Preparation, Characterization, and Chemical Properties of the Flavin Coenzyme Analogues 5-Deazariboflavin, 5-Deazariboflavin 5′-Phosphate, and 5-Deazariboflavin 5′-Diphosphate, 5′→5′-Adenosine Ester†

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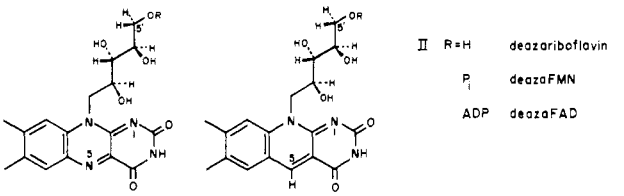
ABSTRACT: In order to facilitate interpretation of the deazaisoalloxazine system as a valid mechanistic probe of flavoenzyme catalysis, we have examined some of the fundamental chemical properties of this system. The enzymatic synthesis, on a micromole scale, of the flavin coenzyme analogues 5-deazariboflavin 5′-phosphate (deazaFMN) and 5-deazariboflavin 5′-diphosphate, 5′→5′-adenosine ester (deazaFAD), has been achieved. This latter synthesis is accomplished with a partially purified FAD synthetase complex (from *Brevibacterium ammoniagenes*), containing both phosphorylating and adenylylating activities, allowing direct conversion of the riboflavin analogue to the flavin adenine dinucleotide level. The structure of the reduced deazaflavin resulting from enzymatic and chemical reduction is established as the 1,5-dihydrodeazaflavin by proton magnetic resonance. Similarly, the C-5 position of the deazaflavins is demonstrated to be the locus for hydrogen transfer in deazaflavin redox reactions. Preparation of 1,5-dihydrodeazaflavins by sodium borohydride reduction stabilized them to autoxidation ($t_{1/2} \approx 40$ h, 22 °C) although dihydrodeazaflavins are rapidly oxidized by other electron acceptors, including riboflavin, phenazine methosulfate, methylene blue, and dichlorophenolindophenol. Mixtures of oxidized and reduced deazaflavins undergo a rapid two-electron disproportionation ($k = 22\ M^{-1}\ s^{-1}$, 0 °C), and oxidized deazaflavins form transient covalent adducts with nitroalkane anions at pH <5. Generalized methods for the synthesis of isotopically labeled flavin and deazaflavin coenzymes and their purification by adsorptive chromatography are given.

The chemical synthesis of the 5-deaza analogue of vitamin B_2, riboflavin (I, R = H), was reported by Cheng and his colleagues (O'Brien et al., 1970). In 1972, Tollin's laboratory (Edmondson et al., 1972) reported conversion of 5-deazariboflavin to 5-deazaFMN[1] by chemical phosphorylation, and they determined that this analogue would bind to the apoflavodoxin from *Azotobacter vinelandii* in place of the normal FMN but could report no data on the key question of whether the deazaFMN could replace FMN for normal redox function. Since then our laboratory has reported briefly on the successful enzymatic reduction and reoxidation of 5-deazariboflavin (Fisher and Walsh, 1974; Spencer et al., 1976), of 5-deazaFMN (Averill et al., 1975; Spencer et al., 1976), and of 5-deazaFAD (Spencer et al., 1976; see also Cromartie and Walsh, 1976) by appropriate apo forms of various flavoenzymes. Concurrently Hersh and Jorns documented similar successes for deazaFMN (Jorns and Hersh, 1975) and for deazaFAD (Hersh and Jorns, 1976). In the accompanying paper on detailed enzymatic studies with the deazaflavin coenzymes, we outline the types of mechanistic information these analogues may provide about flavoenzyme oxidative catalysis.

Given the biological activity of these deazaflavins as an impetus, we outline in this paper a combination of chemical and enzymatic methods for preparing pure samples, nonradioactive and radioactive, of 5-deazariboflavin (II, R = H),



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5-deazaFMN, and 5-deazaFAD along with the chemical and spectroscopic characterizations establishing their structures. Since much of the information adduced about enzymatic reduction and reoxidation of the 5-deazaflavins revolves around interpretation of the nature of the two electron reduced analogues, we also report here the structure of the dihydrodeazaflavin molecules formed both by chemical and enzymatic reduction. Finally, we document some of the chemical properties relevant to their enzymatic behavior, including reoxidation of the dihydrodeazaflavins by oxygen or by riboflavin, disproportionation between reduced and oxidized deazaflavins, and reactivity of deazaflavins with nitroalkane carbanions. With these key chemical patterns established, the companion paper details how flavoenzymes can use these reactivities in catalysis.

