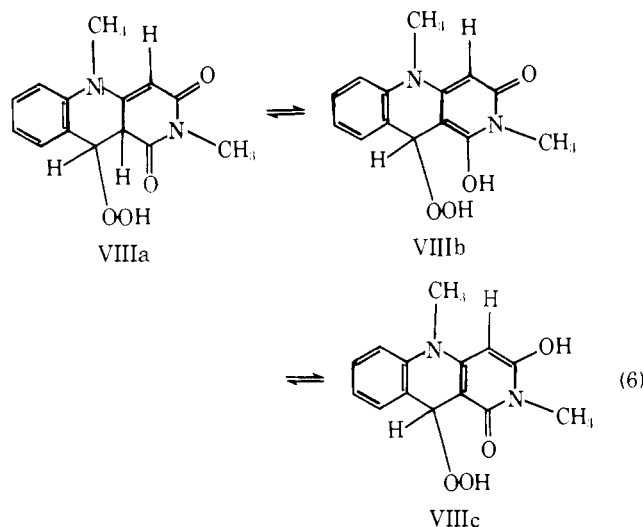
peroxy derivative of II, we proposed[5] a 1,5 adduct Xa on account of its NMR spectrum and the similarity of its UV spectrum with those of the other 1,5 adducts of II. However, it is also feasible that this compound exists as the tautomer Xb
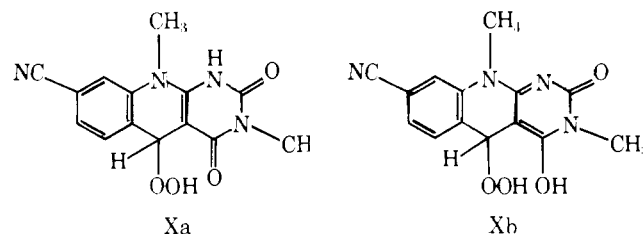
since these two structures would be indistinguishable by their NMR spectrum (solvent Me$_2$SO-d_6).

As is expected of hydroperoxides in general, compound VIII is unstable at elevated temperatures. For this reason we were unable to obtain satisfactory microanalytical combustion data for its characterization. The mass spectrum of VIII is rather complex, showing strong peaks at m/e 256, 240, 199 (base peak), and 170. The first two peaks presumably arise by loss of H$_2$O and H$_2$O$_2$ moieties from the molecular ion at m/e 274. The molecular ion itself does not appear in the spectrum owing to its instability at high temperatures. The base peak at m/e 199 is attributable to a retro-Diels–Alder decomposition of the ion at m/e 256 as depicted in Scheme I.

Table I. Comparison of the Properties of 5-Deazaisoalloxazines I and II and 1,5-Dideazaisoalloxazines III and IV

	I	II	III	IV
	NMR of Oxidized Form			
δ C(5) H^a, ppm	8.95	9 83^c	8.51	8.41
δ C(5) H^b, ppm		8.80	7.82	8.24
δ C(1) H, ppm			5.47^a	5.53^a
			5.00^b	5.37^b
	NMR of Reduced Form			
δ C(5) H$_2$, ppm	3.63^{d,4a}	3.83^{d,5}	3.50^e	3.64^e
pK_a of Reduced Form	7.4^5	6.9^5	6.8	6.3
$E°$, mV^f	−380^{16}	−390^5	−429^g	−368^g
	Reactivity with Nucleophiles^5			
KCN	reversible	irreversible	slow and require high pH (10–11)	
K$_2$SO$_3$	rapid equilibrium		very slow	
H$_2$O$_2$	form adducts		form adducts	
thiols	no reaction	rapid equilibrium	no reaction	
Hydroperoxy Adducts				
NMR δ C(5) H^d, ppm	5.28	5.40	5.13	
δ C(1) H^d, ppm			5.85	
k_2 (KI), M^{-1} s^{-1} ^{h}	0.13	0.62^5	0.005	0.02
k_2 (thioxane), M^{-1} s^{-1} ^{h}	0.013	0.09	i	i
	Reoxidation of Reduced Form^5			
k_2 (with *N*-methylacridinium chloride)	1.7 × 10^3 M^{-1} s^{-1}	k	3.6 × 10^3 M^{-1} s^{-1}	5.8 × 10^2 M^{-1} s^{-1}
k_2 (with K$_3$Fe(CN)$_6$)	1 × 10^2 M^{-1} s^{-1}	7.3 × 10^2 M^{-1} s^{-1}	8 × 10^3 M^{-1} s^{-1}	1 × 10^4 M^{-1} s^{-1}

