235, 1711.
Cunningham, L. W., and Nuenke, B. J. (1961), *J. Biol. Chem. 236,* 1716.
Ellman, G. L. (1959), *Arch. Biochem. Biophys. 82,* 70.
Fraenkel-Conrat, H. (1955), *J. Biol. Chem. 217,* 373.
Griffith, O. H., and McConnell, H. M. (1966), *Proc. Natl. Acad. Sci. U.S.A. 55,* 8.
Hughes, W. L., Jr., and Straessle, R. (1950), *J. Am. Chem. Soc. 72,* 452.
Hull, H. H., Chang, R., and Kaplan, L. J. (1975), *Biochim. Biophys. Acta 400,* 132.
Jirousek, L., and Cunningham, L. W. (1968), *Biochim. Biophys. Acta 170,* 160.
Jirousek, L., and Pritchard, E. T. (1971), *Biochim. Biophys. Acta 229,* 618.
Jirousek, L., and Soodak, M. (1974), *Biochem. Biophys. Res. Commun. 59,* 927.
Kharasch, N. (1961), in Organic Sulfur Compounds, Kharasch, N., Ed., New York, N.Y., Pergamon Press, p 375.
Klebanoff, S. J. (1967), *J. Exp. Med. 126,* 1063.
Klebanoff, S. J. (1975), *Semin. Hematol. 12,* 117.
Maloof, F., and Soodak, M. (1963), *Pharmacol. Rev. 15,* 43.
Mora, S., Huvos, P., Libor, S., and Elodi, P. (1969), *Acta Biochim. Biophys. Acad. Sci. Hung. 4,* 151.
Morrison, M. (1973), *Ann. N.Y. Acad. Sci. 212,* 175.
Morrison, M., and Bayse, G. S. (1970), *Biochemistry 9,* 2995.
Morrison, M., Bayse, G. S., and Michaels, A. W. (1971b), *Anal. Biochem. 42,* 195.
Morrison, M., Bayse, G. S., and Webster, R. G. (1971a), *Immunochemistry 8,* 289.
Morrison, M., Gates, R. E., and Huber, C. T. (1974), in Membrane Transformations in Neoplasia, Schultz, J., and Block, R. E., Ed., New York, N.Y., Academic Press, p 33.
Morrison, M., and Hultquist, D. E. (1963), *J. Biol. Chem. 238,* 2847.
Morrison, M., and Schonbaum, G. S. (1976), *Annu. Rev. Biochem. 45, fr861.*
Noel, J. K. F., and Hunter, J. M. (1972), J. Biol. Chem. 247, 7391.
Parker, D. J., and Allison, W. S. (1969), *J. Biol. Chem. 244,* 180.
Phillips, D. R., and Morrison, M. (1970), *Biochem. Biophys. Res. Commun. 40,* 284.
Phillips, D. R., and Morrison, M. (1971), *Biochemistry 10,* 1766.
Trundle, D., and Cunningham, L. W. (1969), *Biochemistry 8,* 1919.
Yamazaki, I., and Yokota, D. (1973), *Mol. Cell. Biochem. 2,* 39.

One- and Two-Electron Redox Chemistry of 1-Carba-1-deazariboflavin†

Rob Spencer, Jed Fisher,‡ and Christopher Walsh*

ABSTRACT: 1-Carba-1-deazariboflavin is a purple, nonfluorescent analogue of riboflavin that should prove useful in flavin coenzyme chemical and enzymatic studies. The two-electron-reduced form dihydro-1-deazariboflavin is readily produced by reduction with dithionite, hydrogen over platinum, or light in the presence of (ethylenedinitrilo)tetraacetic acid, but not by sodium borohydride. The hydrogen at position 1 of dihydro-1-deazariboflavin exchanges slowly with solvent protons. Unlike the analogue dihydro-5-carba-5-deazariboflavin, dihydro-1-deazariboflavin is rapidly oxidized by molecular oxygen with appreciable production of superoxide anion. The redox potential of 1-deazariboflavin is −280 mV, some 70 mV more negative than riboflavin. 1-Deazariboflavin is bound to egg-white flavin-binding apoprotein with a dissociation constant of 1.6 nM. Titration of protein-bound dihydro-1-deazariboflavin with oxygenated buffer yields a long-wavelength-absorbing species, which spectral and electron paramagnetic resonance evidence suggests is the bound 1-deazariboflavin semiquinone. Similar semiquinone species are produced from 5-methyl-1-deazariboflavin and the 1-deazariboflavin cation. In contrast to 5-deazaflavins, 1-deazaflavins resemble the parent flavins in possession of readily accessible one- *and* two-electron redox chemistry. An accompanying manuscript describes the coenzymatic activity of 1-deazaflavins with several flavoenzymes.

The use of structurally modified substrates and structurally modified coenzyme analogues has been enlightening in the development of chemical insight of how electrons are transferred at flavoenzyme active sites (Bright and Porter, 1975; Bruice, 1976; Hemmerich, 1976; Walsh, 1977; Walsh et al., 1977a,b). The major barrier to broader application of mechanistically useful flavin analogues has been the lack of suitably modified flavin isosteres. The dearth has been remedied by syntheses of a variety of aza- and deazaflavin analogues by the synthetic chemical group at Merck (Ashton et al., 1977). We report here and in the following paper of this issue some of the chemical properties and enzymatic redox activities of one of these new analogues, 1-carba-1-deazariboflavin, and its coenzyme forms 1-deazaFMN[1] and 1-deazaFAD. A priori, this analogue is of special interest in that it is complementary

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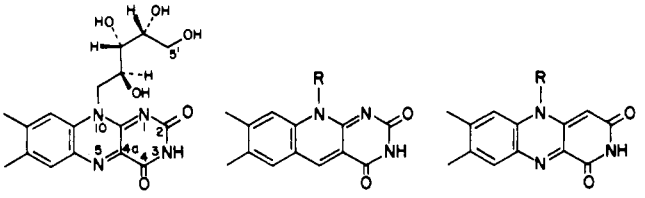
to the 5-deazaflavin isostere: now N-1, the other end of the enediamine electron sink in flavins, has been replaced by carbon. Comparisons between riboflavin, **1,** 5-deazariboflavin, **2,** and 1-deazariboflavin, **3,** should be fruitful.



1 2 3

1-Deazariboflavin is a purple-black solid which yields purple solutions at neutral pH possessing λ_{max} values in the visible spectrum of 365 nm (ϵ 4000 M^{-1} cm^{-1}) and 535 nm (6800) (Figure 1). 1-Deazaflavins differ from both parent isoalloxazines and 5-deazaisoalloxazines in that they completely lack a detectable fluorescent emission from any of the electronic transitions of the oxidized state.

The following are the main points we wish to probe in this paper. (1) What are the preferred structures of oxidized and reduced 1-deazaflavins in aqueous solutions? How does their acid–base chemistry differ from that of unmodified isoalloxamines? (2) How readily is 1-deazariboflavin reduced by several known flavin reductants? Is sequential one-electron transfer reduction preferred over two-electron (hydride) reduction, or vice versa? Are both modes of reduction seen? (3) Is reduced 1-deazariboflavin readily oxidized by molecular oxygen, and, if so, what are the oxygen products? (4) What is the redox potential of the 1-deazaRF–1-deazaRFH_2 couple? (5) Is C-1 of reduced 1-deazariboflavin a prochiral center; i.e., does dihydro-1-deazaflavin have a structure where C-1 is a methylene group? Can 1-deazaflavins be used to probe the stereochemistry of hydrogen transfer to and from position 1, as well as the origin of any hydrogen transferred to that carbon? (6) In general, how does substitution of carbon for nitrogen at position 1 affect the ability of a flavin to carry out both one- and two-electron redox reactions?

