The Photochemistry of Riboflavin. IV. The Photobleaching of Some Nitrogen-9 Substituted Isoalloxazines and Flavins

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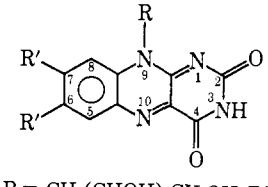
Abstract: A variety of alcoholic type side chains attached at the N-9 position of the isoalloxazine nucleus have been photobleached under anaerobic and aerobic conditions. Anaerobic photolysis in neutral aqueous solution gave primarily two products as ascertained by polarography. One product was alloxazine and the other was an oxygen-sensitive intermediate which must have a ring formed by attachment of the side chain to the isoalloxazine nucleus. All isoalloxazines that underwent the photobleaching reaction produced the cyclic intermediate. The product ratio of alloxazine to intermediate is dependent on the length of the side chain. Linear ω-hydroxylic chains of three or more carbon atoms gave predominately the intermediate, whereas two carbon-atom side chains gave a mixture. Primary, secondary, and tertiary alcoholic groups on the side chain under anaerobic irradiation gave aldehydes, ketones, and the regenerated alcohol, respectively, from the dark oxidation of the cyclic intermediate. Quantum efficiencies varied from 0.2 to 2.0% for the various isoalloxazines and flavins. Aerobic photobleaching of the isoalloxazines gave similar products to the anaerobic photobleaching as determined by thin layer chromatography, but the rate processes were complicated by the competitive physical and chemical steps involving oxygen. Deuterium substitution at various locations on the ω-hydroxyalkyl side chain has conclusively shown that the carbon to hydrogen bond at the hydroxyl containing carbon controls the rate of the photobleaching process if such an α hydrogen is present. If an α hydrogen is not present on the hydroxyalkyl side chain, the photobleaching is initiated by abstraction of the hydroxyl hydrogen. Other alkyl hydrogens on the side chain are unreactive in the photobleaching process. These results substantiate a mechanism of intramolecular hydrogen abstraction from the N-9 substituted hydroxyalkyl side chain to the isoalloxazine nucleus during the primary photoprocess of photobleaching.

Karrer and his coworkers were the first to study the photochemistry of isoalloxazines other than riboflavin.[1,2] Several different N-9 substituted isoalloxazines were prepared in an attempt to locate the active sites on the ribityl side chain of riboflavin I. They photolyzed the compounds 9-(2′,3′-dihydroxypropyl)- (II), 9-(2′-hydroxyethyl)- (III), and 9-(1′-hydroxymethyl-2′-hydroxyethyl)isoalloxazine (IV). The only definite product identified from the irradiation was alloxazine (V). Some leuco compounds were formed as indicated by a color return upon dark oxidation of the solutions. It was Karrer's contention, as a result of these studies, that the flavins were altered at the 2′-carbon position. It was thought that the alcoholic group was oxidized to a carbonyl group which subsequently decomposed to form an alloxazine. Later, Karrer and Meerwein[3] showed that the photolysis of 9-(2′-hydroxy-2′-methylpropyl)-isoalloxazine (VI), which contains a tertiary alcoholic group, was very slow by comparison with the other isoalloxazines studied. This indicated to them the necessity of an α hydrogen on the hydroxylic carbon.

Yang and McCormick[4] studied the anaerobic photobleaching of a series of flavins[5] bearing N-9 substituted polyhydroxyalkyl and ω-hydroxyalkyl groups. The relative rates of photobleaching were in the decreasing order polyhydroxyalkyl-, 3′-hydroxypropyl-, 5′-hydroxypentyl-, 6′-hydroxyhexyl-, 4′-hydroxybutylflavin. Paper chromatograms of the photoproducts from aerobic photobleaching indicated two or more products for most of the flavins but only lumichrome was definitely identified. Halwer[6] restudied some of the same isoalloxazines used by Karrer. He verified that photolysis of VI was indeed slow in neutral solution but the photobleaching was strongly acid catalyzed and VI reacted faster than other isoalloxazines under these conditions. He concluded that it was the hydroxyl group and not the α hydrogen that was important to the photochemistry. Metzler and McBride[7] found that formylmethylflavin (IX) photobleached more rapidly under anaerobic conditions than any other flavin or isoalloxazine. This flavin is now accepted as the mysterious deuteroflavin of the old literature.[8]



I, R = $CH_2(CHOH)_3CH_2OH$; R′ = CH_3
II, R = $CH_2(CHOH)CH_2OH$; R′ = H
III, R = CH_2CH_2OH; R′ = H
IV, R = $CH(CH_2OH)_2$; R′ = H
V, R = H(at *N*-1 position); R′ = H
VI, R = $CH_2(COH)(CH_3)_2$; R′ = H
VII, R = $CH_2CH_2CH_2OH$; R′ = H
VIII, R = $(CH_2)_3CH_2OH$; R′ = H
IX, R = CH_2CHO; R′ = CH_3
X, R = $CH_2(CHOH)CH_3$; R′ = H
XI, R = $CH_2(CHOH)CH_2OH$; R′ = CH_3
XII, R = $CH_2CH_2CH_2OH$; R′ = CH_3

(1) P. Karrer, T. Köbner, H. Salomon, and F. Zehender, *Helv. Chim. Acta*, **18**, 266 (1935).
(2) P. Karrer and H. F. Meerwein, *ibid.*, **18**, 480 (1935).
(3) P. Karrer and H. F. Meerwein, *ibid.*, **18**, 1126 (1935).
(4) C. S. Yang and D. B. McCormick, *J. Amer. Chem. Soc.*, **87**, 5763 (1965).
(5) Throughout this paper 6,7-dimethylisoalloxazines containing N-9 substitutents will be termed flavins.
(6) M. Halwer, *J. Amer. Chem. Soc.*, 73, 4870 (1951).
(7) M. M. McBride and D. E. Metzler, *Photochem. Photobiol.*, 6, 113 (1967).
(8) E. C. Smith and D. E. Metzler, *J. Amer. Chem. Soc.*, **85**, 3285 (1963).

Moore and coworkers[9] have shown that the photochemistry of at least one isoalloxazine, 9-(2′-hydroxyethyl)isoalloxazine (III) can be explained on the basis of an intramolecular exchange of hydrogen from the side chain to the isoallozazine nucleus. A photochemical study of several N-9 substituted isoallozaxines was undertaken to determine if some more generalized statements could be made concerning this intriguing type of photoprocess. The isoalloxazines included in the study were II, III, VI, VII, VIII, IX, and X. Also the 6,7-dimethyl derivatives of III and VII, and XI and XII were studied to assess the effect of these substituents.

Experimental Section

Instrumentation. Infrared spectra were recorded with a Beckman IR-8 spectrometer and ultraviolet spectra were recorded with a Cary Model 15 spectrometer. Nuclear magnetic resonance spectra were taken with a Varian A-60 spectrometer. Trifluoroacetic acid and deuterium oxide were used as nmr solvents with tetramethylsilane as an external standard. Polarographic data were obtained with a Polarecord Model E261R. Electrodes were a vibrating dropping mercury electrode and a silver-silver chloride reference electrode. The polarographic vessel was placed in the light path of a photochemical reactor. The photochemical reactor consisted of a 800C SAH Westinghouse mercury arc at the focal length of a convex quartz lens (diameter, 2.5 in.; focal length, 6 in.). The collimated light beam passed down the length of a Cenco optical bench containing suitable filters, solution holders, and thermopile. Filtered light intensities of approximately 10^{16} quanta sec^{-1} cm^{-2} were obtained with the system.

Procedures. Thin-layer chromatographic analyses were done on commercially prepared E. Merck precoated silica gel F-254 glass plates. The compounds were spotted as aqueous solutions and the plates were placed in a closed developing chamber. The solvent systems used were butanol–acetic acid–water and butanol–trifluoroacetic acid–water in volume proportions 4:1:5. The organic layer was used for the developing solvent. The tlc spots were observed by fluorescence using 366 nm irradiation. The reported R_f values are given to represent approximate movement. Self-consistent data were obtained on either the freshly prepared silica gel plates or the commercial plates. We have recently standardized procedures for tlc including temperature control but the reported study was already completed. All of the reported R_f values were obtained with the butanol–acetic acid–water solvent system.