Experimental Section

Materials. Riboflavin, FMN, and FAD were supplied by Sigma. Riboflavin 4′-phosphate was a gift from Hoffman-La Roche, Basle. Riboflavin 5′-phosphate was from phos-

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[1] Abbreviations used are: deazaRF, 5-deazariboflavin; deazaRFH$_2$, 1,5-dihydrodeazariboflavin; deazaRFH$^-$, 1,5-dihydrodeazariboflavin anion; deazaFMN, 5-deazariboflavin 5′-phosphate; deazaFAD, 5-deazariboflavin 5′-diphosphate, 5′→5′-adenosine ester.

phodiesterase cleavage of FAD. Sodium borodeuteride (98%) was from Merck, and chloroform-*d* (99.8%) and dimethyl-d_6 sulfoxide (100.0%) were from Stohler Isotopes. Nitroethane was purified by distillation and converted to its anion by dissolving in 1 M NaOH a minimum of 30 min before use. Platinum (5% on asbestos) was obtained from Pfaltz and Bauer. Sodium borotritiide (163 Ci/mol) was from New England Nuclear, and [2-^{14}C]riboflavin (26 Ci/mol) from Amersham. [^{14}C]Urea (7.5 mg, 2.0 Ci/mol) was diluted with 52 mg of carrier and [2-^{14}C]barbituric acid was synthesized from this material utilizing the procedure of Haley and Lambooy (1954). The labeled barbituric acid was then condensed without further dilution with *N*-ribityl-2-aminobenzaldehyde to yield [2-^{14}C]deazariboflavin by the usual procedure (O'Brien et al., 1970). Unlabeled deazariboflavin and 10-methyldeazaisoalloxazine were also prepared by this procedure. The Bio-Gel P2 used for adsorption chromatography was 200–400 mesh and obtained from Bio-Rad. All remaining organic chemicals (highest available purity) and inorganic chemicals (reagent grade) were from commercial suppliers. Isolation and assay of the *Beneckea harveyi* NAD(P)H:flavin oxidoreductase is elaborated in the accompanying paper (Fisher et al., 1976).

Instrumentation. Reaction kinetics were monitored on a Gilford Model 222 spectrophotometer and absorption spectra were obtained on a Cary 14 spectrophotometer. Fluorescence measurements were made on a Farrand Mark 1 spectrophotometer. Scintillation counting was done on a Beckman LS-100C, utilizing a cocktail (Cocktail D) consisting of 5 g of 2,5-diphenyloxazole and 100 g of naphthalene dissolved in 950 ml of dioxane. Counting efficiency is essentially constant in the range 0–1.0 ml of water/10.0 ml of cocktail and has been determined to be 41% for ^{3}H and 93% for ^{14}C. Specific activities in the text expressed as cpm/nmol represent observed counts and are uncorrected for this efficiency. The Fourier transform ^{1}H nuclear magnetic resonance (NMR) spectra of the deazaflavins were obtained on an interfaced 60 MHz Perkin-Elmer R20S spectrometer, using a 10-μs pulse width followed by a 1.0-s acquisition of the free induction decay for a resolution of 1.0 Hz. Typical samples were 2–4 mg of deazaflavin in a solvent system of 0.50 ml of 0.67 Me_2SO-d_6/0.33 $CDCl_3$ with chloroform or tetramethylsilane as internal reference. Under these conditions adequate spectra were obtained in 2 h.

Enzyme Isolation. The FAD synthetase complex was purified as follows. *Brevibacterium ammoniagenes* (ATCC 6872) were grown in the medium of Nakamura and Tanaka (1968), consisting of glucose, 10% (w/v); KH_2PO_4, 0.5%; K_2HPO_4, 0.5%; Difco meat extract, 0.3%; Difco yeast extract, 1%; $MgSO_4 \cdot 7H_2O$, 0.5%; $CaCl_2 \cdot 2H_2O$, 0.01%; $FeSO_4 \cdot 7H_2O$, 0.001%; and urea, 0.6%; for 21 h at 30 °C with vigorous shaking. The cells were harvested by centrifugation at 13 000*g* for 1 h at 4 °C; the pellets were pooled and frozen in liquid nitrogen. Approximately 7 g of cells (wet weight) was obtained per liter of culture medium.

Since sonication of freshly harvested bacteria was found to give poor cell breakage, the frozen cells were forced through a Hughes' press at −20 °C. The broken cells were brought up in minimal potassium phosphate buffer (0.1 M, pH 7.5) and the debris was removed by centrifugation at 17 000*g* for 30 min. This and all subsequent steps were performed at 0–4 °C. The crude extract was brought to 50% saturation in ammonium sulfate at 0 °C and centrifuged at 40 000*g* for 20 min. Additional ammonium sulfate was added to bring the supernatant to 80% saturation, and this was centrifuged at 40 000*g* for 20 min. This pellet contained both riboflavin kinase and FAD synthetase activities and could be stored at −20 °C with no apparent loss of activity.