Experimental Section

Materials. 1-Carba-1-deazariboflavin and 3-methyl-1-carba-1-deazariboflavin were the very generous gifts of the synthetic chemistry group of Merck, Sharp and Dohme Research Laboratories, Rahway, New Jersey. The synthesis of this analogue, as accomplished by that Merck group, is presented in a separate publication (Ashton et al., 1977). The egg-white flavin-binding protein was isolated from fresh, locally available eggs by the procedures of Becvar (1973).

Methods. Visible and ultraviolet absorption spectra were recorded with a Perkin-Elmer Model 200 spectrophotometer. Anaerobic spectra were recorded in cells fitted with rubber septa and maintained under argon which had been scrubbed of trace oxygen by bubbling through a solution of vanadous chloride.

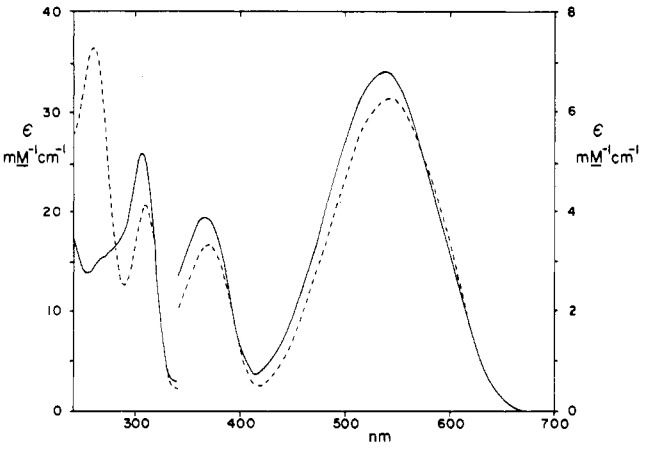


FIGURE 1: Visible and ultraviolet absorption spectra of (—) 1-deazariboflavin and (- - -) 1-deazaFAD, pH 7.

Rapid kinetic experiments were performed with a Durrum stop-flow spectrophotometer. Solutions of riboflavin and 1-deazariboflavin, each 18 μM in flavin and 20 mM in EDTA, were reduced with light and hydrogen/plantinized asbestos, respectively. These reduced flavin solutions were mixed in the stop-flow apparatus with the appropriate air-saturated buffer (20 °C). Oxidation of dihydroriboflavin or dihydro-1-deazariboflavin was monitored at 445 or 535 nm, respectively.

Polarography of 20 to 50 μM solutions of 1-deazariboflavin and riboflavin, in 50 mM phosphate, pH 7.0, and nitrogen saturated, was carried out in both direct current and differential pulse modes with a Princeton Applied Research Model 174A polarographic analyzer. The same instrument with hanging mercury drop electrode Model 9323 was used for cyclic voltammetry at 500 mV s^{-1}.

Fourier transform ^1H NMR spectra were recorded with the 270-MHz Bruker instrument at the Southern New England High Field NMR Facility at Yale University.

Electron spin resonance spectra were obtained in a Varian E9 spectrometer at 20 °C, at 9.120 GHz, modulation frequency 100 kHz, modulation amplitude 2.5 to 20 G, power 5 mW, and scan time 8 min.

All other materials and methods are identical to those in preceding publications (Spencer et al., 1976; Fisher et al., 1976).

Syntheses. In order to measure the rate of solvent proton exchange into the C-1 position, the 1-deazariboflavin sample was prepared as follows. A solution of 5 mg of 1-deazaRF in 25 mL of a ^2H_2O buffer, 10 mM sodium acetate adjusted to pD 5.5 with ^2HCl, was degassed with argon and then reduced with a minimum quantity of sodium dithionite. The yellow solution was kept at ambient temperature for 40 min following which the 1-deazaRF was oxidized with a stream of oxygen. The now purple solution was flash evaporated to dryness and redissolved in minimal hot ^2H_2O, and the 1-deazaRF was precipitated by chilling. The solid was collected by centrifugation, resuspended in ^2H_2O, and repelleted to remove all traces of buffering salts. The sample was then lyophilized twice from ^2H_2O, dried in vacuo, and dissolved in 0.3 mL of dimethyl-d_6 sulfoxide and 0.1 mL of chloroform-*d*, and the ^1H NMR obtained. The control was treated identically except that the dithionite was omitted.

The alkylation of dihydro-1-deazaflavin was done on 20 mL of an approximately 100 μM 1-deazaRF solution in 0.10 M sodium phosphate, pH 7.0, buffer. This solution was degassed with nitrogen and the flavin reduced by the addition of approximately 2 mg of sodium dithionite. Dimethyl sulfate (25

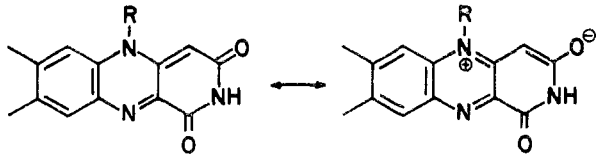
[1] Abbreviations used are: 1-deazariboflavin and 1-deazaRF, 1-carba-1-deazariboflavin; 1-deazaFMN, 1-carba-1-deazariboflavin 5′-phosphate; 1-deazaFAD, 1-carba-1-deazariboflavin 5′-diphosphate, 5′ → 5′-adenosine ester; 1-deazaRFH$^-$, two-electron-reduced 1-carba-1-deazariboflavin anion; 1-deazaRFH·, 1-carba-1-deazariboflavin semiquinone, Fl_{ox}, oxidized flavin (general); FlH$^-$, two-electron-reduced flavin anion (general); EDTA, (ethylenedinitrilo)tetraacetic acid; Tris-HCl, 2-amino-2-hydroxymethyl-1,3-propanediol; NAD, nicotinamide adenine dinucleotide; NADH, reduced NAD; NADPH, NADH phosphate; NMR, nuclear magnetic resonance; EPR, electron paramagnetic resonance.

μL) was added anaerobically and the solution stirred vigorously for 15 min. While still anaerobic, the solution pH was adjusted to approximately 2.5 with 2 N acetic acid, and then aerated to generate a green product. This material was rapidly separated from small amounts of unreacted 1-deazaRF by adsorptive chromatography on Bio-Gel P2 equilibrated and developed with 5 mM sodium citrate, pH 3.0.

Tetraacetyl-1-deazariboflavin was prepared after McCormick (1970). The product gave a single spot on thin-layer chromatography, with mobility distinct from the starting 1-deazariboflavin but identical to tetraacetylriboflavin.

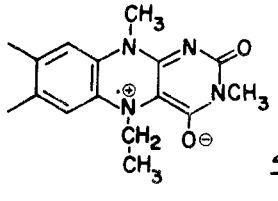
Results and Discussion

Chemical Reduction and Oxidation. As an introduction to this section on the elementary redox chemistry of 1-deazaisoalloxazines, it is appropriate to begin with the consideration of the structure of the oxidized 1-deazaflavin. This point is raised by the observation that the absorption spectrum of 1-deazalumichrome (**3**, with R = H) is very similar to that of 1-deazariboflavin (**3**, R = D-ribityl) (Ashton et al., 1977), so that both molecules have the same preferred tautomeric structure. In contrast, lumichrome and riboflavin have quite different spectra, with the former known to prefer the alloxazine structure (N-10-C-10a unsaturation). The structural question, then, is whether 1-deazariboflavin and 1-deazalumichrome exist as the zwitterionic **3b** or the uncharged **3a.**