Kinetic studies were performed using the described photochemical reactor. The photolysis cell consisted of a 1-cm square spectrophotometer cell connected to a degassing chamber by a Pyrex to quartz seal. The cell contained a side arm midway which terminated in a ground-glass joint for connection to the vacuum system. Three milliliters of 10^{-4} *M* solution were placed in the cell and it was attached to the vacuum system. Air was removed by 4 to 5 freeze–thaw cycles at a pressure of 10^{-3} Torr. The cells were sealed under vacuum and removed from the system. The solutions were protected from light during the degassing procedures. The photolysis cell was placed in the reactor and photolyzed for short intervals after which the absorbance was measured on a spectrophotometer. Light intensities were determined chemically by ferrioxalate actinometry[10] and relative measurements were made with an Epply thermopile connected to a L & N recorder. Values of first-order rate constants were calculated from the slope of plots of the logarithm of absorbance *vs.* time. The reactions were followed for a minimum of one half-life. Photochemical reactions are not normally followed kinetically to this degree of completion. Kinetic isotope-effect data were obtained by irradiating both a deuterated and nondeuterated sample simultaneously in the photochemical reactor. The integrated cross section of the collimated light beam was found to be uniform to within 3% as determined by thermopile measurements and chemical actinometry. Also, the sample positions were interchanged from one trial to the next so that variations in the light beam would be cancelled in the determinations. A Beckman DU cell holder was used to locate reproducibly the cells in the light beam.

Syntheses of Isoalloxazines. The isoalloxazines were synthesized by the basic method of Karrer, *et al.*[1,2,11,12] The amine of the desired side chain was condensed with *o*-chloronitrobenzene or 2-chloro-4,5-dimethylnitrobenzene to produce the N-substituted *o*-nitroaniline. This aniline was catalytically hydrogenated over platinum oxide. Alloxan was condensed with the reduced material to produce the desired isoalloxazine. Initially the process of Tishler, *et al.*,[13] was attempted. The process consisted of treating an N-substituted amine with a diazonium salt to produce an *o*-amino azo compound; the azo group is then split by a carbanion in the condensation with barbituric acid to produce the isoalloxazine. However, in the step that should have formed an *o*-aminoazo compound only a diazoamino compound resulted. Finally, the *o*-aminoazo compound was reached by a diazoamino–aminoazo rearrangement. Since the procedure had only limited value in the preparation of the compound we desired, the method of Karrer was predominately used. It was limited by the availability of the aliphatic amine required.

9-(3′-Hydroxypropyl)isoalloxazine. A solution of 4.5 g (60 mmol) of 3-amino-1-propanol in dry pyridine was heated with 8 g (114 mmol) of 2-chloronitrobenzene for 24 hr. The excess chloronitrobenzene and pyridine were removed by steam distillation. The residue was extracted with ether and the ether extract was evaporated. The product was recrystallized from an acetic acid–ligroin mixture to give pure N-(3′-hydroxypropyl)-2-nitroaniline in a yield of 42.6% (5.0 g). The golden crystals had a melting range 63–64° (lit.[1] mp 65°); $\lambda_{max}(CHCl_3)$ 3625 (OH), 3390 (NH), and 1350 cm^{-1} (NO_2); λ_{max} (EtOH) 232, 280, and 425 nm.

The aniline, 2.00 g (7.3 mmol), was dissolved in 75 ml of absolute ethanol, catalytically reduced with hydrogen over platinum oxide (0.1 g) at room temperature and atmospheric pressure. Three mole equivalents of hydrogen were absorbed after 2 hr. The hydrochloride of the crude product was condensed with 1.17 g (7.4 mmol) of alloxan monohydrate to produce 1.31 g (66.2%) of 9-(3′-hydroxypropyl)isoalloxazine. Three recrystallizations from water containing a few drops of Chlorox yielded pure compound, mp 302–305° dec (lit.[1] mp 297° dec). The ultraviolet spectrum was characteristic of an isoalloxazine with strong peaks at 215, 263, 346, and 430 nm in aqueous solution.[14] The nmr spectrum is discussed in another section.

The corresponding flavin, 6,7-dimethyl-9-(3′-hydroxypropyl)-isoalloxazine, was prepared by the same method. However, the substituted chloronitrobenzene had to be synthesized. Nitration of 3,4-dimethylaniline gave a 45.6% yield of 4,5-dimethyl-2-nitroaniline, mp 138–140° (lit.[15] mp 140°). The 2-chloro-4,5-dimethyl nitrobenzene was prepared from the nitroaniline according to the procedure of Adams, Weisel, and Mosher[16] in 67% yield, mp 57–60°. 3-Amino-1-propanol was heated with excess 2-chloro-4,5-dimethylnitrobenzene to give the desired substituted nitroaniline. The nitroaniline was reduced with hydrogen and condensed with alloxan as described above to produce a 30% yield of the flavin, 6,7-dimethyl-9-(3′-hydroxypropyl)isoalloxazine, mp 295–297° dec. The ultraviolet spectrum in aqueous solution showed strong maxima at 222, 266, 373, and 444 nm. The nmr spectrum in TFA–D_2O solution consisted of peaks at 7.92 (1 H, singlet), 7.63 (1 H, singlet), 4.78 (2 H, triplet, J = 6 cps), 3.63 (2 H, triplet, J = 6 cps), 2.37 (3 H, singlet), 2.23 (3 H, singlet), and 2.14 ppm (2 H, multiplet).

9-(3′-Hydroxypropyl-3′,3′-d_2)isoalloxazine. β-Alanine was reduced with lithium aluminum deuteride in anhydrous tetrahydrofuran.[17] The excess deuteride was destroyed with water and the mixture was extracted with ether. The precipitate was placed in a Soxlet extractor and treated with ether for 96 hr. The ether extracts were combined and evaporated leaving a residue of crude deuterated amino alcohol. A solution of the amino alcohol and dry pyridine

(9) W. M. Moore, J. T. Spence, F. A. Raymond, and S. D. Colson, *J. Amer. Chem. Soc.*, **85**, 3367 (1963).

(10) C. G. Hatchard and C. A. Parker, *Proc. Roy. Soc.* (London), **A235**, 518 (1956).

(11) P. Karrer, H. Salomon, K. Schöpp, and E. Schlittler, *Helv. Chim. Acta*, **17**, 1165 (1934).

(12) P. Karrer, E. Schlittler, K. Pfaehler, and F. Benz, *ibid.*, **17**, 1516 (1934).

(13) M. Tishler, K. Pfister, R. D. Babson, K. Landenburg, and A. J. Fleming, *J. Amer. Chem. Soc.*, **69**, 1487 (1947).

(14) The ultraviolet spectra of all the reported isoalloxazines are identical in shape and very characteristic. Maxima are only slightly affected by N-9-substitution but maxima for the 6,7-dimethyl analogs (flavins) are shifted to longer wavelengths.

(15) I. I. Levkoev, N. N. Sveshnikov, N. S. Barvyn, and M. P. Pashin, *Zh. Obshch. Khim.*, **22**, 516 (1952).

(16) R. R. Adams, C. A. Weisel, and H. S. Mosher, *J. Amer. Chem. Soc.*, **68**, 883 (1946).

(17) O. Vogl and M. Pöhm, *Monatsh.*, **84**, 1097 (1953).

was heated with 2-chloronitrobenzene for 24 hr. The excess 2-chloronitrobenzene and pyridine were removed by steam distillation. The residue was extracted with ether, the ether was evaporated, and the product was recrystallized from a glacial acetic acid–ligroin mixture to give N-(3′-hydroxypropyl-3′,3′-d_2)-2-nitroaniline. The deuterated nitroaniline gave a good mixture melting point with the nondeuterated nitroaniline, mp 63–64° (lit.[1] mp 65°). The infrared spectrum of the deuterated nitroaniline in chloroform was identical with the nondeuterated aniline except for a doublet at 2090 cm^{-1}, which is the C–D stretching frequency. The deuterated nitroaniline was dissolved in absolute ethanol and catalytically reduced with hydrogen over platinum oxide under ambient conditions. The hydrochloride of the crude product was condensed with alloxan monohydrate to yield 9-(3′-hydroxypropyl-3′,3′-d_2)isoalloxazine, mp 314–315° dec. The nmr spectrum of this compound is discussed later.