The last ammonium sulfate pellet was brought up in minimal buffer (as above) and applied to a 5 × 105 cm Sephadex G-100 column, equilibrated, and run in the same buffer. Flavokinase and FAD synthetase activities coeluted at 1.6 times the elution volume of blue dextran 2000, corresponding to a molecular weight of approximately 40 000. All fractions showing activity were pooled and concentrated by ultrafiltration (with an Amicon PM-10 filter) to a concentration of 0.44 mg ml^{-1} of protein (after Lowry, 1951). This solution was quick frozen in liquid nitrogen in aliquots for later preparative use.

Enzyme activity is assayed by addition of 50–100 μg of protein to riboflavin or FMN (final concentrations), 50 μM; ATP, 2 mM; $MgCl_2$, 8 mM; and 50 mM (pH 7.5) potassium phosphate buffer in 100 μl total volume. These are incubated from 4 to 18 h at 37 °C, during which time aliquots are removed and spotted on silica gel TLC plates. Development is with 1-butanol–acetic acid–water, 12:3:5 (v/v) with the flavins visualized in ultraviolet light and compared with known standards: riboflavin, R_f 0.50; FMN, R_f 0.17; FAD, R_f 0.05. [5-^{3}H]deazaRF and [2-^{14}C]riboflavin were used for quantitative kinetics.

Syntheses

Dihydrodeazariboflavin. Several milligrams of deazariboflavin are dissolved in a minimum amount of hot distilled water (≈70 °C) and with the solution still warm an excess of sodium borohydride (ca. 1 mg) is added. Upon completion of the reaction (30–60 s) the excess borohydride is consumed with acetone. The pH is adjusted to between 4 and 5 with acetic acid and the solution allowed to stand at 0 °C for several hours. The dihydrodeazariboflavin appears as a fine white precipitate and is collected by centrifugation. The substantial portion of the supernatant is discarded, leaving the deazaRFH_2 as a loose slurry in the aqueous acetic acid. The deazaRFH_2 may be stored as this slurry for several weeks at 4 °C with very minimal oxidation or stored indefinitely frozen. Substitution of NaB^3H_4 or NaB^2H_4 for the protio reagents gives the appropriately labeled deazaRFH_2 while reduction of [5-^{2}H]deazariboflavin with NaB^2H_4 yields dihydrodeazariboflavin-*5,5-d*$_2$.

[*5-^{3}H*]*Deazariboflavin* is prepared analogously to [5-^{2}H]deazariboflavin. [5^{3}H]Deazariboflavin is very much less stable with respect to radiochemical decomposition than [5-^{3}H]deazaRFH_2, and is therefore made only as is needed.

[*5-^{2}H*]*Deazariboflavin.* A solution of chemically prepared deazaFMN in distilled water is reduced with sodium borodeuteride; acetone is added and the deaza$FMNH_2$ oxidized with a small amount of riboflavin. This single cycle results in greater than 80% retention of deuterium at C-5. This sequence is repeated two additional times, following which the deazaFMN is absorbed onto DEAE-cellulose and thoroughly washed with distilled water. The [5-^{2}H]deazaFMN is then removed with a 0.20 M $(NH_4)HCO_3$ solution and treated with alkaline phosphatase. After completion of this reaction the solution is diluted and passed through a second DEAE-cellulose column to absorb unreacted deazaflavin phosphates. The [5-^{2}H]deazaRF is desalted as necessary on Bio-Gel P2.

Deazariboflavin Phosphate. A total of three chemical

procedures were duplicated: phosphorus oxychloride as the phosphate donor (Flexser and Farkas, 1952). Since this is the most commonly used procedure, the deazaFMN was further purified by ion exchange (Moffat and Khorana, 1958) and P2 adsorption chromatography. Secondly, there was catechol cyclic phosphate as the phosphate donor (Ukita and Nagasawa, 1959), followed up to, but not including the electrophoresis. Finally, a slightly modified procedure of the pyrophosphoryltetrachloride of Honjo et al. (1971) was also tried. Only the Flexser and Farkas procedure yielded acceptable material (optimal amount of the 5′ isomer).

Deazariboflavin 5′-Phosphate. Initially, deazaFMN was prepared in micromole quantities using partially purified ATP:riboflavin 5′-phosphotransferase (EC 2.7.1.26) (McCormick, 1971). However, we have since found it more convenient to obtain deazaFMN by phosphodiesterase cleavage of deazaFAD, since it is possible to obtain deazaFAD in much larger quantities.