^{a} N(3) methyl derivatives in CDCl$_3$. ^{b} N(3) sulfopropyl derivatives in D$_2$O. ^{c} In TFA. ^{d} In Me$_2$SO-d_6. ^{e} Reduced by sodium borohydride in D$_2$O. ^{f} pH 8.0. ^{g} Measured by equilibration of the reduced form with I. ^{h} In methanol. ^{i} Reaction in methanol was very slow. ^{j} pH 7.5 phosphate buffer. ^{k} The rate was not determined because a precipitate came out upon standing.

Scheme I

m/e 274

m/e 256

m/e 199

$-CHO·$ or $-(NCH_3)·$ → (*m/e* 170)
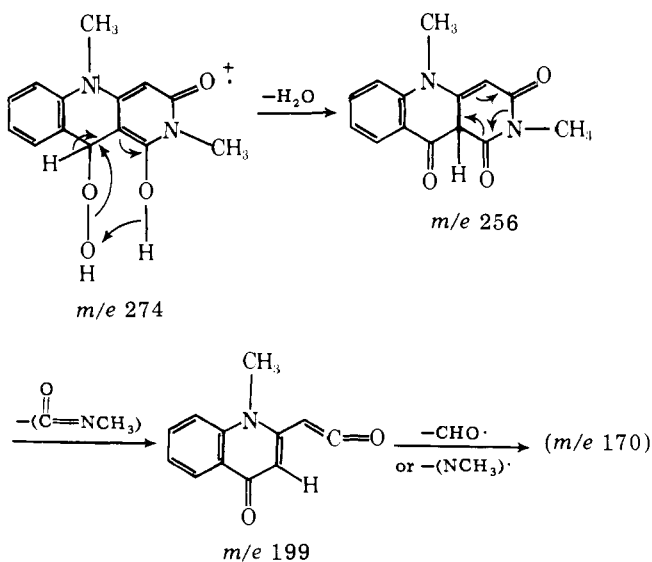
The molecular weight of VIII was established by its reaction with excess potassium iodide (eq 7). Thus a known quantity

$$\text{VIII} \xrightarrow{\text{KI}} \quad + \text{ I}_3^- \qquad (7)$$
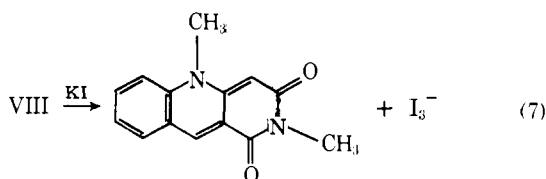
of compound VIII was allowed to react with a 0.1 M solution of KI in methanol at 30 °C. The second-order rate constant was 5 × 10^{-3} M^{-1} s^{-1}. From the known amount of VIII employed and from the final concentration of III produced, the molecular weight was determined to be 276 ± 10, in good agreement with the value of 274 expected of the structure VIII.

The amount of I$_3^-$ generated during the reaction (eq 7) cannot be used for the calculation of the molecular weight of VIII because of the slowness of the reaction which tends to allow further reaction of the formed [I$_3^-$]. Though VIII reacts readily with thioxane in an aqueous medium, its reaction with thioxane in methanol was too slow to be conveniently followed so that no comparison of the rate constant could be made with the reaction of the C(5) hydroperoxides of the 5-deaza analogue (X).^{5} It is obvious, however, that the less polarized peroxide (VIII) is the less reactive with thioxane to provide the corresponding sulfoxide.

The hydroperoxy adduct of II (i,e., X), but not of I, was previously reported.^{5} In this study we succeeded in isolating the C(5) hydroperoxide of I which is also a 1,5 adduct. However, unlike X, which is stable indefinitely at room temperature, this hydroperoxy adduct loses H$_2$O$_2$ on standing, reverting to I in but a few weeks. Rate constants for the reaction of the hydroperoxide of I with KI and thioxane were obtained (Table I).