9-(3′-Hydroxypropyl-2′,2′-d_2)isoalloxazine. Ethyl cyanoacetate was shaken with anhydrous ether and deuterium oxide containing potassium carbonate for 77 hr.[18] The cyano ester was recovered from the ether layer after drying over anhydrous sodium sulfate. The exchange was not complete as determined from the nmr spectrum. The deuterium exchange procedure was repeated and the yield was 5.0 g of ethyl cyanoacetate-α,α-d_2. The nmr spectrum showed essentially complete exchange. The dideuterio ethyl cyanoacetate was slowly added to a solution of lithium aluminum hydride in ether and the mixture was refluxed for 1 hr.[19] The mixture was hydrolyzed by successive additions of wet ether, water, 20% sodium hydroxide, and water to yield 3-amino-1-propanol-2,2-d_2. The nmr spectrum of the neat material showed peaks at 4.12 (3 H, singlet), 3.50 (2 H, singlet), and 2.62 ppm (2 H, singlet) with respect to an external TMS standard. 3-Amino-1-propanol-2,2-d_2 was treated with 2-chloronitrobenzene as described previously to yield yellow crystals, mp 63–64°. The infrared spectrum for N-(3′-hydroxypropyl-2′,2′-d_2)-2-nitroaniline was identical with N-(3′-hydroxypropyl-3′,3′-d_2)-2-nitroaniline except for a 10-cm^{-1} shift in the peak at 2200 cm^{-1}. The deuterated nitroaniline was reduced with hydrogen and condensed with alloxan as described previously to give yellow-orange crystals, mp 302–305° dec. The nmr spectrum will be discussed later.

9-(2′,3′-Dihydroxypropyl)isoalloxazine. 3-Amino-1,2-propanediol was condensed with excess 2-chloronitrobenzene and the product was isolated to yield yellow crystals, mp 117–118° dec (lit.[11] mp 120° dec). The nitroaniline was reduced with hydrogen and condensed with alloxan as described previously to give a 52.4% yield of the isoalloxazine, mp 287–289° dec. Its structure was verified by nmr and ultraviolet spectroscopy.

The corresponding flavin, 6,7-dimethyl-9-(2′,3′-dihydroxypropyl)isoalloxazine, was prepared as described previously, mp 291–295° dec (lit.[12] mp 294° dec). Its structure was verified by nmr and ultraviolet spectroscopy.

9-(2′-Hydroxypropyl)isoalloxazine. Following the general procedure described, 1-amino-2-propanol was condensed with 2-chloronitrobenzene to produce in 49.9% yield the nitroaniline. The nitroaniline was reduced and condensed with alloxan to give a 55.6% yield of the isoalloxazine, mp 295° dec. Its structure was verified by nmr and ultraviolet spectroscopy.

9-(2′-Hydroxy-2′-methylpropyl)isoalloxazine. 2-Cyano-2-propanol in ether was reduced with lithium aluminum hydride. The product was fractionally distilled under reduced pressure and the fraction boiling at 52–57° (5 Torr) was collected. The yield was 9.5% of the amino alcohol. The nitroaniline was prepared in 37.6% yield from the reaction of 1-amino-2-methyl-2-propanol with 2-chloronitrobenzene, mp 79–81° (lit.[3] mp 83°). The nitroaniline was reduced and condensed with alloxan to produce 1.27 g (46.8%) of 9-(2′-hydroxy-2′-methylpropyl)isoalloxazine, mp 243–295° dec (lit.[3] mp 285° dec). The nmr spectrum showed peaks at 8.18 (1 H, doublet), 7.86 (3 H, singlet), 4.72 (2 H, singlet), and 1.18 ppm (6 H, singlet).

9-(2′-Hydroxypropyl)isoalloxazine. Following the general procedure described, 1-amino-2-propanol was condensed with 2-chloronitrobenzene to produce the nitroaniline which was reduced and then condensed with alloxan. The resulting isoalloxazine had a mp 295° dec.

9-(4′-Hydroxybutyl)isoalloxazine. This isoalloxazine was produced in the standard procedure beginning with 4-aminobutanol.

9-(2′-Hydroxyethyl)isoalloxazine. This isoalloxazine had been prepared previously and the synthesis was described in detail.[9]

9-(Formylmethyl)isoalloxazine. The aldehyde was prepared by the procedure of Fall and Petering[20] starting with 343 mg of 9-(2′,3′-dihydroxypropyl)isoalloxazine and 327 mg of periodic acid. 9-(Formylmethyl)isoalloxazine was produced in 92% yield, mp 240° dec.

9-(4′-Hydroxybutyl-4′,4′-d_2)isoalloxazine. The procedure followed parallels that used for the preparation of 9-(3′-hydroxypropyl-3′,3′-d_2)isoalloxazine. 4-Aminobutanoic acid was added to a solution of lithium aluminum deuteride to produce the amino alcohol. The amino alcohol was treated with 2-chloronitrobenzene as previously described. Attempts to recrystallize the deuterated nitroaniline from glacial acetic acid were unsuccessful. Following the general procedure, the crude nitroaniline was reduced with hydrogen and the product was then condensed with alloxan monohydrate to give 9-(4′-hydroxybutyl-4′,4′-d_2)isoalloxazine. The nmr spectrum showed peaks at 8.03 (1 H, doublet), 7.84 (3 H, broad singlet), 4.64 (2 H, doublet) and 1.78 ppm (4 H, multiplet). This contrasts with the nondeuterated analog which showed peaks at 8.12 (1 H, doublet, J = 9 cps), 7.70 (3 H, broad singlet), 4.70 (2 H, triplet, J = 6 cps), 3.69 (2 H, triplet, J = 5 cps), and 1.75 ppm (4 H, multiplet). The 3.69-ppm peak is missing for the deuterated isoalloxazine which is indicative of the terminal hydroxyl containing C′ protons.

Nuclear Magnetic Resonance Spectra of Isoalloxazines. It was realized that an nmr study of the isoalloxazines would develop a basis for the identification of the photodegradation products of riboflavin. Recently the nmr spectra of some 6,7-dimethylisoalloxazines have been published and assignments have been made for the aromatic 5- and 8-hydrogens and the 6- and 7-methyl groups. Bullock and Jardetzky[21] were able to demonstrate the reactivity of C-7 methyl protons by deuterium exchange. However, no attempt was made to analyze the N-9 substituents. The portion of the nmr spectrum of riboflavin which can be attributed to the aliphatic protons of the N-9 ribityl group are poorly resolved in deuterium oxide, dimethyl sulfoxide, or trifluoroacetic acid. The nmr spectra published by Bullock and Jardetzky[21] illustrate the problem. However, it was observed by accident that a dilute solution of deuterium oxide in trifluoroacetic acid (TFA) provides a suitable medium for well-resolved nmr spectra of N-9 substituents and a moderately resolved spectrum of riboflavin.

A typical nmr spectrum[22] of riboflavin has been shown to consist of a doublet centered at 2.25 ppm, an unresolved multipet at 3.34–4.17 ppm, and singlets at 6.88 and 7.10 ppm in a neutral aqueous solution. The assignments corresponding to increasing frequency are 6- and 7-methyl protons, 9-ribityl group, and 8- and 5-aromatic protons. The over-all spectrum is relatively unchanged when riboflavin is dissolved in trifluoroacetic acid. However, the intensities of certain nmr peaks of the ribityl side chain of riboflavin I were observed to undergo a downfield shift when allowed to stand in TFA. The nmr spectrum of riboflavin was examined 2 hr after the initial observation and the amplitude of the 3.75-ppm peak was greatly diminished while the 4.07-ppm peak had diminished only half as much. The 4.42-ppm peak, which was initially the least intense, now was the most intense. Addition of deuterium oxide reversed the effect and the original spectrum was produced but spin–spin splittings which were not resolved in TFA were apparent in the D_2O–TFA mixture as shown in Figure 1. An esterification reaction explains the reversible spectral shifts. The hydroxyl groups of the ribityl side chain were slowly acetylated forming a tetratrifluoroacetyl ester which produced the downfield shift of the absorption peaks of the aliphatic protons.

A comparison of the nmr spectra of riboflavin with that of 3′-hydroxypropyl and 2′,3′-dihydroxypropylisoalloxazine led to the assignment of the peaks of the side chain in riboflavin. The assignment of the hydrogens α to the primary hydroxyl group of 3′-hydroxypropylisoalloxazine at 3.69 ppm was verified by comparison of its spectrum with that of the 2′- and 3′-deuterated analogs (see Figure 2). The spectrum of the 3′-deuterated analog showed the absence of a triplet at 3.69 ppm. Indeed, this gives conclusive evidence for assignment of the triplet at 3.69 ppm in 3′-hydroxy-

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(19) W. G. Nystrom and W. G. Brown, *J. Amer. Chem. Soc.*, **69**, 2548 (1947).
(20) H. H. Fall and H. G. Petering, *ibid.*, **78**, 377 (1956).
(21) F. J. Bullock and O. Jardetzky, *J. Org. Chem.*, **30**, 2056 (1965).
(22) Spectra were taken on a Varian A-60 spectrometer and are reported based on 60 Mc and are expressed relative to tetramethylsilane as an external standard. High sensitivity was required for these spectra since isoalloxazines have low solubility in almost any solvent. A spectrum amplitude of 200 was usually necessary.