Deazariboflavin 5′-Diphosphate, 5′→5′-Adenosine Ester (DeazaFAD). Four milligrams of deazariboflavin (10.5 μmol) was thoroughly dissolved in 250 ml of 30 mM potassium phosphate (pH 7.5) buffer by warming on a steam bath. The solution was cooled to 37 °C and 250 mg of ATP, 225 mg of $MgCl_2$, and approximately 3.5 mg of partially purified FAD synthetase complex were added. Incubation at 37 °C for 48 h results in greater than 90% conversion to deazaFAD. The solution was loaded onto a 3.5 × 16 cm column of DEAE-cellulose, and the column developed after washing with distilled water with a gradient of 0.0–0.2 M NaCl in 50 mM KP_i (pH 7.0) buffer. The yellow deazaFAD fractions were pooled and concentrated under reduced pressure to approximately 25 ml. One-fifth portion of this was loaded onto a 2.6 × 100 cm Bio-Gel P2 column, and was developed at 4 °C with distilled water (10 ml/h). The deazaFAD thus obtained is homogeneous by TLC and essentially salt free [deazaFAD, λ_{max} 228 nm (ε 32 000), 256 (33 200), 340 (10 500), and 399 (11 500)]. This material is stored frozen at −20 °C. [5-^{3}H]DeazaFAD, [2-^{14}C]deazaFAD, and [2-^{14}C]FAD are prepared from the appropriate labeled flavins. DeazaFAD with tritium localized in the adenosine moiety may be prepared from [2,8-^{3}H]ATP.

Results and Discussion

Preparation and Characterization of the Oxidized Forms of 5-Deazariboflavin, 5-DeazaFMN, and 5-DeazaFAD. (A) Preparation of 5-Deazariboflavin. We (Fisher and Walsh, 1974) as well as Jorns and Hersh (1975) have repeated the multistep literature synthesis of O'Brien et al. (1970) without incident and have previously published a proton magnetic resonance spectrum (Fisher and Walsh, 1974) providing spectroscopic confirmation that we have indeed prepared 5-deazariboflavin. Starting from [^{14}C]urea, we have now prepared [2-^{14}C]-5-deazariboflavin at a specific activity (530 cpm/nmol) useful in both chemical and enzymatic studies. Reduction by sodium borodeuteride or sodium borotritiide followed by reoxidation are efficient methods for generating [5-^{2}H]- and [5-^{3}H]deazariboflavin, respectively, introducing other isotopes of hydrogen at this key redox locus of the molecule. We have prepared [5-^{3}H]deazariboflavin at 20 000 cpm/nmol in this manner and among other virtues, this provides a sensitive method superior to those previously available for assaying flavokinase and FAD synthetase activities.

(B) Preparation of 5-DeazaFMN. Since riboflavin and 5-deazariboflavin are not the coenzymatically active forms of vitamin B_2 (with the notable exception of the bacterial NADH oxidase discussed in the accompanying paper), we needed methods for preparation of 5-deazaFMN and then 5-deazaFAD for evaluation of coenzymatic function. Initial supplies of deazariboflavin were limited so efficient microscale syntheses were in order.

Several chemical procedures for phosphorylation of riboflavin to riboflavin 5′-phosphate (FMN) have been reported, yet commercial samples of chemically synthesized FMN are known to be contaminated with as much as 10% phosphorylated impurities such as the 4′-phosphate and the 4′,5′-cyclic phosphate (Mayhew et al., 1976; Lee and Murphy, 1975). Clearly the presence of isomeric deazaflavin phosphates might alter the reconstitution characteristics of various apoenzymes. We thus reexamined several chemical procedures to determine which would lead to maximal phosphorylation of the (terminal) 5′-hydroxyl group of the ribityl side chain. In all cases, the deazaflavin phosphate obtained reacted at a diminished rate in an enzymatic assay (*B. harveyi* NAD(P)H:flavin oxidoreductase) relative to authentic deazariboflavin 5′-phosphate. Only the deazariboflavin phosphate obtained from the Flexser and Farkas (1952) phosphorus oxychloride method appeared to yield predominantly deazaFMN (relative V_{max} of 0.87). However, in order to eliminate all possible doubt, alternative, enzymatic syntheses were developed.

For the enzymatic synthesis of deazaFMN, we first turned to the riboflavin kinase of rat liver, an enzyme whose isolation and preliminary characterization had been reported by McCormick (1971) and whose thermal stability is enhanced in the presence of substrates (Rivlin and Langdon, 1966). Using crude liver extracts and prolonged incubation times, it was possible to accumulate up to several micromoles of deazaFMN, sufficient for the stoichiometric reconstitution of apo flavoenzymes. The major disadvantage of this enzymatic synthesis was that it obviously could not provide the quantities of deazaFMN needed for a chemical synthesis of deazaFAD. This problem was nicely circumvented with the development of an alternative enzymatic system, which has allowed the direct enzymatic conversion of deazariboflavin to deazaFAD in a single pot.