3a **3b**

That the latter structure **3a** is the major contributor (and that 1-deazariboflavin is completely structurally analogous to riboflavin) is suggested by several observations. First, the spectrum of 2′,3′,4′,5′-tetraacetyl-1-deazariboflavin shows only subtle changes as the solvent dielectric is lowered (H_2O λ_{max} 365 nm (4000 M^{-1} cm^{-1}), 535 (6800); chloroform 350 (4000), 523 (6800); dioxane 340 (sh, 3500), 494 (6400)). These shifts completely parallel those observed for tetraacetylriboflavin (H_2O 372 (10700), 445 (12500); chloroform: 352 (9300), 449 (13600); dioxane: 336 (9100), 439 (13000). By comparison, the zwitterionic flavin semiquinone **4** exhibits dramatic spectral



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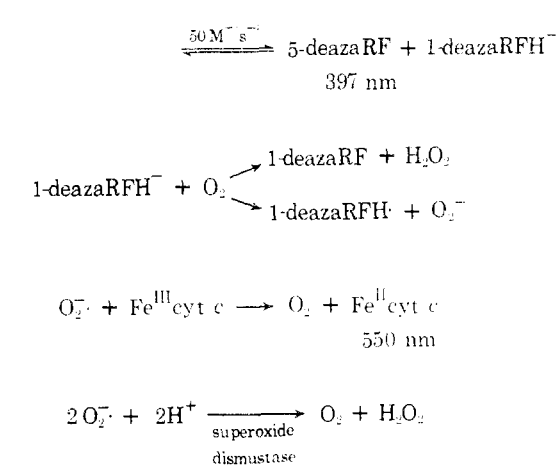
changes (H_2O 502 (3900), 580 (3600); chloroform 490 (2350), 603 (4000), 642 (4400), Müller et al., 1972). Secondly, 1-deazariboflavin has a low aqueous solubility, and, like riboflavin, remains immobile at the origin during paper electrophoresis at pH 3. At this pH one would anticipate protonation of O^2 of **3b** to generate a cationic species. The immobility in an electric field suggests that this is not the case. The NMR data of Ashton et al. (1977) confirm that 1-deazalumichrome also exists as the isoalloxazine tautomer (**3a**, R = H).

In aqueous solutions, 1-deazariboflavin is rapidly reduced by sodium dithionite to yield a species bleached at 535 nm. Subsequent experiments have indicated that his reduction method yields a dihydro-1-deazaisoalloxazine, spectrally identical to the species produced by enzymatic 2 e^- oxidoreduction (Spencer et al., 1977). This dihydro-1-deazaflavin is, in turn, rapidly oxidized by molecular oxygen (vide infra) with quantitative regeneration of the oxidized 1-deazariboflavin and (ultimate) production of equimolar quantities of hydrogen peroxide. The discrete addition of air aliquots to a previously anaerobic solution of dihydro-1-deazariboflavin at pH 7.9 generates a series of isosbestic spectra, indicating the absence of a stable intermediate. 1-Deazariboflavin is alternatively photoreduced in neutral, 50 mM EDTA degassed solution, though more slowly than riboflavin under the same conditions, to this same dihydro species. Reduction of neutral aqueous 1-deazariboflavin with hydrogen over platinized asbestos also proceeds readily to the same dihydro-1-deazariboflavin.

In marked contrast to the susceptibility of riboflavin and 5-deazariboflavin to reduction by borohydride is the resistance of aqueous 1-deazariboflavin solutions to reduction by sodium borohydride. In order for this reductant to reduce 1-deazariboflavin at any significant rate, a decrease in solvent polarity with 25% (v/v) dimethylformamide is required; under these conditions (pH 7.0), reduction does proceed to yield as sole product an oxygen-stable, reduced 1-deazariboflavin compound (λ_{max} 440 nm (3900)). This product is distinct from the dihydro-1-deazaflavin which is produced by sodium dithionite, photoreduction, and enzymatic action. Since this species has no obvious biological relevance, a structural elucidation has not yet been made, leaving us only to suggest the possibility of preferential sodium borohydride reduction occurring at either the C-2 or C-4 carbonyls or to the 10a-1 double bond, similar to what occurs on exhaustive borohydride reduction of isoalloxazines (Müller et al., 1971). This borohydride-produced material is capable of further photoreduction with loss of the 440-nm absorption, which reappears on aeration. It is unclear why aqueous sodium borohydride reduction is not facile, and it is conceivable that these preliminary observations may have bearing on the mechanism of electron input into the 1-deazaflavin system in that both sodium dithionite (Mayhew and Massey, 1973; Lambeth and Palmer, 1973) and light/EDTA (Massey and Palmer, 1966) are believed to reduce isoalloxazines by sequential one-electron transfers.

Reduction by Dihydro-5-deazariboflavin. 1-Deazariboflavin is readily reduced by dihydro-5-deazariboflavin in the uppermost reaction of Scheme I. In aerobic buffer, dihydro-

Scheme I



1-deazariboflavin is rapidly oxidized back to 1-deazariboflavin, so that the reaction proceeds exclusively to the right as shown. The reaction is first order in 1-deazariboflavin and dihydro-

5-deazariboflavin, with a rate constant of 50 M^{-1} s^{-1} (pH 8.3, 30 °C). Implicit in this facile reaction is that 1-deazaflavins can undergo *concerted* two-electron redox processes: the very low potential for formation of 5-deazaflavin radicals (−650 mV; Blankenhorn, 1976) precludes a mechanism involving sequential one-electron transfers.

Reoxidation of Dihydro-1-deazaflavin by O_2. Examination of the stoichiometry of dihydro-1-deazaflavin oxygen oxidation provides an additional approach to the question of sequential one-electron transfers in 1-deazaflavin oxidoreduction. The oxygen oxidation of 1,5-dihydroisoalloxazines is known to be mechanistically complex (Massey et al., 1973; Hemmerich and Wessiak, 1976; Kemal et al., 1977), involving an intermediate peroxyflavin adduct that subsequently decomposes by pH-dependent pathways involving either homolytic (generating semiquinone and superoxide) or heterolytic (generating dihydroflavin and peroxide) bond cleavage. By analogy with the stable semiquinone states of the parent flavins, if such semiquinones were also stable in the 1-deazaflavin series one might anticipate this to be reflected in (at least some) production of superoxide anion. The assay system of Massey et al. (1969b) involving superoxide-dependent cytochrome *c* reduction was used to investigate this process. Thus, light/EDTA photoreduction of 1-deazariboflavin in air-saturated buffer resulted in cytochrome *c* reduction. No such reduction was observed in the absence of either light or 1-deazaflavin, or most importantly, in the presence of superoxide dismutase. Substitution of 1,5-dihydro-5-deazariboflavin for light/EDTA as the reductant (thus generating dihydro-1-deazaflavin by the comproportionation reaction; Scheme I) gave identical results and allowed quantitation of the stoichiometries by examination of the kinetics at 397 (oxidized 5-deazariboflavin production) and 550 nm (cytochrome *c* reduction). Assay by this method in the presence and absence of superoxide dismutase gave a mean value of 1.1 mol of cytochrome *c* reduced per mol of dihydro-1-deazariboflavin oxidized (0.1 M KPP_i, pH 8.3, 30 °C), indicating that superoxide anion is a significant product of dihydro-1-deazaflavin oxygen oxidation at this pH.[2] The production of 1 mol of hydrogen peroxide per mol of dihydro-1-deazariboflavin (noted in a preceding paragraph) was observed in the absence of a superoxide-trapping system, and thus approximately 50% of this H_2O_2 must arise by superoxide dismutation.

Kinetics of Dihydro-1-deazariboflavin Oxidation. Since dihydro-1-deazariboflavin reacts rapidly with oxygen with production of superoxide, a kinetic analysis of this reaction is warranted for a better understanding of the chemistry of the oxygen reactivity of reduced flavins, and for the interpretation of the kinetics of 1-deazaflavin-substituted flavoprotein oxidases (Spencer et al., 1977). We have undertaken a preliminary investigation of this reaction with the assistance of Dr. Robert Presswood.