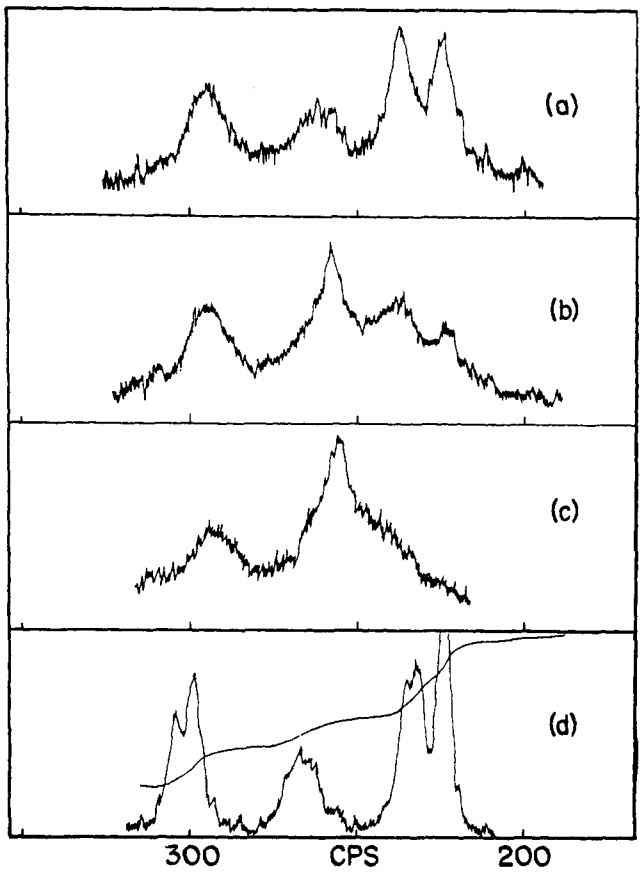


Figure 1. Nmr spectra of riboflavin in trifluoroacetic acid: (a) initial; (b) after 62 min; (c) after 188 min; (d) after addition of deuterium oxide.

propylisoalloxazine to the C-3 protons. Similarly, the 2′-deuterated analog showed two singlets, one at 4.85 and another at 3.70 ppm, with the unresoived multiplet at 2.14 ppm missing. These spectra not only verify the assignment of the triplet at 3.69 ppm in 3′-hydroxypropyl flavin, but also permit assignment of the singlet of riboflavin at 3.75 ppm to the C-5′ protons. Also, the peak at 4.85 ppm must be assigned to the C-1′ methylene.

The spectrum of 9-(2′,3′-dihydroxypropyl)isoalloxazine consists of a doublet at 4.91 ppm, an unresolved multiplet at 4.34 ppm, and a doublet at 3.70 ppm. Comparison with the spectrum of riboflavin led to the assignment of the 4.42-ppm peak of riboflavin to the C-2′ protons since the 3.75- and 4.90-ppm peaks must be assigned to the C-5′ protons and C-1′ methylene, respectively. The remaining doublet of riboflavin at 4.07 ppm with a relative intensity of two protons must be assigned to the C-3′ and C-4′ protons. This suggests that the C-2′ proton is in close proximity to the 1-nitrogen and it is deshielded in comparison to the C-3′ and C-4′ protons which are removed from the vicinity of the 1-nitrogen. In acid medium two peaks occur for the methyl groups, one at 2.43 and another at 2.30 ppm, relative to external TMS. It was shown by Bullock and Jaradetzky that in basic medium the aromatic protons occur as two singlets in which the upfield peak has been assigned to the C-8-H. In TFA–D_2O only one peak, whose relative intensity corresponds to two protons, is observed. As a result we assign the singlet at 7.98 ppm in riboflavin to the C-5 H and C-8 H. Table I summarizes the assignments of all nonexcyangeable protons in riboflavin and the spectrum is shown in Figure 3, This spin–spin splitting pattern observed for riboflavin in TFA–D_2O was not as well resolved as those of the other N-9 substituted isoalloxazines. For a highly resolved spectrum, one would expect a doublet at 3.73, a multiplet at 4.07, and a quartet at 4.42 ppm. Only the 5.04 ppm doublet of the ribityl side chain appears as would be expected.

The nmr spectrum of formylmethylflavin IX shows an additional feature that was useful for product identification. The formyl proton peak is located in an unusual region of the spectrum at 6.50 ppm. This is a large shift from the normal position at 10 ppm, and it suggests protonation followed by interaction with the isoalloxazine nucleus. Suelter and Metaler[23] found that the pK_a for formylmethylflavin was 3.5 as compared to 0.12 for riboflavin, and they proposed such an interaction to explain the high value. Polarographic data also indicates that the formyl group interacts with the isoalloxazine nucleus during reversible photoreduction.[24]

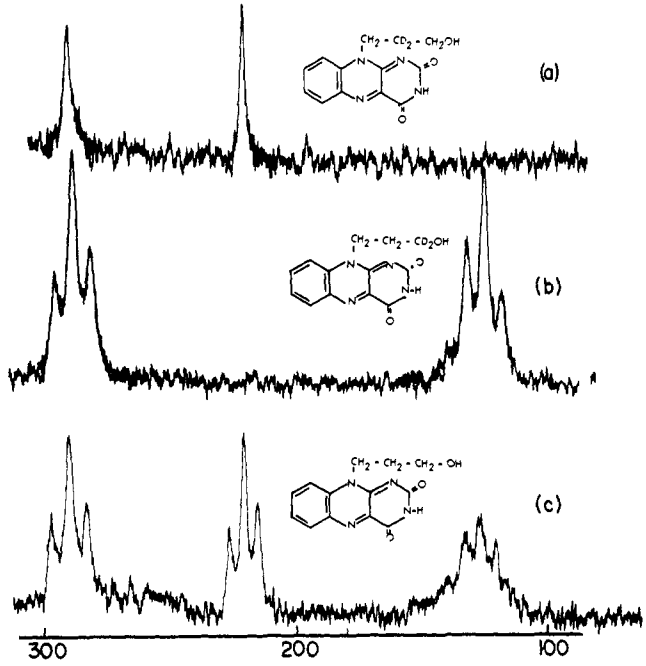


Figure 2. Nmr spectra of isoalloxazine derivatives in trifluoroacetic acid and deuterium oxide: (a) 9-(3′-hydroxypropyl-2′,2′-d_2)isoalloxazine; (b) 9-(3′-hydroxypropyl-3′,3′-d_2)isoalloxazine; (c) 9-(3′-hydroxypropyl)isoalloxazine.

Table I. Summary of Nmr Spectral Assignments for Riboflavin in TFA–D_2O

Proton assignment	Ppm	No. of protons
C-5,8	7.98	2(s)[a]
C-1′	5.04	2(d)
C-2′	4.42	1(u)
C-3′,4′	4.07	2(u)
C-5′	3.75	2(s)
C-6 or 7	2.43	3(s)
C-6 or 7	2.30	3(s)

[a] Symbols: s, singlet; d, doublet; u, undetermined multiplet.

Results

The isoalloxazines and flavins were studied in much the same way that was described in previous papers.[9,24] The rate data were taken from spectrophotometric observations. Quantitative determinations of alloxazine (or lumichrome) and cyclic intermediate were made by polarography. Product identification where possible was made by thin layer chromatography and nmr. The quantum yields were calculated from the initial zero-order rates, and they are given for relative comparisons. The purity of the isoalloxazines and the extent of oxygen removal affect this determination.

9-(2′-Hydroxy-2′-methylpropyl)isoalloxazine (VI). A preliminary note on the photochemistry of this interesting isoalloxazine has been presented.[25] The rate of

(23) C. H. Suelter and D. E. Metzler, *Biochem. Biophys. Acta*, **44**, 23 (1960).

(24) M. M. Mcbride and W. M. Moore, *Photochem. Photobiol.*, **6**, 103 (1967).

(25) W. M. Moore and Charles Baylor, Jr., *J. Amer. Chem. Soc.*, **88**, 5677 (1966).