Owing to the insolubility of IVb, the hydroperoxy adduct could not be prepared. However, the C(5) hydroperoxide of the 3-sulfopropyl derivative (IVc) was isolated in much the same manner as was VIII. This compound was pure by TLC and was not recrystallized. Its structure is similar to that of VIII. Some of its properties are listed in Table I.

Formation of 3,10-Dimethyl-1-hydroxy-1,5-dideazaisoalloxazine from VIII. A very interesting change was observed when the hydroperoxy adduct VIII was incubated anaerobically in aqueous buffers between pH 7 and 9.5. A purple coloration appeared slowly. Figure 4 is a repetitive scan of a solution of VIII in phosphate buffer, pH 8. The formation of the purple coloration (λ_{max} 535 nm, ε 7200 M^{-1} cm^{-1}) is associated with a lag phase, followed by a first-order change. Increase in pH accelerates the increase in A_{535}. However, at pH 10 or above, the initially rapidly formed purple color soon faded, indicating that hydrolysis has occurred. The formation of the purple compound is independent of the presence or absence of fluorescent room light.
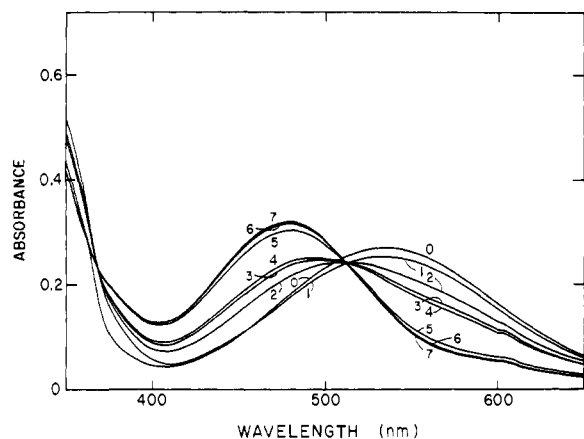
Figure 5. Equilibration of 3,10-dimethyl-1-hydroxy-1,5-dideazaisoalloxazine (IX) with dihydro-10-methyl-3-sulfopropyl-1,5-dideazaisoalloxazine (III-H_2) anaerobically at 30 °C, pH 7.9 (phosphate). Curve 0 was a 6.6 × 10^{-5} M solution of IX in the buffer. Lines 1-7 were recorded 5, 33, 57, 66, 210, and 1080 min, respectively, after addition of III-H_2 to a concentration of 8.4 × 10^{-5} M.

Scheme II

IX
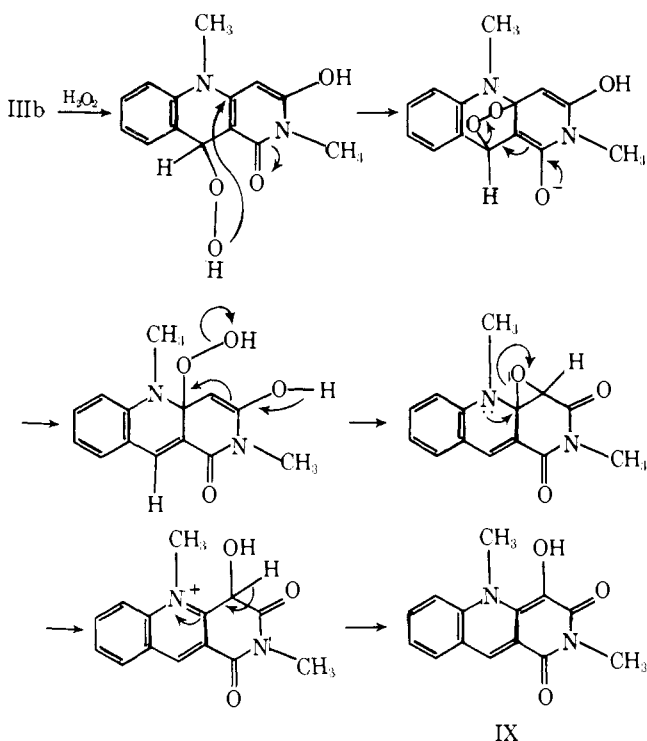
We succeeded in obtaining a pure sample of the purple, almost black, compound in solid form. It dissolves readily in chloroform but is only moderately soluble in other organic solvents. The NMR spectrum ($CDCl_3$) of this compound is rather simple (Figure 3). The peak at δ 6.03 ppm disappeared upon shaking with D_2O. The IR spectrum definitely shows the presence of a hydroxyl group. The mass spectrum is fairly straightforward, with an intense ion at *m/e* 256 (molecular ion) and some minor fragmentations, reflecting the aromaticity of the ring structure. The compound has no detectable EPR signal. Microanalytical data show that there are three oxygen atoms per molecule. We propose that this purple compound has the structure of 3,10-dimethyl-1-hydroxy-1,5-dideazaisoalloxazine (IX). The assignment of the OH group to position 1 follows from the NMR spectrum (Figure 3) in which the 8.19-ppm peak indicates that C(5) has a vinyl proton whereas there is no C(1) vinyl proton. The NMR spectrum also shows that the aromatic ring is intact and so are the two *N*-methyl