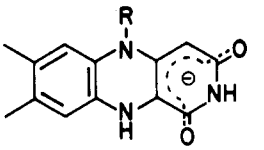
The kinetics of oxidation of dihydro-1-deazariboflavin appear to be fully as complex as those of dihydroriboflavin (Gibson and Hastings, 1962; Massey et al., 1973; Kemal et al., 1977), in that the oxidation is autocatalytic, shows evidence of long-wavelength intermediates, and is strongly dependent on the presence of superoxide anion. Under all conditions studied, dihydro-1-deazariboflavin is oxidized between one- and twofold as rapidly as dihydroriboflavin (Table I). Several parallels with dihydroriboflavin oxidation are worth noting.

TABLE I: Rates of Oxidation of Dihydroriboflavin and Dihydro-1-deazariboflavin by Molecular Oxygen: Pseudo-First-Order Initial Rates.[a]

	s^{-1}			
	Dihydroriboflavin		Dihydro-1-deazariboflavin	
pH	−SOD[b]	+SOD	−SOD	+SOD
4.0	0.3	*c*	0.5	*c*
7.0	6.5	1.9	7.0	3.9
9.0	2.1	1.1	2.7	2.0

[a] 20 °C in 0.1 M buffers (acetate, phosphate, and pyrophosphate), extrapolated to mixing time due to autocatalytic kinetics. [b] Superoxide dismutase, ca. 10^{-7} M when present. [c] Not determined, as SOD superfluous below the pK_a of superoxide anion.

(1) At pH 4, 1-deazaRFH_2 is oxidized tenfold more slowly than at neutrality. Thus, it is the anion 1-deazaRFH^- (structure **5**) that is the more reactive species, as is the case for



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dihydroriboflavin (for oxidation by oxygen) and dihydro-5-deazariboflavin (for oxidation by riboflavin) (Blankenhorn, 1976; Spencer et al., 1976).

(2) At all pH values studied, and in the presence or absence of superoxide dismutase, dihydro-1-deazariboflavin oxidation is autocatalytic; i.e., the oxidation rate accelerates throughout the course of the reaction. Thus, as with dihydroriboflavin, some initial product of the reaction is more reactive with oxygen than dihydro-1-deazariboflavin itself. Such reactive species could be the 1-deazariboflavin semiquinone (vide infra) or an intermolecular complex of 1-deazariboflavin redox species.

(3) The addition of superoxide dismutase at pH 7 and 9 slows the oxidation up to twofold, with the effect being greater at pH 7. This again parallels dihydroriboflavin, and implicates superoxide anion as a kinetically important product in both oxidations. This observation confirms the existence of superoxide anion as product in dihydro-1-deazariboflavin oxidation.

(4) We have observed a small (0.005 optical density), reproducible absorbance transient at 780 nm in 1-deazaRFH^- oxidation at pH 7, which is maximal between 10C and 200 ms after mixing. Such an absorbance also implicates 1-deazariboflavin semiquinones or, less likely, intermolecular complexes as intermediates in the reaction.

Kemal et al. (1977) have recently demonstrated the correlation between the redox potentials of a series of flavin analogues and the logs of their pseudo-first-order rate constants for oxidation by molecular oxygen in the presence of superoxide dismutase. Their findings predict a twofold increase in oxidation rate for every 100 mV more negative flavin redox potential. Dihydro-1-deazariboflavin shows just such an increase over dihydroriboflavin, indicating that the 1-carba, 1-deaza substitution offers no kinetic barrier to the oxygen reactivity of reduced flavins. This last point must be emphasized in consideration of the possible structures involved in dihydroflavin oxidation by oxygen.

Structure of Dihydro-1-deazaisoalloxazines. The preceding results and discussions have been undertaken without

[2] Under the same conditions, riboflavin was observed to produce between 0.3 and 0.6 mol of superoxide per mol of dihydro-5-deazariboflavin oxidized, consistent with previous determinations. If superoxide were the sole oxygen product and completely trapped by cytochrome *c*, one would observe 2.0 mol of superoxide per mol of dihydroflavin.

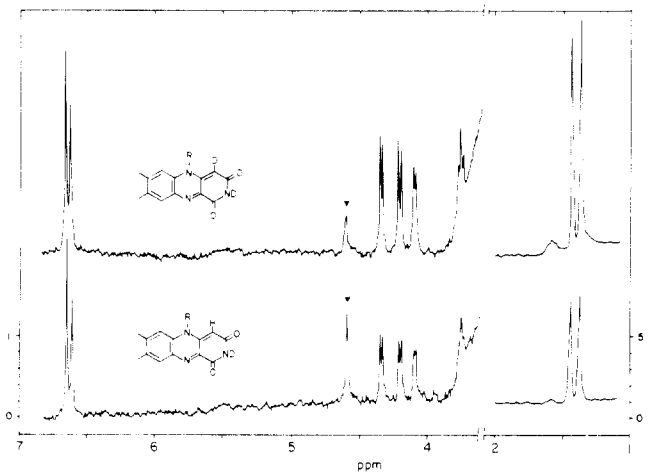
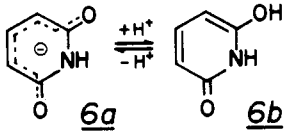


FIGURE 2: 270-MHz Fourier transform ^1H NMR spectra of 1-deazariboflavin. Upper spectrum: after 40 min of incubation of *dihydro*-1-deazariboflavin in ^2H_2O, pD 5.5 (see Syntheses). Lower spectrum: after incubation of *oxidized* 1-deazariboflavin in ^2H_2O. Parts per million are downfield from tetramethylsilane. Assignments: (▼) C_1H (s), δ 4.60; C_6H, C_9H (d), δ 6.67, 6.63 (2 protons); ribityl protons (d), δ 4.34, 4.20, 4.09 (1 proton each); C_7-CH_3, C_8-CH_3 (d), δ 1.44, 1.37 (6 protons; note change in vertical scale). In the upper spectrum, C_1H integrates to 0.31 proton; in the lower spectrum to 1.06 protons.

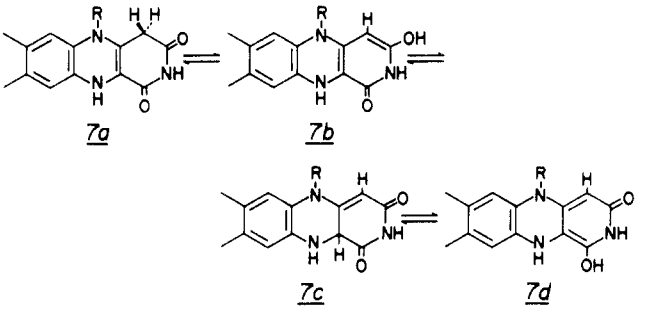
comment on the actual structure of the dihydro-1-deazaflavin undergoing oxidation; this point requires clarification before proceeding further. Dihydroisoalloxazines, the parent dihydro compounds, exist in a 1,5-dihydro form characterized by a pK_a = 6.5 corresponding to proton dissociation from N-1 to leave a (predominantly) N-1 localized anion. Dihydro-5-deazaisoalloxazines have both the 1,5-dihydro form and a pK_a (7.2) for N-1 dissociation as well (Spencer et al., 1976; Blankenhorn, 1976). By analogy then, a likely candidate for the dihydro-1-deazaisoalloxazine is a similar 1,5-dihydro form. To this point is the observation of a dihydro-1-deazariboflavin pK_a of 5.6 as manifested in a broad visible absorption change. The predominant species at alkaline pH has no visible λ_{max} and is identifiable as an anion by its adherance to ion-exchange resins, while the neutral species has λ_{max} 480 nm, ϵ 2000 M^{-1} cm^{-1}. Both the spectral and pK_a evidence are completely consistent with structure **5** for the dihydro-1-deazaflavin anion. Also in support of **5** as the anion structure is the acid–base chemistry of pyridine-2,6-diol **6a,b** (1-deazauracil; Spinner and White,



6a **6b** $pK_a = 4.52$

1966). The possibility of proton dissociation having occurred from N-3 is precluded by titration of dithionite-reduced *N*-3-methyl-1-deazariboflavin (pH 7.8, no λ_{max}; pH 4.8, λ_{max} 460 nm, ϵ 2700 M^{-1} cm^{-1}; pK_a = 6.3).