(C) Preparation of 5-DeazaFAD. A search of the patent literature revealed that strains of *Brevibacterium ammoniagenes* excrete FAD into the medium (Tanaka and Nakamura, 1971) and that cell extracts contain an FAD synthesizing activity which survives column chromatography on Sephadex G-100 (Nakamura and Tanaka, 1968). We have confirmed these results and also have partially purified the FAD synthetase activity as indicated in the Experimental Section. Interestingly, assays with riboflavin in place of FMN as substrate indicated the presence of flavokinase activity as well, with both activities coprecipitating on ammonium sulfate addition and subsequently exactly coeluting on gel filtration. To test the idea of a functional complex of activities converting riboflavin to FAD, we performed polyacrylamide disc gel electrophoresis on duplicate gels, staining one for protein and slicing the other into sections and assaying for activity. As expected, the protein stain indicated at least ten bands and the enzymatic activities do not correspond to any of the major bands visualized with Coomassie blue. However, the assay of the parallel gel shows that riboflavin kinase and FAD synthetase activities have the same mobility (within experimental accuracy).

Using [2-^{14}C]riboflavin or [2-^{14}C]FMN as substrates

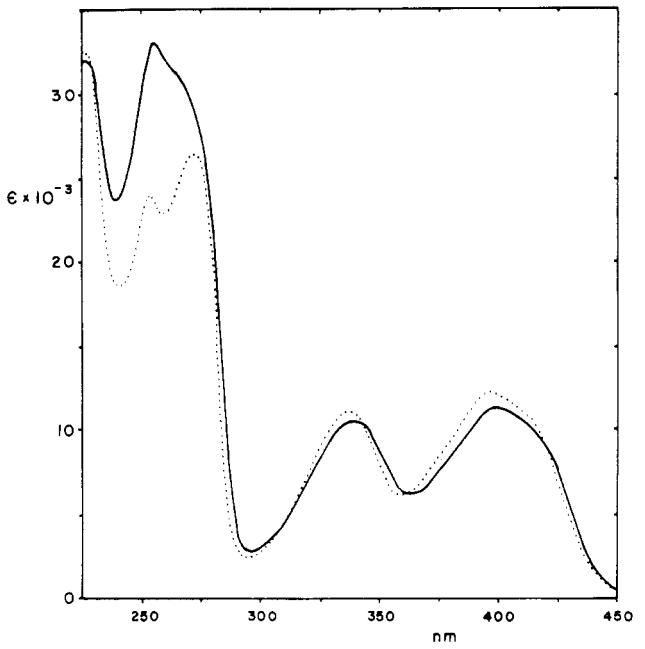


FIGURE 1: The absorption spectra (water) of deazaFMN (···) and deazaFAD (—).

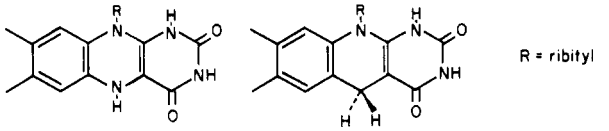
and measuring [2-^{14}C]FAD synthesis, the specific activities are 0.23 nmol min^{-1} mg^{-1} radioactive riboflavin and 4.9 nmol min^{-1} mg^{-1} with radioactive FMN as substrate. The flavokinase activity is the rate-limiting step in FAD formation. Despite the low specific activity values, further purification is not mandatory for use in the synthesis of FAD analogues since the combined enzyme activities are active for 18 h at 37 °C and, more importantly, there is no evidence of phosphatase or phosphodiesterase contamination at this stage.

The combined riboflavin kinase/FAD synthetase converts deazariboflavin to deazaFAD at the same rate as the natural isoalloxazine. Thus, 10 μmol of deazaFAD can be prepared using 3.5 mg of protein over a 48-h incubation. Since the FAD synthetase activity is 20-fold faster than riboflavin kinase activity, deazaFMN remains at a low steady-state concentration (ca. 1–2 μM) while deazaFAD accumulates quantitatively. A welcome bonus of this enzymatic system is that we can prepare authentic 5′-deazaFMN quantitatively from the purified deazaFAD by brief phosphodiesterase treatment and this is currently our preferred method of deazaFMN synthesis. Coupled with our synthesis of [5-^{3}H]deazariboflavin, this enzyme system affords [5-^{3}H]deazaFMN and [5-^{3}H]deazaFAD easily in the range of 20 000 cpm/nmol. In addition to 5-deazariboflavin the kinase/synthetase will convert dihydrodeazariboflavin to dihydrodeazaFAD. Since quantitative conversion of added riboflavin or riboflavin analogue to FAD or the corresponding FAD analogue is achievable, the *B. ammoniagenes* enzymes should be of general interest in preparing the coenzyme forms, including radiolabeled ones on a microscale, for direct evaluation of biological activity.[2] The flavokinase/FAD synthetase is, of course, interesting on its own right as an apparent multienzyme complex. Further purification and characterization of the enzymatic activities is currently under way.