Scheme III

XI
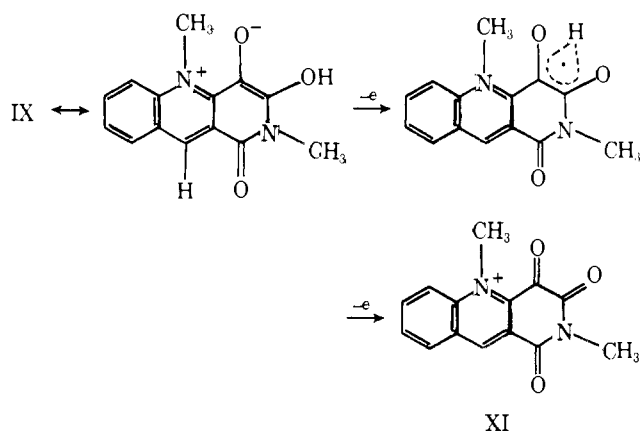
groups. Note that the extinction coefficient of the 535-nm peak of IX is not too different from that of the parent compound III (ϵ_{475} 9200 M^{-1} cm^{-1}) and the shift of λ_{max} to higher wavelength is expected of the enol structure of IX.

A possible mechanism for the formation of IX from VIII is shown in Scheme II. We have not attempted to verify the scheme. However, the fact that the reaction is faster at higher pH and the presence of a lag phase (Figure 4) are consistent with the proposed mechanism.

The enol structure IX is expected to be fairly readily oxidized and indeed we found that in aqueous solutions, IX was oxidized almost instantaneously by $K_3Fe(CN)_6$ and more slowly by oxygen or the nitroxide 4-hydroxy-2,2,6,6-tetramethyl-4-piperidine-1-oxy.[13] We were able to show, by subtraction of the spectra of the excess nitroxide or $K_3Fe(CN)_6$ from the spectra of the oxidized solutions, that all three oxidizing agents used gave the same oxidized product of IX. The UV spectrum of IX after oxidation by O_2 is shown in Figure 4 inset. By anaerobic titration of a dilute aqueous solution of IX (5 × 10^{-5} M), we found that 1 mol of IX requires about 2 mol of $K_3Fe(CN)_6$ for complete oxidation. Oxidation of IX in aqueous solutions is likely to occur by the pathway shown in Scheme III, though XI was not isolated and characterized. When $NaCNBH_3$ was added to XI, the purple color was restored.

An aqueous solution of IX equilibrates with dihydro-10-methyl-3-sulfopropyl-1,5-dideazaisoalloxazine as shown in Figure 5. This is in fact an oxidation–reduction equilibrium. Using eq 2 and the known initial concentrations of both reactants and the concentration of IX at equilibrium (from absorbance at 570 nm where III does not absorb), the redox potential of IX was estimated to be 15 mV more positive than that of III.

A similar color change (indicating the formation of a compound similar to IX) occurred when the hydroperoxy adduct of IVc was dissolved in an anaerobic aqueous buffer. The solution possessed a λ_{max} at 560 nm and the color disappeared when exposed to air. In this instance the 1-hydroxy compound was not isolated because the hydroperoxy adduct was prepared from the water-soluble 3-sulfopropyl derivative IVc. No absorbance change was observed when the hydroperoxy adducts of I and II were added to anaerobic buffers. This is not surprising since these compounds have a nitrogen atom at position 1 and hence a reaction pathway similar to that of Scheme II is precluded.