To obtain the neutral dihydro-1-deazariboflavin one can protonate the anion at C-1, C-4a, O^2, or O^4 to obtain the tautomers **7a–d.** The determination of the preferred structure is most readily approached by assay of the acidity of the hydrogen at C-1. Tautomer **7a** is the only one of the four with any acidity at C-1; thus, if this were the majority species one would expect rapid and complete equilibration of C-1 hydrogen with solvent protons, and only if **7a** were a minor contributor to the neutral dihydro-1-deazaflavin would one expect only a slow equilibrium of the C-1 hydrogen with the solvent.



7a **7b** **7c** **7d**

As assay for such acidity at C-1, dihydro-1-deazariboflavin was incubated in ^2H_2O at pD 5.5 for 40 min and allowed to air oxidize, and the resulting 1-deazariboflavin was assayed for proton content at C-1 by Fourier transform NMR. As is clear in Figure 2, the sample showed a decrease, but *not* complete loss, of protium from the C-1 singlet at δ 4.60 ppm, from 1.06 to 0.31 atoms. No exchange of C-1 hydrogen occurs from oxidized 1-deazariboflavin under the same conditions (Figure 2, lower spectrum). This finding of only slow and incomplete exchange eliminates **7a** as a major contributor to the structure of neutral dihydro-1-deazaflavins, and favors the enols **7b** and **7d** as the most likely structures, in agreement with the structure of neutral pyridine-2,6-diol **6b** as determined by Spinner and White (1966). A differentiation between O^2 and O^4 protonation is not possible with the data to date.

In a companion experiment, 1-deazariboflavin was reduced with dithionite in ^3H_2O, equilibrated, and then oxidized to 1-[1-^{3}H]deazariboflavin. This material could be purified to constant specific activity in its oxidized form, but upon reduction at pH 8.5 lost its label to solvent with a half-time of approximately 20 min, consistent with the above conclusions.

Redox Potential Determination. The oxidation–reduction potential of 1-deazariboflavin was determined by the independent methods of (1) anaerobic equilibration with a redox indicator, (2) polarography at the dropping mercury electrode, and (3) cyclic voltammetry at the hanging mercury drop electrode. For the measurement under equilibrium conditions, NADH/NAD^+ was used as the redox indicator (E_0' = −320 mV; Loach, 1968). Equilibrium between the nicotinamide and 1-deazariboflavin was catalyzed by the *B. harveyi* NAD(P)H: flavin oxidoreductase (Spencer et al., 1977) under anaerobic conditions, 0.1 M pyrophosphate, pH 8.3, 25 °C. The equilibrium constant for this reaction, as shown in Scheme II, is 22 ± 7 (six determinations). Since at the experimental pH this reaction does not involve net proton transfer with solvent, the equilibrium position is approximately pH independent. This permits calculation of the E_0' of 1-deazariboflavin: −281 ± 4 mV.

SCHEME II

$$\text{1-deazaRF} + \text{NADH} \overset{\text{enzyme}}{\rightleftharpoons} \text{1-deazaRFH}^- + \text{NAD}^+$$

Polarography of aqueous solutions of 1-deazariboflavin was performed in the differential pulse mode due to the limited solubility of the analogue. The $E_{1/2}$ at pH 7.0, extrapolated to zero modulation voltage, is −280 ± 5 mV vs. the normal hydrogen electrode. 1-Deazariboflavin equilibrates at the mercury surface as readily as riboflavin, as shown by the comparable diffusion currents for solutions of the same concentration. As for riboflavin at −208 mV, the $E_{1/2}$ of 1-deazariboflavin at −280 mV represents a two-electron reduction, since both compounds have the same differential pulse widths at half peak height, 87 mV. Polarographic investigations of $E_{1/2}$ vs. pH are currently under way.[3]

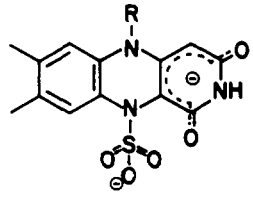
TABLE II: Comparative Properties of Flavins, 5-Deazaflavins, and 1-Deazaflavins.

	Flavins	1-Deazaflavins	5-Deazaflavins
Redox potential, pH 7.0	−208 mV	−280 mV	−311 mV
pK_a of FlH^-	6.5	5.6	7.2
Rate of oxygen oxidn of dihydro form[a]	1.9 s^{-1}	3.9 s^{-1}	$<2 \times 10^{-6}$ s^{-1}
Electrophilicity of position 5 of Fl_{ox}: K_D of sulfite[b]	1.0 M	16 M	5.3×10^{-3} M
Nucleophilicity of position 5 of FlH^-: facile alkylation[c]	Yes	Yes	No
Accessible, obsd semiquinone	Yes	Yes	No[d]
Redox equiv reactivities	1 and 2 e^-	1 and 2 e^-	2 e^- only
Incorp of stable ^3H into Fl_{ox} on redn/oxidn	No	Yes[e]	Yes
Fluorescence of Fl_{ox}	Yellow-green	None	Blue

[a] pH 7.0, 20 °C, with superoxide dismutase. [b] pH 8.3, 20 °C, all at riboflavin level (5′-OH). [c] With dimethyl sulfate, aqueous solution, pH 7-8. [d] Seen only on irradiation of 5-deazaFAD D-amino acid oxidase (Hersh et al., 1976). [e] Only by slow equilibration with ^3H_2O (see text).

Cyclic voltammograms of aqueous 1-deazariboflavin at pH 7.0 show symmetric reduction and oxidation waves of equal height centered at −278 mV, demonstrating the complete reversibility of this two-electron redox reaction. This $E_{1/2}$ is in excellent agreement with those found polarographically and by enzyme-catalyzed equilibration with NAD^+/NADH.

The determination of flavin redox potentials has also been approached through the equilibrium constant for sulfite addition (Müller and Massey, 1969). At pH 8.5, 20 °C, 1-deazariboflavin forms an adduct with a K_D for total sulfite of 16 ± 1 M. Since this adduct is bleached at 535 nm, and by analogy to other flavins (Müller and Massey, 1969), we suggest the formation of an N-5 adduct with structure **8**.



8

Compared to the series of exocyclically substituted flavins of Müller and Massey (1969), 1-deazariboflavin is somewhat more electrophilic toward sulfite than its redox potential would suggest; this may be due to the greater anion stability in the adduct **8** than is present in the corresponding riboflavin–sulfite adduct, as is apparent in the dihydro-1-deazariboflavin pK_a of 5.6 vs. 6.5 for dihydroriboflavin. In any case, 1-deazaflavins are clearly more like flavins in their electrophilicity than are 5-deazaflavins: the latter have the high electrophilicity at C-5 more characteristic of nicotinamides than flavins (Table II; Jorns and Hersh, 1976; Blankenhorn, 1976).

1-Deazaflavin–Egg-White Binding Protein Association. Although this protein has no known catalytic activity, it is well suited for flavin structural characterization in that the thermodynamics and kinetics of flavin association for a large number of flavin analogues have been determined (Becvar, 1973). The behavior of bound riboflavin places this binding site as a dehydrogenase type (Massey et al., 1969a), by its inertness toward addition of sulfite and its stabilization of the zwitterionic (blue) flavin semiquinone (vide infra; V. Massey, personal communication).