Properties of the Deazaflavins. As noted by O'Brien et al. (1970) the prominent uv-visible absorption peaks in deazariboflavin are blue shifted some 50 nm compared to the peaks in riboflavin (338 and 396 nm compared to 375 and 452 nm). Figure 1 shows the absorption spectra for deazaFMN [λ_{max} 396 nm (ϵ 12 000)] and deazaFAD [λ_{max} 399 nm (ϵ 11 500)]. It has long been known that hydrolysis of FAD to FMN increases fluorescence some tenfold, explicable on the release of self-quenching due to the intramolecular interaction of the planar purine and isoalloxazine ring systems (Weber, 1950). Similarly, phosphodiesterase conversion of our purest samples of deazaFAD to deazaFMN increases fluorescence 11-fold, indicating similar conformations in solution and good purity of the deazaFAD (excitation at 365 nm, deazaFMN emission maximum at 459 nm, deazaFAD emission maximum 462 nm).

At this point we wish to comment on the behavior of the deazaflavins when subjected to gel filtration on the polyacrylamide Bio-Gel P2 since this is of enormous practical importance in their separation and purification. The fact that polyacrylamide gels such as Bio-Gel P2 weakly adsorb aromatic species allows a rapid and facile purification of deazaflavins that would not be possible by ion exchange or molecular sieve techniques alone. While knowledge of this adsorptive ability has existed for some time, it has only infrequently been applied to purifications of this sort. Bio-Gel P2 does not separate deazaFMN and deazaFAD but will desalt them (salts elute immediately before deazaFMN and deazaFAD) and separate away other aromatics. Interestingly, this process occurs with self-concentration as the deazaFMN and deazaFAD travel the column. Flow rates that are adequate for deazaFMN or deazaFAD will, however, excessively broaden any tailing deazaRF. At slower flow rates intimate mixtures of deazariboflavin and riboflavin may be efficiently separated. The purification of deazaFMN after *Naja naja* phosphodiesterase cleavage of deazaFAD is facilitated by the ability of P2 to completely separate deazaFMN and AMP, a separation that would be much less satisfactory with ion exchange. These instances illustrate the potential value of this technique.

Preparation and Structural Characterization of Reduced Deazariboflavin as the 1,5-Dihydro Species. By analogy with riboflavin which is reduced to the 1,5-dihydroisoalloxazine (III), we expected that 5-deazariboflavin



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would undergo two-electron reduction to the 1,5-dihydrodeazariboflavin (IV). However, explicit proof of this structure is critical to the recent interpretations about the nature of enzyme-catalyzed direct hydrogen transfer to the deazaflavin coenzymes. Such structural proof is offered in this section.

Both flavins and deazaflavins are reduced by dithionite, sodium borohydride, and hydrogen/platinum catalyst. With deazaflavins reduction proceeds with loss of the blue fluorescence to give a much paler fluorescent dihydro form. The 396-nm absorption of the oxidized deazaflavin is completely

[2] However, riboflavin 4′-phosphate is not converted to the corresponding 4′-FAD, allowing among other things the possibility to use isomeric phosphorylated mixtures of deazaFMN (or other analogues) as substrates to adenylylate only the correct 5′ isomer to the FAD level.