Oxidation of Dihydro-1,5-dideazaisoalloxazines. The oxidation of IIIc-H_2 was carried out with a number of reagents: oxygen, 4-hydroxy-2,2,6,6-tetramethyl-4-piperidine-1-oxy,[13] potassium ferricyanide, and *N*-methylacridinium chloride. Only the last two reagents were used for the oxidation of IVc-H_2.
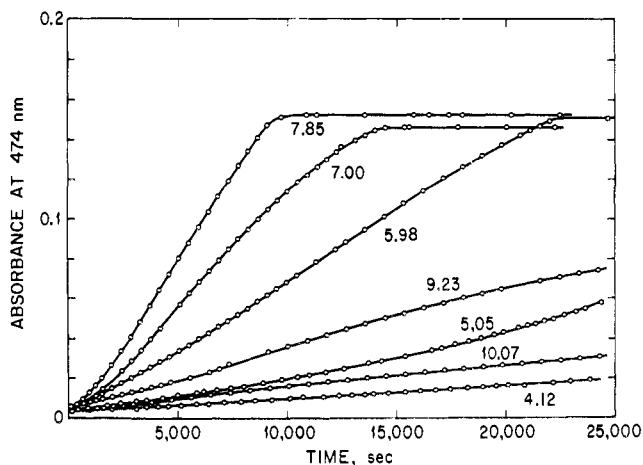
Figure 6. Plots of absorbance (474 nm) vs. time for the oxidation of di-hydro-10-methyl-3-sulfopropyl-1,5-dideazaisoalloxazine (IIIc-H_2) in oxygen-saturated buffers (pH 5-6, acetate; pH 6-8, phosphate; pH 9-10, carbonate) at 30 °C. [III-H_2] = 1.7×10^{-5} M. [O_2] ≈ 8×10^{-4} M. Numbers on the curves indicate the pH value.

Figure 7. Dependence of pseudo-first-order rate constants k_{obsd} for the reaction of IIIc-H_2 with $K_3Fe(CN)_6$ on concentration of $K_3Fe(CN)_6$.

The time course for the oxidation of III-H_2 by molecular oxygen (A_{475}) shows a sigmoidal appearance of III and is thus characteristic of an autocatalytic process, as previously found for the oxidation of dihydroflavins[14] and dihydro-5-deaza-flavins. There could be obtained no evidence of any long wavelength absorbing intermediates which might be indicative of the accumulation of radical species. Typical absorbance traces for the oxidation of III-H_2 in oxygen-saturated buffers are shown in Figure 6. These traces are not easily reproducible. Slight variations in curvature of the absorbances traces are observed when the oxidation was carried out under apparently identical conditions. This is a consequence of the autocatalytic nature of the reaction. The rate of oxidation increases with pH up to about pH 8. At pH 9 or above, the rate of appearance of III decreases, which may possibly be attributed to hydrolysis. At a given pH, the reaction rate is also sensitive to the buffer species employed. In the presence of the oxidized form (IIIc), the oxidation of IIIc-H_2 by O_2 was somewhat accelerated, as was the case with oxidation of reduced 5-deazaflavins and dihydroflavins. We did not attempt to make any detailed kinetic analysis of the oxidation of IIIc-H_2 by O_2^-. The slowness of the reaction implies that it does not depend on superoxide anion O_2^- since dismutation of O_2^- is fast. The hydroperoxide VIII cannot be an intermediate in the oxidation process since a mixture of III-H_2 and VIII did not yield any appreciable amount of III. It is clear that if a hydroperoxy adduct is an intermediate in the oxidation process, it must possess a different structure than VIII.

Non-first-order kinetics (similar to that obtained upon O_2 oxidation) was observed when IIIc-H_2 was oxidized by the nitroxide 4-hydroxy-2,2,6,6-tetramethyl-4-piperidine-1-oxy. With a nitroxide concentration of 0.05 M, the rate of oxidation is greater than that for O_2 oxidation ([O_2] ≈ 8×10^{-4} M) by only about fivefold (pH 7). A much shorter lag phase exists with the nitroxide oxidation. Here again the absorbance traces (not shown) tend to be quantitatively irreproducible and no detailed analysis of the reaction was made. The rate of oxidation of III-H_2 by the nitroxide is faster at lower pH values. This is in accord with the stronger oxidizing properties of nitroxides at lower pH values.[15]