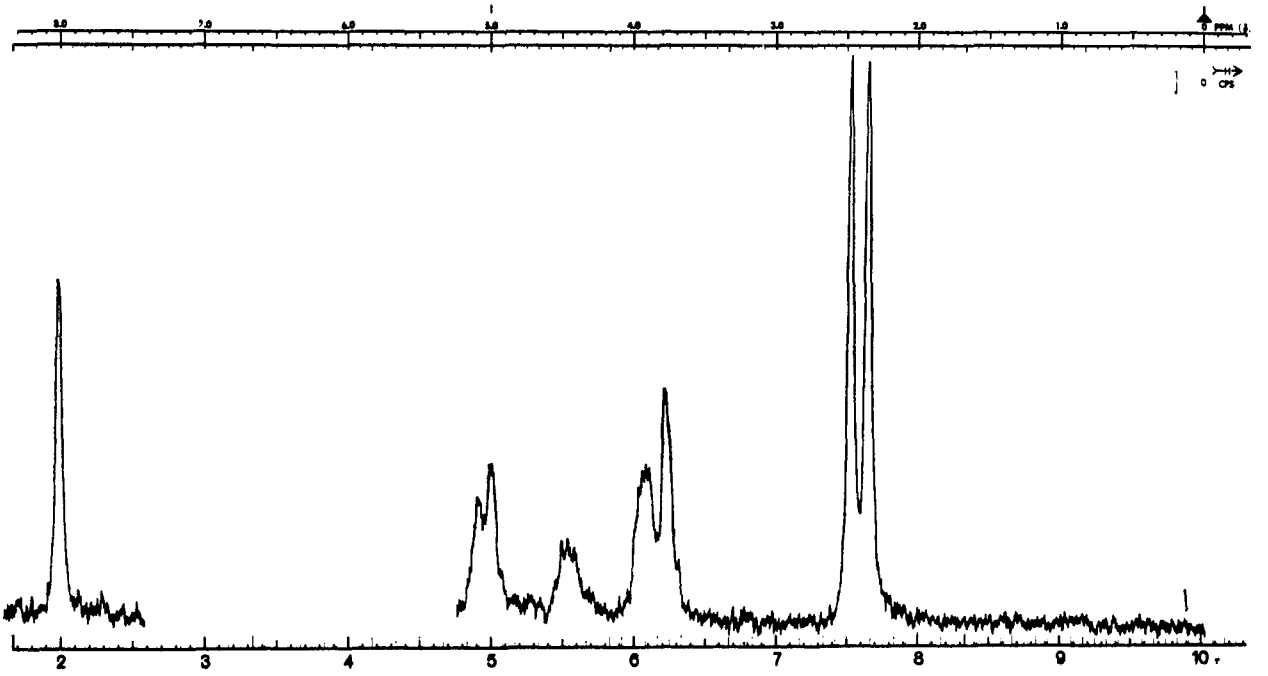


Figure 3. Nmr spectrum of riboflavin in trifluoroacetic acid and deuterium oxide.

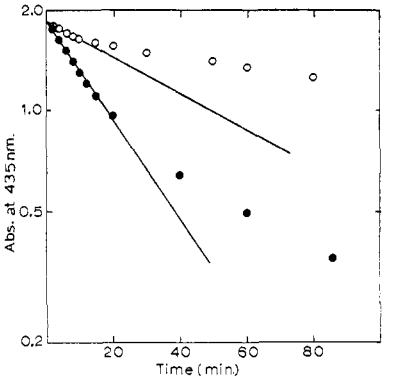


Figure 4. Rate data for the anaerobic photolysis of 9-(2′-hydroxy-2′-methylpropyl)isoalloxazine in H_2O (●) and in D_2O (○).

anaerobic photobleaching of an aqueous solution (pH 6.4), 1.5×10^{-4} *M* of VI, followed pseudo-first-order kinetics during one half-life. The initial rate gave a quantum yield of 3.9×10^{-3}. In the aerobic photolysis, the absorbance decreased very slowly. Compared to the photoreduction of riboflavin with EDTA, the aerobic photobleaching of VI in a Warburg apparatus indicated that no oxygen was consumed. This demonstrated that a photoreduction reaction followed by air oxidation was not responsible for the apparent inactivity of VI. The anaerobic photobleaching rate was estimated to be more than thirty times the aerobic photobleaching rate.

Simultaneous irradiation of VI in water and deuterium oxide gave pseudo-first-order rate constants of 3.40×10^{-2} min^{-1} and 1.22×10^{-3} min^{-1}, respectively, for an average kinetic isotope effect of 2.8. This is lower than the value of 4.9 previously reported.[25] However, several factors are responsible for the uncertainty in the value and the lower number represents a limit. Figure 4 shows that the deuterated system deviates from the first-order rates before one half-life of the reaction. This is not the usual situation. Compound VI is completely unreactive to aerobic irradiation and this is unusual for isoalloxazines. Therefore, the anaerobic photobleaching reaction is particularly sensitive to traces of oxygen and the scatter of our results would indicate this.[26] All of the isoalloxazines that we have synthesized have been irradiated anaerobically in deuterium oxide, however, 9-(2′-hydroxy-2′-methylpropyl)-isoalloxazine is the only one which has been found to exhibit a kinetic isotope effect. It is also the only isoalloxazine that has contained a tertiary alcohol group.

Irradiation of VI anaerobically produced alloxazine and a reduced flavin in a 50:50 ratio as determined from spectrophotometric and polarographic data. Color return from oxidation of the photolyzed solution is a measure of intermediate or reduced isoalloxazine concentration. Polarographic studies on the photobleaching of VI in 0.1 *M* KCl confirmed the spectrophotometric results. The measured half-wave potentials were −0.58, −0.42, and −0.22 V for alloxazine, VI, and reduced species. These data show that the reduced isoalloxazine is not the 1,10-dihydro form of VI, but a cyclic-reduced isoalloxazine since the oxidation potential is shifted. Photoreduction of VI with EDTA verified that the 1,10-dihydro form of VI did have the same potential as VI itself.[27]

Thin layer chromatograms of the anaerobically irradiated solutions showed only two spots which were identical with those of alloxazine (R_f 0.75) and VI (R_f 0.30). Preparative tlc was performed on the photolyzed mixture and the nmr spectrum of the eluted spot with R_f 0.30 was shown to be identical with VI.

The possibility that small amounts of a flavin aldehyde or ketone were formed and had an identical R_f value was checked. Addition of hydroxylamine hydrochloride or semicarbazide hydrochloride to the photo-

(26) In some more recent kinetic studies, 9 to 10 degassing cycles have been necessary to achieve consistent results. The number varied from 3 to 5 in the present study.

(27) See ref 9 and 12 for a discussion of the photoreduction reaction.

lyzed mixture produced no additional fluorescence spots. This was a modified procedure used successfully by McBride and Metzler[7] to show the presence of flavin aldehydes.

From the photochemical experiments it was concluded that the original isoalloxazine VI was regenerated from the cyclic-reduced isoalloxazine. Such a reaction sequence had been previously shown to occur only for formylmethylflavin.[11] However, VI has both a cyclic intermediate and a 1,10-dihydro species whereas FMF has only the cyclic-reduced intermediate.

The Photobleaching of 9-(2′-Hydroxypropyl)isoalloxazine. Both the anaerobic and aerobic photobleaching of X followed pseudo-first-order kinetics over approximately two half-lives. A quantum efficiency of 8.7×10^{-3} was found for the anaerobic photobleaching in neutral aqueous solution as determined spectrophotometrically at 445 nm. Polarographic analysis of the anaerobically photobleached solution showed that alloxazine and a reduced species were produced in a 70:30 ratio. The measured half-wave potentials were -0.58 and -0.17 V for alloxazine and the reduced species. Dark oxidation of the solution converted the reduced species to an isoalloxazine species with an $E_{1/2}^{\circ}$ of -0.38 V.

The new isoalloxazine species was isolated by preparative tlc and the nmr spectrum was determined. Singlets were found at 6.64 and 2.07 ppm. Thin layer chromatograms of the photolyzed solution gave three spots: alloxazine (R_f 0.77); original flavin (R_f 0.38); and the new flavin (R_f 0.30). Treatment of the photolyzed mixture with either hydroxylamine or semicarbazide caused the spot at R_f 0.30 to disappear and a new spot was found. R_f values of 0.45 and 0.20 were found for the oxime and semicarbazone, respectively. The reaction mixture was also treated with base to determine if the product might be an aldehyde. No change in the product could be assessed as determined by tlc, hydroxylamine, or semicarbazide treatment. These results indicated that the new photoproduct probably was 9-(2′-ketopropyl)isoalloxazine. The photoproduct was extremely stable to light.