Riboflavin, the most tightly bound of any flavin tested, has a dissociation constant of 1.3 nM and shows considerable spectral shifts and vibronic resolution on binding (Becvar, 1973). 1-Deazariboflavin shows more subtle spectral changes on binding: slight red shifts and extinction decreases for both visible peaks (bound spectrum, λ_{max} 369 nm (ϵ 3400 M^{-1} cm^{-1}), 550 nm (6400), shouldering at 820–830 and 590–600 nm). Since 1-deazaflavins are nonfluorescent, a direct measurement of the K_d by flavin fluorescence quenching was not possible; the indirect techniques of competition equilibrium dialysis and kinetic assay of free 1-deazaRF were used. For the former method a series of equilibrium dialysis cells with fixed concentrations of [2-^{14}C]riboflavin and binding apoprotein was equilibrated with varying quantities of 1-deazariboflavin, and the K_d then calculated relative to the riboflavin K_d. The value obtained for 1-deazariboflavin was 1.6 nM (0.1 M Tris-HCl, pH 7.5, 20 °C) and is virtually identical to that for riboflavin. Secondly, and as an independent check, several solutions of varying binding apoprotein and 1-deazaRF were equilibrated, and the equilibrium concentrations of free 1-deazaRF measured from the steady-state velocity of NADH consumption catalyzed by the *B. harveyi* NAD(P)H:flavin oxidoreductase. Since the K_m for 1-deazaRF (20 nM) is as nearly as low as the dissociation constant, an accurate K_d determination is possible, with this method yielding a value of 2 ± 1 nM. That 1-deazariboflavin binds as well as riboflavin indicates the absence of a major stabilizing protein contact directed to the 1 position and is therefore in agreement with Becvar's observation of tight binding for N-1 methylated alloxazines.

The real value of this binding apoprotein is in the examination of the flavin analogue binding in the two other flavin oxidation states: dihydro and semiquinone. Although no quantitative information is available on the binding of dihydroisoalloxazines, we have observed that 1,5-dihydro-5-deazariboflavin is more poorly bound than 5-deazariboflavin, perhaps by three orders of magnitude. With dihydro-1-deazaflavin the binding protein association is easily detected by dramatic spectral and pK_a perturbations. The spectral pK_a of bound dihydro-1-deazaRF is 7.9 ± 0.2, an upward shift of 2.3 pH units from the unbound flavin, a stabilization of neutral species consistent with the hydrophobic nature of the protein-binding site. While the spectrum of the bound anion is similar to that for the free anion (no λ_{max} above 340 nm), the bound neutral species possesses a totally altered spectrum, being red shifted by 70 nm (λ_{max} 524 nm (2100); K_d = 1.4 μM (pH 6.0)). A shift of this magnitude cannot be accounted for by a selective stabilization of a vibrational state within an electronic transistion but only by the stabilization of a structurally different neutral dihydro-1-deazaflavin. We have previously expressed the likelihood of either **7b** or **7d** representing the structure of the uncomplexed neutral dihydro-1-deazaflavin but could not differentiate between them. In addition to these we suggest **9** as a possible structure for the

[3] Preliminary studies from pH 4 to 9 show a single linear correlation of $E_{1/2}$ vs. pH with a slope of 50 mV/pH and no apparent discontinuities.

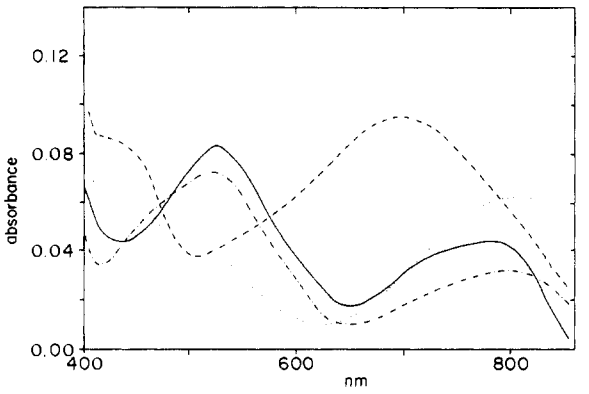
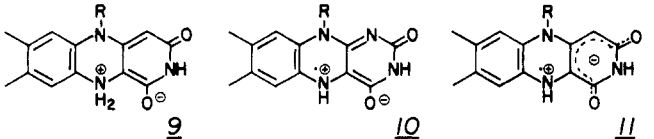


FIGURE 3: Long-wavelength-absorbing species obtained from 1-deazariboflavin. (—) Putative 1-deazaRFH· on binding protein, pH 7, obtained on air titration of 1-deazaRFH$_2^-$ binding protein. (- - -) Putative 5-methyl-1-deazaRFH$^+$ (green material), pH 2, aerobic. (---) Putative 5-methyl-1-deazaRFH·, end product of anaerobic solvolysis (pH 3) of green material. (•••) Putative 5-methyl-1-deazaRFH· on binding protein, pH 6.5, aerobic.



9 10 11

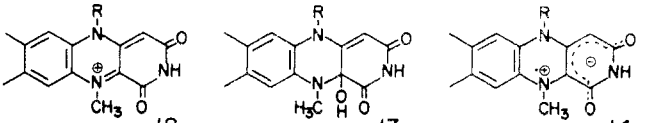
neutral, dihydro-1-deazariboflavin bound to the egg-white protein. This structure is attractive in that it has the same charge distribution as the neutral, blue flavin semiquinone **10** which is stabilized by this protein and as a charge-separated structure could have a very different visible spectrum from **7b** or **7d** which would predominate in free solution.

With respect to the semiquinone states, dithionite titration of the riboflavin-binding protein complex generates the zwitteronic, blue semiquinone **10,** which may also be obtained by full reduction with dithionite and back-titration with oxygen. The radical is made by either method in approximately 50% yield. An identical experiment with 1-deazariboflavin proved to be nonproductive in that the bound 1-deazaRF was inert to any dithionite reduction. This inertness to dithionite reduction is probably a combined effect of steric hindrance and very low redox potential of the bound 1-deazariboflavin. From the dissociation constants of oxidized and reduced 1-deazaRF, this bound redox potential can be estimated at −390 mV.

If, however, an anaerobic solution of the binding apoprotein is added to the dihydro-1-deazariboflavin to form the λ_{max} 524 complex (pH 7), one can titrate with oxygenated buffer to generate a new, long-wavelength absorbing species (λ_{max} 521 nm, 784 nm; Figure 3). In the latter half of the oxygen titration this species is isosbestic at 662 nm with the oxidized 1-deazaRF–binding protein complex. On the basis of the spectrum and the data from the reductive alkylation chemistry of 1-deazariboflavin (vide infra), we tentatively propose this species as a 1-deazariboflavin semiquinone which is kinetically stabilized by the binding protein and which is oxidized on the binding protein via discrete one-electron transfers to oxygen. The likely structure corresponding to this spectrum is the 1-deazaflavin semiquinone zwitterion **11.**

Reductive Methylation of 1-Deazariboflavin. Since alkylation at N-5 of flavins is known to stabilize their neutral semiquinone forms (Müller et al., 1972; Ghisla et al., 1973; Kemal and Bruice, 1976), we have carried out preliminary reductive methylation studies of 1-deazariboflavin. The small amounts of 1-deazariboflavin available to date have required micromole scale syntheses and have precluded the quantitation of yields and purity of the species described below. Nonetheless, it is evident that this is a facile route to the chemistry of the 1-deazaflavin radicals.