The oxidation of IIIc-H_2 and IVc-H_2, as well as I-H_2 and II-H_2, by $K_3Fe(CN)_6$ and N-methylacridinium chloride occurred readily and gave excellent pseudo-first-order kinetics. The observed first-order rate constants are proportional to the concentrations of $K_3Fe(CN)_6$ or N-methylacridinium chloride (e.g., see Figure 7). In the case of oxidation of the reduced
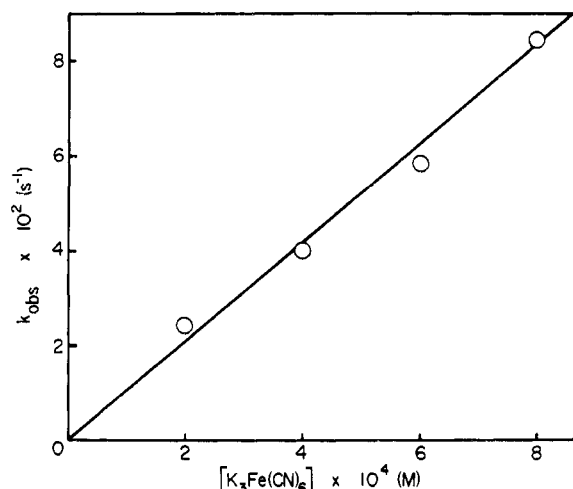
deazaisoalloxazines by $K_3Fe(CN)_6$, this observation would be in accord with the rate-determining formation of radical followed by its rapid comproportionation (eq 8). (If this were so,
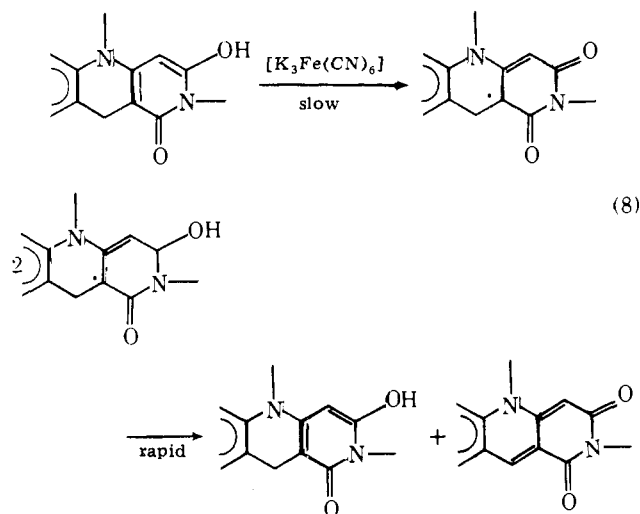
the observed second-order rate constant (Table I) would be one-half that for the rate-determining $1e^-$ transfer process.) Alternatively, rate-determining formation of 1,5-deazaflavin radical may be followed by a more rapid transfer of one electron or a hydrogen atom to yield the oxidized dideazaflavin directly. The determined second-order rate constants are shown in Table I. In these reactions, oxidized deazaflavins, potassium ferrocyanide, and N-methylacridan were shown spectrally or by TLC to be the products. The rate constants of the reaction of N-methylacridinium chloride with reduced 5-deaza- and 1,5-dideazaisoalloxazines fall within the range of rate constants for N-methylacridinium salts with various NADH analogues.[17] Thus, the second-order rate constants for 1,4-dihydro-N-ribose-5-phosphate nicotinamide and 1,4-dihydro-N-propylnicotinamide are 42 (pH 8) and 2000 M^{-1} s^{-1}, respectively.[17] On the other hand, the dihydro-1,5-dideaza-isoalloxazines react with $K_3Fe(CN)_6$ at least an order of magnitude faster than the reduced 5-deazaisoalloxazines which in turn react with this reagent much faster than NADH analogues. The $1e^-$ oxidation of NADH analogues by $K_3Fe(CN)_6$ has been investigated[18] and has been shown to be slow,[19] though no rate constants were reported.