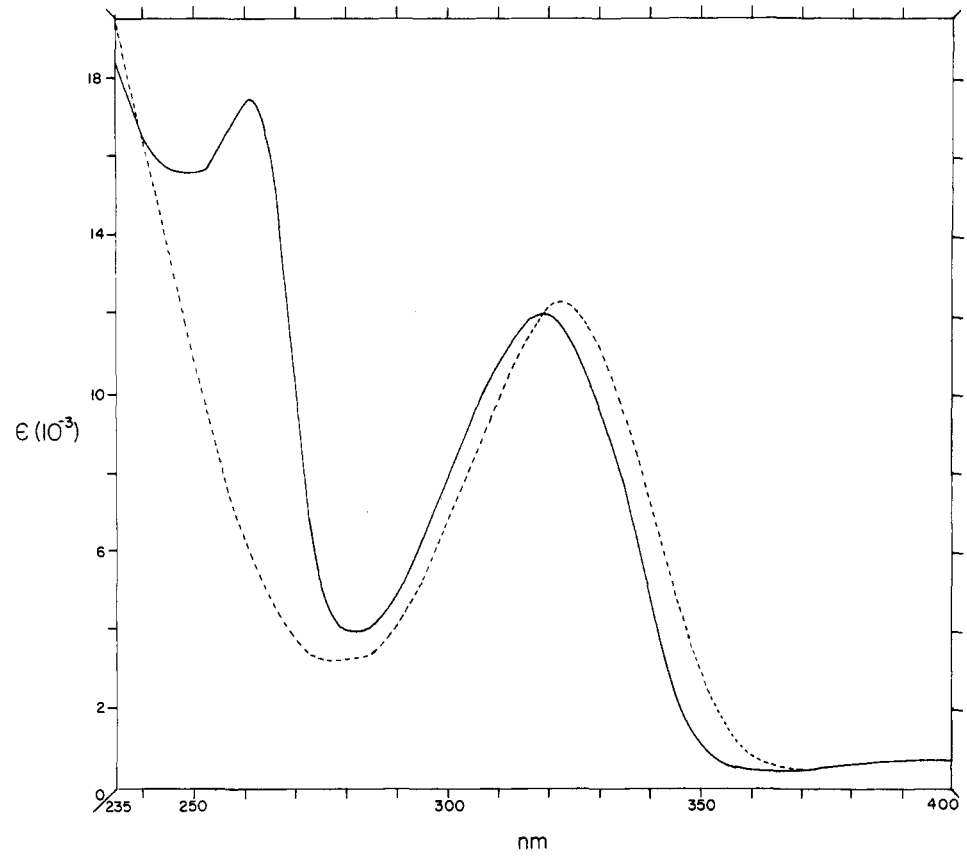


FIGURE 2: The absorption spectrum of dihydrodeazariboflavin at pH 5.7 (- - -) and as its anion at pH 8.6 (—).

abolished on reduction, with the 338-nm peak shifting to 320 nm with no loss in intensity. Enzymatic reduction catalyzed by the *B. harveyi* oxidoreductase with NADH yields the absorption spectra of Figure 2, identical with that obtained from chemical reduction. Correspondingly, all the chemically prepared dihydrodeazariboflavin samples undergo oxidoreductase catalyzed oxidation by NAD^+.

While encouraging that only a single dihydro form is produced, it remained to be determined exactly what structure this compound possessed. Brüstlein and Bruice (1972) have assigned a 1,5-dihydrodeazaflavin structure to the product of the nonenzymatic reduction of 10-methyldeazaisoalloxazine by NADH, from interpretation of its ^1H NMR spectrum. Unfortunately, the absorption spectrum of this compound was not reported and as such a direct comparison with the ribityl containing deazaisoalloxazines was prohibited. A similar lack of spectral information existed in a recent report outlining a synthetic sequence to these dihydroquinolines (Fenner and Michaelis, 1972). Consistent with a 1,5-dihydro structure is our finding that dihydrodeazariboflavin possesses a $pK_a = 7.2 \pm 0.1$ [deazaRFH$_2$, λ_{max} 323 nm (ϵ 12 400); deazaRFH$^-$, λ_{max} 319 nm (ϵ 12 000); and 262 nm (ϵ 17 500)]. A similar pK_a near neutrality in dihydroisoalloxazine structures is indicative of saturation at the 10a-1 carbon-nitrogen bond as would occur in the 1,5-dihydrodeazaisoalloxazines (pK_a would correspond to ionization of N-1) (Ghisla et al., 1973).

However, ^1H NMR analyses offer the more direct corroboration of this structure as well as for a straightforward determination that C-5 is the position to which hydrogen is transferred on reduction. To compare our findings with those of Brüstlein and Bruice, we reduced 10-methyldeazaisoalloxazine with both sodium borohydride and sodium borodeuteride. The reduced form of this protio compound also has the characteristic 320-nm absorption and possessed a ^1H NMR spectrum similar to that reported previously (Brüstlein and Bruice, 1972). As shown in Figure 3, the two-proton singlet at δ 3.57 (C-5 hydrogens) is consistent only with the 1,5-dihydro structure and its marked diminution with sodium borodeuteride as reductant confirms direct hydrogen transfer to the C-5 position by this reagent. We have similarly examined the ^1H NMR spectrum of deazariboflavin and its borohydride reduction product (Figure 4) and can also assign a 1,5-dihydro structure. Reduction results in increased shielding of the aromatic protons, and collapse of the aromatic methyl doublet into a six hydrogen singlet as is also observed in the ^1H NMR spectra of dihydroisoalloxazines (Ghisla et al., 1973). On borodeuteride reduction the δ 3.27 singlet is somewhat reduced in intensity (Figure 4) but explicit identification of transfer to C-5 of the deuteron is prevented by adventitious water protons absorbing in this same region. Nevertheless, the fact that both the 10-methyldihydrodeazaisoalloxazine and dihydrodeazariboflavin have identical ultraviolet spectra provides the necessary correlation. To substantiate this beyond question, the dihydrodeazariboflavin sample prepared from borodeuteride was catalytically oxidized by riboflavin (vide infra) and the ^1H NMR spectrum of the oxidized deazariboflavin examined (Figure 5). As predicted, the C-5 hydrogen signal, which is easily observed at δ 8.83, is significantly reduced in intensity. The C-5 hydrogen now integrates to <0.20 atom of hydrogen compared to the internal 1.0 hydrogen signals at C-7 and C-9 of the benzene nucleus. The high deuterium content, greater than the 0.5 atom expected for random loss of ^1H or ^2H from C-5 on reoxidation, indicates a product isotope effect has occurred. This is correlat-