9-(4′-Hydroxybutyl)isoalloxazine (VIII). The rate of disappearance of VIII followed pseudo-first-order kinetics over 1 half-life. The initial rate gave a quantum yield of 12×10^{-3}. The anaerobic photobleaching rate was 12 times the aerobic photobleaching rate. Compounds VIII and VIII-4′,4′-d_2 were irradiated under anaerobic conditions simultaneously in the usual fashion and the pseudo-first-order rate constants were 5×10^{-2} min^{-1} and 2.46×10^{-2} min^{-1}, respectively, for three determinations. This gives an average isotope effect of 2.03. Aerobic photobleaching of VIII and VIII-4′,4′-d_2 gave a kinetic isotope of 2.0, almost identical with the anaerobic isotope effect although the overall rates were 12 times faster for the anaerobic process.

The yellow color return in the reoxidized anaerobically photolyzed solution was very high. Repetition of the sequence also gave good color return. During irradiation the original light yellow solution became orange-yellow. The color remained until air was admitted to the solution. Table II summarizes the results of this experiment.

Polarographic analysis of the anaerobically photobleached solution of VIII showed that principally one product was formed. The reduction wave for VIII disappeared and the oxidation wave of a cyclic reduced isoalloxazine appeared. Of particular importance was that *no alloxazine was formed.* Upon reoxidation, the original reduction wave was regenerated.

Table II. Per Cent of Original Absorbance at 435 nm

Run	After photolysis 1	After aeration 1	After photolysis 2	After aeration 2
1	27.3	88.7	38.2	75.0
2	27.5	93.7	42.7	76.0
3	24.8	90.4		

Examination of the thin-layer chromatograms of solutions from the polarographic cell as well as from the spectrophotometric cell showed them to be identical. Products with R_f values at 0.39 and 0.43 were found in addition to VIII at 0.30 and traces of alloxazine at 0.75. It was apparent that spectrophotometry and polarography had made the system appear less complex than was the case.

To determine the origin of these products, thin-layer chromatograms of the photolyzed mixture were taken as a function of time. In this manner it was established that the major product (R_f 0.43) increased with time. However, recycling of the deaeration–irradiation–aeration procedure resulted in further production of the minor product (R_f 0.39) at the expense of VIII and the major photoproduct. No other fluorescent spots were detected.

Five liters of a 1.5×10^{-4} *M* solution of VIII were completely photolyzed in the immersion reactor. After aeration 1 l. of this solution was concentrated and the nmr spectrum of the residue was measured. The nmr spectrum was not well resolved, but it did suffice for identification purposes. The spectrum was characterized by a decrease in the relative intensity of the multiplet at 1.75 ppm. A new broad peak appeared at 2.80 ppm which can be attributed to C-3′ protons that are adjacent to a carbonyl group. In addition, two other broad bands of intensity equal to the 2.80-ppm band were observed at 4.83 and 2.41 ppm. These correspond to the 4.71- and 1.75-ppm peaks for VIII and are assigned to the C-1′ and C-2′ protons, respectively. The 4′-formyl proton was obscured by the HDO peak which had shifted due to residual water in the sample.

The photolyzed mixture was treated with hydroxylamine hydrochloride and semicarbazide hydrochloride and then examined by tlc. The hydroxylamine-treated solution made the spots at R_f 0.39 and 0.43 appear as one. The semicarbazide treated solution gave a new spot at 0.20 for the semicarbazone. The results confirm that the major product contained a carbonyl group in the 4′-position. The stability of the minor product to light suggests that either the 4′-acid was formed or that an alkyl group had been generated. Aerobic photolysis of VIII gave the same products as the anaerobic photolysis plus increased amounts of alloxazine. Also, a trace of a fourth product was observed at R_f 0.24.

9-(3′-Hydroxypropyl)isoalloxazine (VII). The rate of anaerobic photobleaching of an aqueous solution of VII followed pseudo-first-order kinetics during a minimum of two half-lives. The initial rate gave a quantum

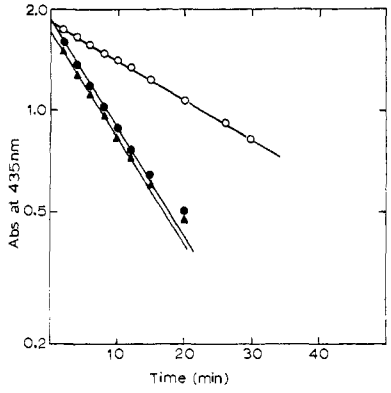


Figure 5. Rate data for the anaerobic photolysis of 9-(3′-hydroxypropyl)isoalloxazine (●); 9-(3′-hydroxypropyl-2′,2′-d_2)isoalloxazine (▲); and 9-(3′-hydroxypropyl-3′,3′-d_2)isoalloxazine (○).

yield of 1.7×10^{-2}. The corresponding flavin XII had a quantum yield of 0.24×10^{-2}. Quantum yields for the aerobic photobleaching of VII and XII were 5.0×10^{-3} and 1.3×10^{-3}, respectively.

The photolysis rates for the anaerobic solutions of VII, VII-2′,2′-d_2, and VII-3′,3′-d_2 are shown in Figure 5. The average of three determinations for each compound gave pseudo-first-order rate constants of 7.39×10^{-2}, 7.67×10^{-2}, and 2.60×10^{-2}, respectively. Kinetic isotope effects of 0.97 and 2.8 were calculated for the two deuterated isoalloxazines as compared to the undeuterated compound. For the aerobic photobleaching, isotope effects of 0.92 and 2.9 were found from an average of three determinations.

The irradiation of VII and XII under anaerobic conditions gave three distinct photoproducts which were easily separated from each other and the starting material by tlc. The major product (VIIa) had an R_f value of 0.45 and the minor product (VIIb) had an R_f value of 0.23. Similar results were obtained with XII. The third product was alloxazine and it was present in only trace amounts. Karrer, *et al.*,[1-3] also found only traces of alloxazine from the photobleaching of VII.

The unknown products VIIa and b were treated with hydroxylamine and semicarbazide. The aldehydic nature of VIIa was established by tlc of the semicarbazone. Five-liter samples of VII were then irradiated and the photolyzed solutions were evaporated to dryness. The residue was examined by nmr. Broad unresolved peaks were observed at 6.00, 4.50, and 2.17 ppm with atomic ratios of 1:2:2. These corresponded to C-3′ formyl (by comparison to the nmr of formylmethylflavin), C-1′ methylene, and C-2′ methylene protons, respectively. The C-3′ protons for VII at 3.63 ppm were completely absent.

Polarographic analysis of the photobleached solutions of VII and XII gave three waves; reduction half-wave potentials of −0.58 and −0.46 V, and an oxidation half-wave potential of −0.06 V. These potentials correspond to alloxazine, VII, and a cyclic intermediate, respectively. The small height of the alloxazine wave confirmed the amounts found in the tlc analysis.

9-(2′,3′-Dihydroxypropyl)isoalloxazine (II). The rates of anaerobic photobleaching for II and the corresponding flavin XI followed pseudo-first-order kinetics over several half-lives. The quantum efficiencies were 7.2×10^{-3} and 1.6×10^{-3}, respectively. Aerobic photobleaching gave lower quantum efficiencies of 3.6×10^{-3} and 0.4×10^{-3}.

Polarographic analysis of anaerobically photobleached solutions of II gave reduction half-wave potentials for alloxazine and II of −0.61 and −0.51 V, and an oxidation half-wave potential of −0.05 V for the cyclic intermediate. The alloxazine to intermediate product ratio was 3 to 1 as determined polarographically and 2.5 to 1 from color-return data.

Thin-layer chromatography showed only two products in addition to the original isoalloxazine. The major product was alloxazine, R_f 0.75. The other product showed the typical yellowish green fluoresence of an isoalloxazine, R_f 0.59. The product was suspected of being an aldehyde and it gave a positive test with hydroxylamine and semicarbazide. Comparison with an authentic sample showed it to be 9-(formylmethyl)isoalloxazine. Only 6,7-dimethyl-9-(2′-hydroxyethyl)isoalloxazine,[7] formylmethylflavin,[24] and riboflavin[3] have yielded the formylmethyl derivative upon irradiation.

Discussion

The quantum yield results for the compounds examined are summarized in Table II. The quantum yields were calculated from initial zero-order rates for the disappearance of the isoalloxazine. Although the reactions do not hold to zero order, at the absorbance values used near two we make that assumption based on an intramolecular rearrangement. Interference by the photoproducts is minimized by using the initial rate data. The quantum-yield values were not used in the isotope effect calculations, but they were used solely for comparison purposes.