Although the use of 5-deazaisoalloxazines for investigating the mechanism of reactions of flavins is well documented,[2-4] the rationale behind the use of these compounds as flavin

models has recently been questioned by a few authors,[19,20] who prefer to consider them as NAD^+ analogues. The 1-deazaisoalloxazines are certainly closer analogues to flavins than the 5-deazaisoalloxazines, In this study, we have demonstrated some interesting chemical properties of 1,5-dideazaisoalloxazines. These compounds resemble the 5-deazaflavins more than the 1-deazaflavins, From the properties of these three kinds of deazaisoalloxazines one may conclude that position 5 of the isoalloxazine ring system is the most important site for flavin-mediated chemical reactions. More specifically, the 1,4-pyrazine ring which incorporates the N(5) and N(10) members provides stability to the radical states of oxidation and this is not realized on replacement of the N(5) moiety by carbon. As we have previously pointed out,[21] the radical stability in isoalloxazines may be attributed to Hoffman orbital splitting,[22] a feature which has found experimental proof in the established stability of radicals generated from 1,4-dihydropyrazines.[23]

Acknowledgment. This work was supported by grants from the National Institutes of Health and the National Science Foundation. We thank Professors Neil Castagnoli (University of California at San Francisco) and George Popjack (University of California at Los Angeles) for mass spectral analysis and for discussion concerning their interpretation.

References and Notes

(1) D. E. O'Brien, L. T. Weinstock, and C. C. Cheng, *J. Heterocycl. Chem.*, **7**, 99 (1970).
(2) (a) R. J. Pollock and L. B. Hersh, *J. Biol. Chem.*, **248**, 6724 (1973); (b) M. S. Jorns and L. B. Hersh, *J. Am. Chem. Soc.*, **96**, 4012 (1974); (c) L. B. Hersh and M. S. Jorns, *J. Biol. Chem.*, **250**, 8728 (1975); (d) B. A. Averill, A. Schonbrunn, R. H. Abeles, L. T. Weinstock, C. C. Cheng, J. Fisher, R. Spencer, and C. Walsh, *ibid.*, **250**, 1603 (1975); (e) M. S. Jorns and L. B. Hersh, *ibid.*, **250**, 3620 (1975); (f) L. B. Hersh, M. S. Jorns, J. Peterson, and M. Currie, *J. Am. Chem. Soc.*, **98**, 865 (1976); (g) T. H. Cromartie and C. T. Walsh, *J. Biol. Chem.*, **251**, 329 (1976); (h) J. Fisher, R. Spencer, and C. Walsh, *Biochemistry*, **15**, 1054 (1976); (i) R. Spencer, J. Fisher, R. Laura, and C. Walsh, in "Flavins and Flavoproteins", T. P. Singer, Ed., Elsevier, Amsterdam, 1976, p 349.
(3) (a) D. E. Edmonson, B. Barman, and G. Tollin, *Biochemistry*, **11**, 1133 (1972); (b) M. S. Jorns and L. B. Hersh, *J. Biol. Chem.*, **251**, 4872 (1976).
(4) (a) M. Brüstlein and T. C. Bruice, *J. Am. Chem. Soc.*, **94**, 6548 (1972); (b) S. Shinkai and T. C. Bruice, *ibid.*, **95**, 7526 (1973); (c) J. Fisher and C. Walsh, *ibid.*, **96**, 4345 (1974); (d) R. Spencer, J. Fisher, and C. Walsh, *Biochemistry*, **15**, 1043 (1976).
(5) R. L. Chan and T. C. Bruice, *J. Am. Chem. Soc.*, **99**, 6721 (1977).
(6) (a) W. T. Ashton, D. W. Graham, R. D. Brown, and E. F. Rogers, *Tetrahedron Lett.*, 2551 (1977); (b) W. T. Ashton, R. D. Brown, and R. L. Tolman, *J. Heterocycl. Chem.*, **15**, 489 (1978); (c) L. T. Weinstock, C. J. W. Wiegand, and C. C. Cheng, *ibid.*, **14**, 1261 (1977).
(7) R. Spencer, J. Fisher, and C. Walsh, *Biochemistry*, **16**, 3586 (1977).
(8) (a) R. Spencer, J. Fisher, and C. Walsh, *Biochemistry*, **16**, 3594 (1977); (b) C. Walsh, J. Fisher, R. Spencer, D. W. Graham, W. T. Ashton, J. E. Brown, R. D. Brown, and E. F. Rogers, *ibid.*, **17**, 1942 (1978).
(9) H. Baron, F. G. P. Remfry, and J. F. Thorpe, *J. Chem. Soc.*, **85**, 1726 (1904).
(10) M. T. Stankovich and V. Massey, *Biochim. Biophys. Acta*, **452**, 335 (1976).
(11) H. N. Rydon and K. Undheim, *J. Chem. Soc.*, 4676 (1962).
(12) F. Müller, V. Massey, and P. Hemmerich, *Methods Enzymol.*, **18**, 474 (1960).
(13) T. W. Chan and T. C. Bruice, *J. Am. Chem. Soc.*, **99**, 7287 (1977).
(14) C. Kemal, T. W. Chan, and T. C. Bruice, *J. Am. Chem. Soc.*, **99**, 7272 (1977).
(15) M. B. Naimann, S. G. Mairanovskii, B. M. Kovarskaya, E. G. Rozantsev, and E. G. Gintsberg, *Bull. Acad. Sci. USSR, Div. Chem. Sci.*, 1424 (1964).
(16) G. Blankenhorn, *Biochemistry*, **14**, 3172 (1975).
(17) D. J. Creighton, J. Hajdu, G. Mooser, and D. S. Sigman, *J. Am. Chem. Soc.*, **95**, 6855 (1973).
(18) T. Okamoto, A. Ohno, and S. Oka, *J. Chem. Soc., Chem Commun.*, 181 (1977).
(19) P. Hemmerich, V. Massey, and H. Fenner, *FEBS Lett.*, **84**, 5 (1977).
(20) G. Blankenhorn, *Eur. J. Biochem.*, **67**, 67 (1976).
(21) T. C. Bruice, *Prog. Bioorg. Chem.*, **4**, 1 (1976).
(22) R. Hoffman, *Acc. Chem. Res.*, **4**, 1 (1971).
(23) E. Heilbronner and A. K. Muszkat, *J. Am. Chem. Soc.*, **92**, 3878 (1970).