The green material obtained on reductive methylation of 1-deazariboflavin (see Syntheses), as purified by adsorptive chromatography at pH 3, has the spectrum of Figure 3 (λ_{max} 700 nm, $\epsilon \geq 2000$ M^{-1} cm^{-1}). This material is moderately stable in acid and shows no change in spectrum from pH 0.5 to 3. On standing in aerobic buffer at pH 3, such green solutions will become purple with a half-time of several hours; by spectrum and thin-layer chromatography this purple product is the starting material, 1-deazariboflavin. In this slow reaction isosbestic points at 455 and 620 nm are preserved. Thus, the reductive alkylation procedure does not result in an irreversible methylation or destruction of the ring system or ribityl side chain. Due to its acid stability and subsequent reactions, we propose that the green product is the 5-methyl-1-deazariboflavin cation **12.**



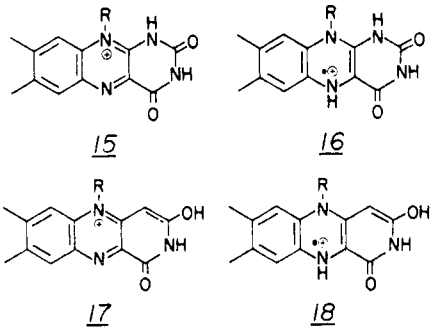
12 13 14

When the green material is reduced with dithionite, taken to neutral pH, and allowed to air oxidize, it yields only unidentified UV-absorbing material and no 1-deazariboflavin. By analogy to the behavior of N-5 methylflavins (Kemal and Bruice, 1976) above pH 8, this may represent the irreversible solvolysis of the 1-deazaflavin ring system, proceeding via the 5-methyl-4a-hydroxy-1-deazariboflavin pseudobase **13.** In contrast, when the same experiment is performed but with anaerobic binding apoprotein added before the oxidation of neutralized, reduced green product, an oxygen-stable species is obtained (Figure 3) with a spectrum remarkably close to that of the unmodified 1-deazariboflavin semiquinone on the binding protein (Figure 3). We suggest that this is the 5-methyl-1-deazariboflavin semiquinone **14,** now protected from solvolytic breakdown by the binding protein.

If a solution of the green product at pH 3 is made anaerobic and allowed to undergo solvolysis under these conditions, another long-wavelength-absorbing species is observed to accumulate (Figure 3). This species is also similar in spectrum to the 1-deazariboflavin semiquinone on the binding protein, and may be the *N*-5-methyl radical **10,** stable (in the absence of binding protein) at pH 3 while anaerobic.

Electron spin resonance signals were observed for a solution of the green species (pH 3, aerobic), as well as the blue flavin radical, from riboflavin taken through the same reductive methylation procedure (pH 7, aerobic). For both samples a single line was observed at $g = 2.004$, $\Delta H_{p\text{-}p} = 23$ G, though, qualitatively, the amount of radical was much less in the 1-deazaflavin sample. This observation of a radical signal for the green material is consistent with the above structural assignments, since a small steady-state concentration of the radical **14** is likely to be an intermediate in the aerobic solvolysis of the cation **12.** For further discussion of these points, we refer the reader to the complex pathway for the solvolysis of *N*-5-methylflavins, as elegantly characterized by Kemal and Bruice (1976).

Cation and Cation Radical Species. The thorough deoxygenation of a solution of riboflavin in 6 N HCl gives quantitative production of the cation radical **16** from the cation **15.** On addition of oxygen aliquots, the radical is readily titrated back to the cation. Similarly, a solution of 1-deazariboflavin in 6 N HCl has a spectrum distinct from that in neutral solu-



15 16

17 18

tion, presumably representing the 1-deazariboflavin cation **17** (λ_{max} 385 nm (5600 M^{-1} cm^{-1}), 505 (7700)). When this solution is thoroughly deoxygenated, a new long-wavelength-absorbing species appears (λ_{max} 360 nm (6100 M^{-1} cm^{-1}), 453 (3800), 521 (3300), 660 (1700)) which gives a positive EPR signal (g = 2.008, ΔH_{p-p} = 20 ± 1 G) and is probably the 1-deazariboflavin cation radical **18** (or tautomers thereof). Addition of oxygen to the long-wavelength-absorbing material gives quantitative return of the 1-deazariboflavin cation spectrum and loss of the EPR signal. This is an additional demonstration of the facile production of 1-deazaflavin radicals.

Conclusion

It is pertinent in conclusion to compare 1-deazariboflavin with its parent riboflavin and with its carba-deaza congener, 5-deazariboflavin, and to summarize how carba-deaza substitution at the opposite ends of the 1,5 redox locus affects the chemistry of flavins. Several of these chemical properties are listed in Table II.

With a redox potential of −280 mV, 1-deazariboflavin is 31 mV more positive than 5-deazariboflavin and 72 mV more negative than riboflavin. Thus, 1-deazaflavins are more difficult to reduce than the parent flavins, and 1-deazaflavin coenzyme substitution of apoflavoenzymes can be expected to slow the flavin-reduction step in catalysis. Indeed, 1-deazaflavin coenzyme substitution of flavoenzymes may be expected to show significant, even preferential, catalysis in the reverse direction to that observed with parent flavin coenzymes. This is also the expectation for 5-deazaflavins, and the reverse enzymatic reaction is indeed preferred for several 5-deaza-substituted flavoenzymes (Fisher et al., 1976; Hersh and Jorns, 1975).

Reduction of 1-deazariboflavin with dithionite, light/EDTA, or H_2/Pt yields the dihydro anion, which has a pK_a of 5.6, presumably representing protonation of the 2- or 4-enolate anion as the pH is lowered. Dihydro-1-deazariboflavin exchanges its C-1 hydrogen only slowly with solvent protons; thus, carbon-1 does not exist significantly as a methylene group (structure **7a**) in dihydro-1-deazaflavins. This has two consequences: (1) The origin of any hydrogen stably incorporated at C-1 during enzymatic redox reactions cannot be studied easily, since hydrogen transfer to this carbon is unlikely. This is in marked contrast to 5-deazaflavins, with which direct and complete hydrogen transfer from substrate is readily detected (Fisher et al., 1976; Hersh and Jorns, 1975). (2) Since a methylene group is not formed readily at C-1 in dihydro-1-deazaflavins, one cannot use this locus as a prochiral center to study the stereochemistry of hydrogen transfer.

In chemistry at position 5, the 1-deazaflavins are clearly more like flavins than 5-deazaflavins. Thus, both 1-deazariboflavin and riboflavin add sulfite at position 5 much less readily than 5-deazariboflavin (Table II), and dihydro-1-deazariboflavin and dihydroriboflavin readily attack dimethyl sulfate to yield N-5 methylated species, while dihydro-5-deazariboflavin is inert to such alkylation.

It is in one-electron chemistry, though, where 1-deazaflavins are most clearly distinct from 5-deazaflavins. 5-Deazaflavins have a redox potential for semiquinone formation of −650 mV (Blankenhorn, 1976) and thus have no thermodynamically accessible one-electron chemistry (except via photoreduction: Hersh et al., 1976). One consequence of this very low potential is that dihydro-5-deazaflavins are essentially inert to oxidation by molecular oxygen. 1-Deazaflavins, however, like parent flavins, have semiquinones stabilized by protein binding or N-5 alkylation. Dihydro-1-deazariboflavin is rapidly oxidized by molecular oxygen, in fact some twofold faster than dihydroriboflavin: both with the autocatalysis, superoxide dependence, and long-wavelength transients indicative of radical intermediates.

Since 5-deazaflavins are restricted to net two-electron processes, the facile oxidation of dihydro-5-deazariboflavin by catalytic amounts of riboflavin or 1-deazariboflavin indicates that both these latter species can undergo concerted two-electron as well as one-electron redox reactions. 1-Deazaflavins, then, have as full and varied a redox chemistry as the parent flavins.