Table III. Summary of Initial Quantum Yields for the Photobleaching of Isoalloxazines

N-9 substituent	Anaerobic ($\Phi \times 10^3$) Iso-alloxazine	Flavin	Aerobic ($\Phi \times 10^3$) Iso-alloxazine	Flavin
2′-Hydroxyethyl	2.6			
2′-Hydroxypropyl	8.7			
2′-Hydroxy-2′-methyl-propyl	3.9		Nil	
Formylmethyl		15.0		
2′-Ketopropyl	0.0			
3′-Hydroxypropyl	17.0	2.4	5.0	1.3
2′,3′-Dihydroxypropyl	7.2	1.6	3.6	0.4
4′-Hydroxybutyl	12.0		1.0	
Riboflavin		12.0		

For the ω-hydroxyalkyl substituents, the longer chain alcohols are more reactive. The flavin analogs are definitely less reactive where comparisons have been made. Generally the reactivity of the groups are: aldehyde hydrogen > α-hydrocarbon hydrogen > hydroxyl hydrogen > alkyl hydrogen. The polarographic and thin layer chromatographic results are summarized in Tables IV and V. It should be emphasized that polarography provides quantitative and qualitative data during the irradiation which cannot be obtained in any other way. Significantly, the polarographic data show the presence of the cyclic intermediate in the photobleaching of every isoalloxazine examined. The color return during oxidation accompanied by the loss of the polarographic wave for the intermediate is very dra-

Table IV. Summary of Polarographic Results from the Anaerobic Photobleaching of Isoalloxazines

N-9 substituent	Reduction potential, V[a] Original isoalloxazine	Reoxidized[b] isoalloxazine	Alloxazine	Oxidation potential, V, reduced species	Ratio of[c] alloxazine to reduced species
2′-Hydroxyethyl	−0.46	−0.44	−0.57	−0.05	5.7
2′-Hydroxypropyl	−0.44	−0.38	−0.58	−0.17	2.3
2′-Hydroxy-2′-methylpropyl	−0.42	−0.42	−0.58	−0.22	1.0
Formylmethylflavin	−0.45	−0.45	−0.57	−0.16	1.0
3′-Hydroxypropyl	−0.46	−0.44	−0.58	−0.06	Small
4′-Hydroxybutyl	−0.46	−0.46	None	−0.21	Very small
2′,3′-Dihydroxypropyl	−0.51	−0.45	−0.61	−0.05	3.0
Riboflavin	−0.47	−0.45	−0.65	−0.19	

[a] Half-wave potentials *vs.* silver–silver chloride electrode. [b] Measured potential of isoalloxazine derived from the reduced species (cyclic intermediate) after oxidation of photobleached solution. [c] Derived from the height of the polarographic waves.

Table V. Summary of Thin-Layer Chromatographic Results from the Anaerobic Photobleaching of Isoalloxazines

N-9 substituent	R_f values for butanol–acetic acid–water[a] Original isoalloxazine	New isoalloxazine	Derivative of new isoalloxazine	Alloxazine	Other products
2′-Hydroxyethyl	0.32	0.59	0.45,[b] 0.15[c]	0.75	None
2′-Hydroxypropyl	0.38	0.30	0.45,[b] 0.20[c]	0.77	None
2′-Hydroxy-2′-methylpropyl	0.30	None		0.75	None
Formylmethylflavin	0.59		0.45,[b] 0.15[c,d]	0.65	
3′-Hydroxypropyl	0.35	0.45	0.22[c]	0.79	0.23
4′-Hydroxybutyl	0.30	0.43	0.20[c]	Trace	0.39
2′,3′-Dihydroxypropyl	0.30	0.59	0.45,[b] 0.15[c]	0.75	None
Riboflavin	0.32	0.59	0.45,[b] 0.15[c]	0.80	0.38

[a] Volume ratio was 4:1:5 and organic layer was used. [b] Oxime of new isoalloxazine. [c] Semicarbazone of new isoalloxazine. [d] Oxime and semicarbazone of formylmethylflavin.

matic. The nature of the oxidized products provides some information on the structure of the intermediate. The ω-hydroxyalkyl groups produce aldehydes, and possibly some acids as secondary products. Secondary alcoholic groups form ketones which are unreactive toward further hydrogen abstraction. The tertiary alcohol amazingly regenerates itself during the oxidation of the cyclic intermediate.

Riboflavin and isoalloxazines participate in several types of photoreactions. Several factors control the pathway of a particular reaction set, and many researchers have been mislead about the photoprocess they were studying. Under anaerobic, or aerobic conditions, almost all of the photoreactions of isoalloxazines in aqueous solution will be accompanied by a loss of yellow color (a decrease in the absorption maximum at 445 nm). Alloxazine, 1,10-dihydroisoalloxazine and the cyclic intermediate have low extinction coefficients in the visible, so color loss alone is not an indication as to the type of photoprocess. There seems to be three distinctive processes for isoalloxazines under normally encountered reaction conditions. They will be identified as photoreduction, photodegradation, and photosensitization.

Photoreduction specifies the transfer of a hydrogen atom (or equivalent) from a donor molecule to the photoexcited acceptor. The photoreaction of riboflavin with EDTA is an example of this type,[9] and the product is 1,10-dihydroriboflavin. Many α-amino acids will serve as hydrogen donors for isoalloxazines.[28] Color loss is usually rapid and air oxidation will completely regenerate the isoalloxazine if the donor concentration is sufficiently large.

(28) W. R. Frisell, C. W. Chung, and C. G. Mackenzie, *J. Biol. Chem.*, **234**, 1297 (1959).

Photodegradation refers to the fragmentation of the photoexcited molecule, and two principal types are possible. If the excitation energy can be localized in a fissionable bond, then fragmentation may result as in the gas-phase photolysis of acetone. However, an internal rearrangement of the atoms during excitation can also occur, and the result may be molecular fragments or an isomer of the original molecule. Photoreactions of the aliphatic ketones display the range of possibilities for photodegradation.

Photosensitization specifies energy transfer from the excited molecule to an acceptor. The acceptor then reacts in some fashion and products are obtained. Isoalloxazines are proven energy-transfer agents as has been demonstrated in the work on singlet oxygen formation,[29] pimaricin decomposition,[30] and potassium iodide quenching.[31] It is difficult to assess the importance of energy transfer in the photobleaching under consideration here. There is evidence that riboflavin sensitizes the degradation of indoleacetic acid simultaneously with the photoreduction of riboflavin with this amino acid.[32] It is conceivable that the products from the photodegradation could be sensitized to further reactivity. We have observed the quenching effects of the photoproducts by adding them to unphotolyzed solutions of riboflavin and measuring the difference in initial photobleaching rates. The sensitization and quenching effects become very important in aerobic systems, and kinetic data must be treated very qualitatively.

(29) C. S. Foote and S. Wexler, *J. Amer. Chem. Soc.*, **86**, 3880 (1964).
(30) J. Posthuma and W. Berends, *Biochim. Biophys. Acta*, **112**, 422 (1966).
(31) B. Holmström and G. Oster, *J. Amer. Chem. Soc.*, **83**, 1867 (1961).
(32) W. M. Moore and J. Hen, unpublished results.

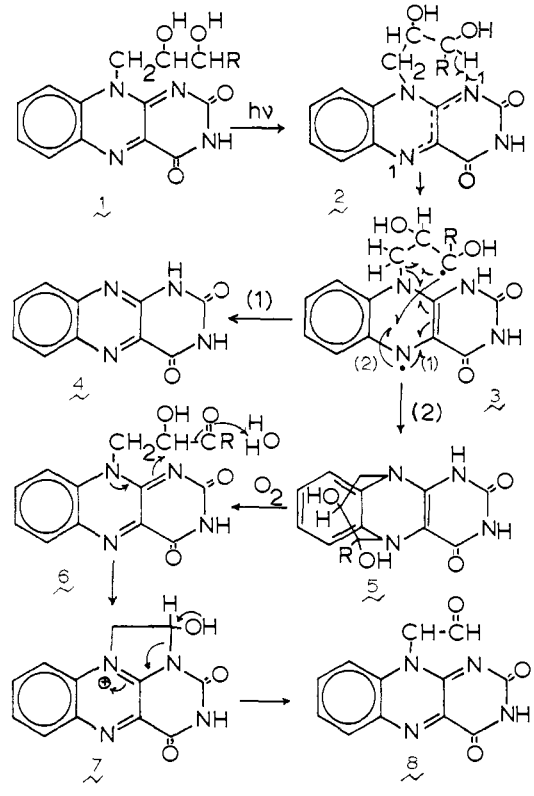
With this background and the results from our study, the evidence supports the hypothesis that the aqueous anaerobic photobleaching of isoalloxazines is mainly a photodegradation reaction resulting from an internal rearrangement of the molecule during excitation. Photoreduction and photosensitization are minor side reactions in this clean system.