Rates of Formation and Decomposition of Tetrahedral Intermediates in the Hydrolysis of Dimethyl Aroylphosphonates. Substituent Effects on a Model for Carboxylate Ester Hydrolysis

Ronald Kluger* and Jik Chin

Contribution from the Department of Chemistry, University of Toronto, Toronto, Ontario, Canada M5S 1A1. Received June 22, 1978

Abstract: Dimethyl aroylphosphonates are known to undergo hydrolysis to form substituted benzoic acids and dimethyl phosphonate: $ArCOPO(OCH_3)_2 + H_2O \rightarrow ArCOOH + HPO(OCH_3)_2$. A detailed kinetic analysis of the reaction for a series of phenyl-substituted dimethyl benzoylphosphonates was performed using spectroscopic techniques. The reaction proceeds in two steps which under certain conditions can be observed separately. Initially, hydration of the carbonyl group occurs (eq 2). Below pH 5 the rate of establishment of this equilibrium can be measured without interference from the subsequent decomposition process, which is proportional to hydroxide concentration and slower than hydration under these conditions. Above pH 6, hydration (k_1) is slow compared to conversion to products. Thus, above pH 6 hydration becomes rate limiting and the observed rate constant approaches k_1. Between pH 5 and 6 the reaction steps are comparable in magnitude and complex kinetic behavior results. Thus a complete profile for a carbonyl cleavage reaction can be obtained. Linear free energy relationships have been used to estimate the values of K_a (eq 4) for the species studied (substituents: 3-bromo, 4-methyl, 4-methoxy, hydrogen). This provides values for k_3 (eq 5) ($\sim 10^4 \text{ s}^{-1}$). The equilibrium and kinetic data obtained in this way give enough information to determine substituent dependencies of each step, solvent isotope effects, and activation parameters. These fit into patterns established for other carbonyl cleavage and hydration reactions which have not been analyzed. It is suggested that since the phosphonate diester anion is about as basic as alkoxide, the reaction serves as a model for the breakup of intermediates in ester hydrolysis. The hydration reaction fits into reactivity schemes suggested for aldehydes. The observed features of acyl phosphate ester hydrolysis can be analyzed in terms of rate-limiting hydration as well.

A reaction sequence that many types of carbonyl group containing compounds undergo involves addition of water to the carbonyl group to form a hydrate followed by expulsion of one of the substituents attached to the hydrated carbon atom (eq 1). Members of this general class of reaction include ester hydrolysis, amide hydrolysis, and ketone cleavage. Although