Having defined some of the basic chemistry of 1-deazaflavins, it is apparent that these compounds are useful and interesting flavin isosteres. In the following paper of this issue we have evaluated the behavior of 1-deazaflavins in several enzymatic redox systems.

Acknowledgments

We thank the Merck group of Ashton et al. (1977) for the gift of 1-deazaflavins which enabled us to undertake this study; Dr. Robert Presswood for his assistance in the rapid kinetics experiments; Peter Demou and Walter Krol for taking the high-field Fourier transform NMR spectra; and Drs. M. Stankovich, V. Massey, and T. C. Bruice for helpful unpublished information, comments, and suggestions.

References

Ashton, W., Brown, R., Graham, D., and Rogers, E. (1977), *Tetrahedron Lett.* (submitted).

Becvar, J. (1973), Ph.D. Thesis, University of Michigan.

Blankenhorn, G. (1976), *Eur. J. Biochem. 67*, 67-80.

Bright, H., and Porter, D. (1975), *Enzymes, 3rd Ed. 12*, 421-505.

Bruice, T. C. (1976), *Prog. Bioorg. Chem. 4*, 1-87.

Fenner, H., Roessler, H., Duchstein, H., and Hemmerich, P. (1976), *Flavins Flavoproteins, Proc. Int. Symp., 5th*, 343-348.

Fisher, J., Spencer, R., and Walsh, C. (1976), *Biochemistry 15*, 1054-1064.

Ghisla, S., Hartmann, U., Hemmerich, P., and Müller, F. (1973), *Justus Liebigs Ann. Chem.*, 1388-1415.

Gibson, O., and Hastings, J. W. (1962), *Biochem. J. 83*, 368-377.

Hemmerich, P. (1976), in Fortschritte der Chemie Organischer Naturstoffe, Grisebach, H., Ed., New York, N.Y., Springer-Verlag, pp 451-526.

Hemmerich, P., and Wessiak, A. (1976), *Flavins Flavoproteins, Proc. Int. Symp., 5th*, 9-22.

Hersh, L., and Jorns, M. S. (1975), *J. Biol. Chem. 250*, 8728-8734.

Hersh, L., Jorns, M., Peterson, J., and Currie, M. (1976), *J. Am. Chem. Soc. 98*, 865-867.

Jorns, M., and Hersh, L. (1976), *J. Biol. Chem. 251*, 4872-

4881.
Kemal, C., and Bruice, T. (1976), *J. Am. Chem. Soc. 98*, 3955-3964.
Kemal, C., Chan, T., Williams, R., and Bruice, T. (1977), *J. Am. Chem. Soc. 99* (in press).
Lambeth, D., and Palmer, G. (1973), *J. Biol. Chem. 248*, 6095-6103.
Loach, P. (1968), in Handbook of Biochemistry, Sober, H., Ed., Cleveland, Ohio, The Chemical Rubber Co., pp J27-J34.
Massey, V., Müller, F., Feldberg, R., Schuman, M., Sullivan, P., Howell, L., Mayhew, S., Matthews, R., and Foust, G. (1969a), *J. Biol. Chem. 244*, 3999-4006.
Massey, V., and Palmer, G. (1966), *Biochemistry 5*, 3181-3189.
Massey, V., Palmer, G., and Ballou, D. (1973), *Oxidases Relat. Redox Syst., 1, Proc. Symp.*, 25-43.
Massey, V., Strickland, S., Mayhew, S., Howell, L., Engel, P., Matthews, R., Schuman, M., and Sullivan, P. (1969b), *Biochem. Biophys. Res. Commun. 36*, 891-897.
Mayhew, S., and Massey, V. (1973), *Biochim. Biophys. Acta 315*, 181-190.
McCormick, D. (1970), *J. Heterocycl. Chem. 7*, 447-458.
Müller, F., Brüstlein, M., Hemmerich, P., Massey, V., and Walker, W. (1972), *Eur. J. Biochem. 25*, 573-580.
Müller, F., and Massey, V. (1969), *J. Biol. Chem. 244*, 4007-4016.
Müller, F., Massey, V., and Hemmerich, P. (1971), *Methods Enzymol. 18B*, 474-479.
Spencer, R., Fisher, J., and Walsh, C. (1976), *Biochemistry 15*, 1043-1053.
Spencer, R., Fisher, J., and Walsh, C. (1977), *Biochemistry 16* (following paper in this issue).
Spinner, E., and White, J. (1966), *J. Chem. Soc. B*, 991.
Stankovich, M., and Massey, V. (1976), *Biochim. Biophys. Acta 452*, 335-344.
Walsh, C. (1977), *Horiz. Biochem. Biophys. 3* (in press).
Walsh, C., Cromartie, T., Marcotte, P., and Spencer, R. (1977a), *Methods Enzymol.* (in press).
Walsh, C., Fisher, J., Spencer, R., Ashton, W., Graham, D., Brown, J., Brown, R., and Rogers, E. (1977b), *Biochemistry*, submitted.

Reconstitution of Flavin Enzymes with 1-Carba-1-deazaflavin Coenzyme Analogues†

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ABSTRACT: The riboflavin analogue 1-carba-1-deazariboflavin has been converted to 1-deazaFAD enzymatically and the coenzymatic activity of the analogue determined with three flavin-dependent enzymes. The NAD(P)H:flavin oxidoreductase from the marine bacterium *Beneckea harveyi* uses 1-deazariboflavin in the catalytic oxidation of NADH. The V_{max} for 1-deazariboflavin reduction by NADH is 0.025 that for riboflavin reduction, reflecting a thermodynamic control of V_{max} since the E_0' for the deaza analogue is 72 mV more negative than E_0' for riboflavin. Experiments using either 4(*R*)-[^{3}H]NADH (tritium in the transferable chiral locus) or ^3H_2O resulted in no incorporation of tritium at carbon-1 during aerobic catalysis involving multiple turnovers, consistent with an enolate structure for the dihydro-1-deazariboflavin anion. 1-DeazaFAD is an effective FAD analogue for apo-D-amino acid oxidase reconstitution and catalysis. Results are presented on binding of the coenzyme analogue and of inhibitors known to produce charge transfers with native holoenzyme. The V_{max} for turnover of several physiological D-amino acids by 1-deazaFAD-D-amino acid oxidase equals the V_{max} for native holoenzyme. The slow step in both instances is product release; the rate-determining physical step masks any effect the more negative redox potential of 1-deazaFAD might have on slowing down the coenzyme reduction step. Catalytic behavior of the 1-deazaFAD-enzyme with 3-haloamino acids, with nitroethane anion and with D-propargylglycine is qualitatively identical to the holoenzyme. 1-DeazaFAD also reconstitutes apoglucose oxidase from *Aspergillus niger*, functioning at a V_{max} = 0.10 that of FAD-glucose oxidase. Reduction of the coenzyme analogue is probably rate determining in turnover, with the lower redox potential of 1-deazaFAD controlling V_{max}. In agreement, [1-^{2}H]glucose shows a V_{max} kinetic isotope effect of 8.5.

Flavin-dependent enzymes catalyze many diverse and important redox transformations of organic and inorganic substrates in biological systems. These include flavoprotein dehydrogenases, many of which are membrane-bound components of electron-transport chains and are not reoxidized physiologically by molecular oxygen. The other broad category of flavoenzymes is one where the dihydroflavins are oxidized by molecular oxygen either functioning as oxidases, reducing O_2 to H_2O_2, or functioning as monooxygenases, activating O_2 for transfer of one oxygen atom to the substrate. Two important features of flavin chemistry relevant to its biological roles are its reduction potential and the reactivity of the dihydro and semiquinone forms for electron transfer to acceptors.

In the preceding paper of this issue we have probed some of the chemical and physical properties of a promising riboflavin analogue, 1-deazariboflavin.[1] In this manuscript we report

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