Hölmstrom[33] has shown that the primary step in the photoreduction of riboflavin is a one-electron process. Through flash photolysis experiments, he found that the semiquinone radical formation did not follow the second-order kinetics required for the reaction of the reduced flavin with the oxidized flavin. Since the initial step in the photobleaching reaction is similar to photoreduction, it seems reasonable to assume a one-electron transfer, or a hydrogen-atom transfer. The excited state responsible for the rearrangement almost certainly has to be the triplet, although its nature is immaterial to a discussion of the chemical mechanism. The results from sensitization,[29,30] and electron-spin resonance studies[34] support the involvement of the triplet state.

It has not been possible in our experiments to distinguish a bimolecular from an intramolecular reaction. Holmström and Oster[31] studied the effect of concentration on the quantum yield for the aqueous anaerobic photobleaching of riboflavin. They found riboflavin to be self quenching, and there was additional inhibition by the photoproducts. It was concluded that the mechanism was intramolecular. McBride and Moore[23] developed a theoretical rate law for the bimolecular mechanism involving formylmethylflavin. The rate data were fit to such a rate law. Along with other results, it was proposed that formylmethylflavin did photobleach by a bimolecular mechanism. The present rate data do not fit any theoretical rate law over an extended range. A first-order rate law would be a good approximation over limited absorbance values, but that experimental relationship holds over several half-lives in some cases as illustrated in Figure 5. None of the mechanisms would predict this behavior; therefore, it is necessary to examine the consequences of each mechanism. The kinetic isotope effect results demand the involvement of the aliphatic side chain in the rate-controlling step. The intramolecular process produces a diradical which can cyclize or split depending upon the location of the radical sites. In a bimolecular process, the photoexcited molecule would be reduced to a semiquinone radical at the expense of another isoalloxazine molecule. The newly created aliphatic-type radical could then produce some of the observed products by disproportionation reactions. The semiquinone radical would produce a yield of at least 25% 1,10-dihydroisoalloxazine. The polarographic data should indicate its presence, but that has not been observed. With the possible exception of formylmethylflavin,[35] the general process seems to be an intramolecular rearrangement.

From the results on the various isoalloxazines, a composite mechanism can be postulated to explain the photobleaching (or photodegradation) of riboflavin and other isoalloxazines. Attack at the 2′-position favors formation of lumichrome (or alloxazine) although some cyclic intermediate can be produced. Such a mechanism was presented in connection with the photobleaching of 9-(2′hydroxyethyl)isoalloxazine.[9] Attack at the 3′-position and positions more remote from the isoalloxazine nucleus favors the formation of the cyclic intermediates **5** as shown in Scheme I. The diradical **3**

Scheme I



has a sufficient lifetime preferentially to couple rather than eliminate the side chain. In the special case of 9-(2′-hydroxy-2′-methylpropyl)isoalloxazine (VI), where the primary process forms an alkoxy radical, no preference is shown for coupling over elimination. The cyclic intermediate **5** formed by the radical coupling (pathway 2) would have the spectral characteristics of 1,10-dihydroriboflavin, but not a reversible electrode potential due to the tertiary amine formed at the 10-nitrogen. The group bridging the N-9 to N-10 position would contain either two carbon or three carbon atoms for the C-2′- or C-3′-hydrogen abstraction, respectively. The cyclic intermediate from VI would have a C–C–O bridge.

Air oxidation of the cyclic intermediate produces an isoalloxazine **6** with an altered side chain. Most of the observed products of the simple isoalloxazine photobleaching have been generated by this stage. The *gem*-diol group on the side chain seems to be necessary for the formation of the formylmethyl derivative **8**. We have no direct evidence for the suggested mechanism **6**–**8** since the α-hydroxyaldehyde or ketone was not isolated. The mechanism is based on the lability of the formyl group at the 2′-position. Formylmethylflavin is unstable in air and it converts to lumiflavin (6,7,9-trimethylisoalloxazine). This reaction is catalyzed by base.[36] It should be stressed that 9-methylisoalloxazine was not observed as a product in any of our reac-

(33) B. Hölmström, *Ark. Kemi*, **22**, 329 (1964).
(34) T. Shiga and L. H. Piette, *Photochem. Photobiol.*, 4, 769 (1965).
(35) Reduced formylmethylflavin is of the cyclic-intermediate structure due to an interaction between the N-1 position and the formyl group.
(36) P. S. Song, E. C. Smith, and D. E. Metzler, *J. Amer. Chem. Soc.*, **87**, 4181 (1965).

tions that did not also produce formylmethylflavin or the isoalloxazine analog. The aldehyde group at the 3′- or 4′-position appears to be relatively stable to air. We suggest that the introduction of a hydroxyl group at the 2′-position would make the 3′-ketone unstable in the environment of the isoalloxazine nucleus. The cleavage of the side chain could have occurred before the cyclization, but we cannot propose a satisfactory mechanism. It is unlikely that hydrogen abstraction at the 2′-position would lead to the aldehyde when the group attached to the 2′-position is not hydrogen. Most oxidations of α-hydroxycarbonyl groups indicate that the hydroxy fragment converts to an aldehyde[37] for a secondary hydroxy function.

A variation of this mechanism has been proposed for VI.[25] Air oxidation of the reduced isoalloxazine nucleus induces a break of the N–O bond at the N-10 position. The resulting alkoxyl radical could abstract a hydrogen from the media (*i.e.*, hydrogen peroxide or side-chain fragments). As in the case of formylmethylflavin, a bimolecular-reaction mechanism provides an appealing alternative. However, unlike the other situation, the cyclic intermediate has an oxidation potential different from the 1,10-dihydro derivative. Therefore, we feel that VI differs from the other isoalloxazines by the nature of the hydrogen-abstraction process, and it does not follow a bimolecular path.

(37) R. Stewart, "Oxidation Mechanisms," W. A. Benjamin, Inc., New York, N. Y., 1964, pp 27, 99, 105.

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Relaxation Spectra of Ribonuclease. VI. The Interaction of Ribonuclease with Uridine 3′-Monophosphate[1]

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Abstract: Kinetic studies of the interaction of ribonuclease with uridine 3′-monophosphate were performed using the temperature-jump method. Changes in both ultraviolet absorption and pH were measured. A partial reinvestigation of the relaxation spectra of ribonuclease–cytidine 3′-monophosphate binding was also carried out. For both nucleotides two relaxation processes are observed which can be attributed to an initial association–dissociation of the enzyme and nucleotide followed by an isomerization of the enzyme–nucleotide complex. These two processes were studied at 25° in the pH range 4.5–7.5. The interaction of ribonuclease with both uridine and cytidine 3′-nucleotides has qualitatively similar relaxation characteristics as a function of pH. However, the dissociation rate constant for uridine 3′-monophosphate binding is approximately two times that for cytidine 3′-monophosphate. A minimal mechanism consistent with all of the data involves three ionizing groups on ribonuclease and two isomeric states of the ribonuclease–3′-nucleotide complex. One of the ionizing groups on ribonuclease is postulated not to interact directly with the 3′-nucleotides, but instead reflects a change in conformation associated with the isomerization of the free enzyme *and* the enzyme–product complex. These results can be correlated with the known three-dimensional structure of the enzyme.

Previous papers in this series have included relaxation studies on the interaction of ribonuclease with cytidine 3′-monophosphate,[3,4] cytidine 2′:3′-cyclic phosphate,[5] and cytidylyl 3′:5′-cytidine.[6] Since the apparent association constants of ribonuclease with cytidine 3′-monophosphate are greater than those with uridine 3′-monophosphate,[7] a comparison of the relaxation spectra of ribonuclease with these nucleotides is of interest. During the course of the current investigation, which utilized an improved temperature-jump apparatus having a substantially greater signal-to-noise ratio, some discrepancies were found with previous data; therefore a partial reinvestigation of the ribonuclease–cytidine 3′-monophosphate interaction was undertaken. The effects designated τ_2[3,4] and τ_3,[3,4] which were previously observed by measurement of pH changes with a pH indicator, were corroborated in the present study by direct observation of the change in the ultraviolet difference absorbance which accompanies ribonuclease–nucleotide binding.[7] The results indicate that two relaxation processes are associated with ribonuclease–nucleotide binding: one is associated with an initial association–dissociation reaction and the other with an isomerization of the enzyme–product complex. The pH dependence of the relaxation times was determined, and a mechanism is discussed which attempts to correlate these results with the known chemical and structural features of ribonuclease.

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Experimental Section

Bovine pancreatic ribonuclease A was obtained as a phosphate-free lyophilized powder from Worthington Biochemicals Corp. and was used without further purification. The concentration of