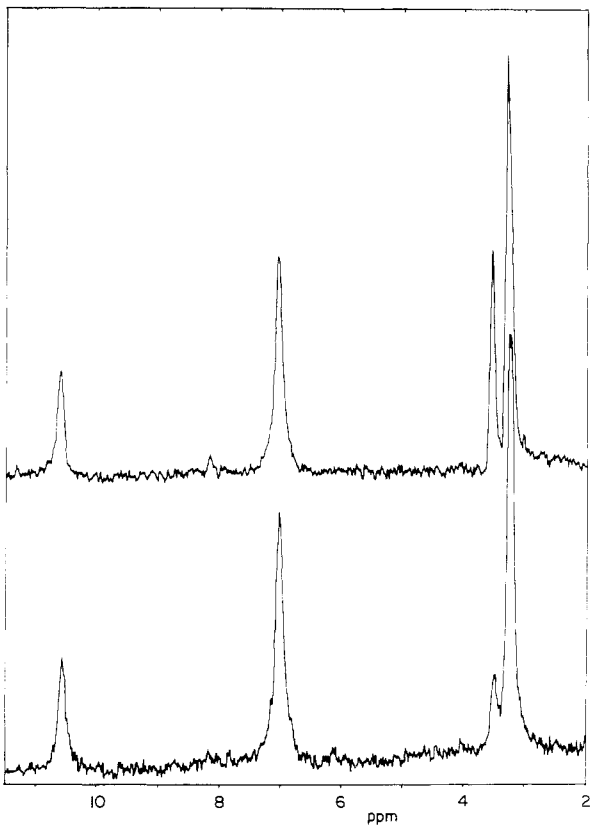


FIGURE 3: The proton magnetic resonance spectrum of 10-methyldihydrodeazaisoalloxazine. (Top) Prepared with sodium borohydride; (bottom) prepared with sodium borodeuteride. Chemical shifts in these and other spectra are given relative to tetramethylsilane (δ 0.00).

ed with kinetic isotope effects on reoxidation in a subsequent section of this paper.

As will be demonstrated in succeeding sections, we have been able to utilize deuterium kinetic isotope effects and tritium transfer data to demonstrate C-5 as not only the recipient position of hydrogen transfer on reduction from borohydride but identically as the locus for hydrogen transfer in the enzymatic redox reactions of deazaflavins.

Reoxidation of 1,5-Dihydrodeazaflavins by Molecular Oxygen. Among other categorizations flavoenzymes can be broadly classified as oxidases or dehydrogenases depending on whether molecular oxygen is the electron acceptor from the reduced coenzyme or some other reoxidant such as NAD^+ or an electron transport chain component is functional. The intrinsic rapid autoxidation of 1,5-dihydroflavins ($t_{1/2} \sim 1$ ms) is thus suppressed when bound to the apoproteins of dehydrogenases. Knowledge of the chemical reactivity of the 1,5-dihydrodeazaflavins is requisite in evaluating analogue functions when bound to apooxidases.

Edmondson et al. (1972) reported that the rate of autoxidation of reduced deazaFMN was much slower than that of dihydroflavins, showing a $t_{1/2}$ of about 3 h. This lowered reactivity to molecular oxygen is a direct consequence of the loss of the nitrogen lone pair at the five position in the carbon analogue. Simple dihydropyrazines are unstable to one-electron oxidation by oxygen and are prone to ready rearrangement (Lown et al., 1974; Tauscher et al., 1973). In contrast, 1,4-dihydropyridines with mild electron-withdrawing substituents (e.g., NADH) require few precautions in handling and are decomposed by oxygen very slowly (Fowler, 1972). The central ring of dihydrodeazaflavins is a dihydropyridine and not a dihydropyrazine and their oxygen lability is more in line with the structurally analogous dihydropyridines.

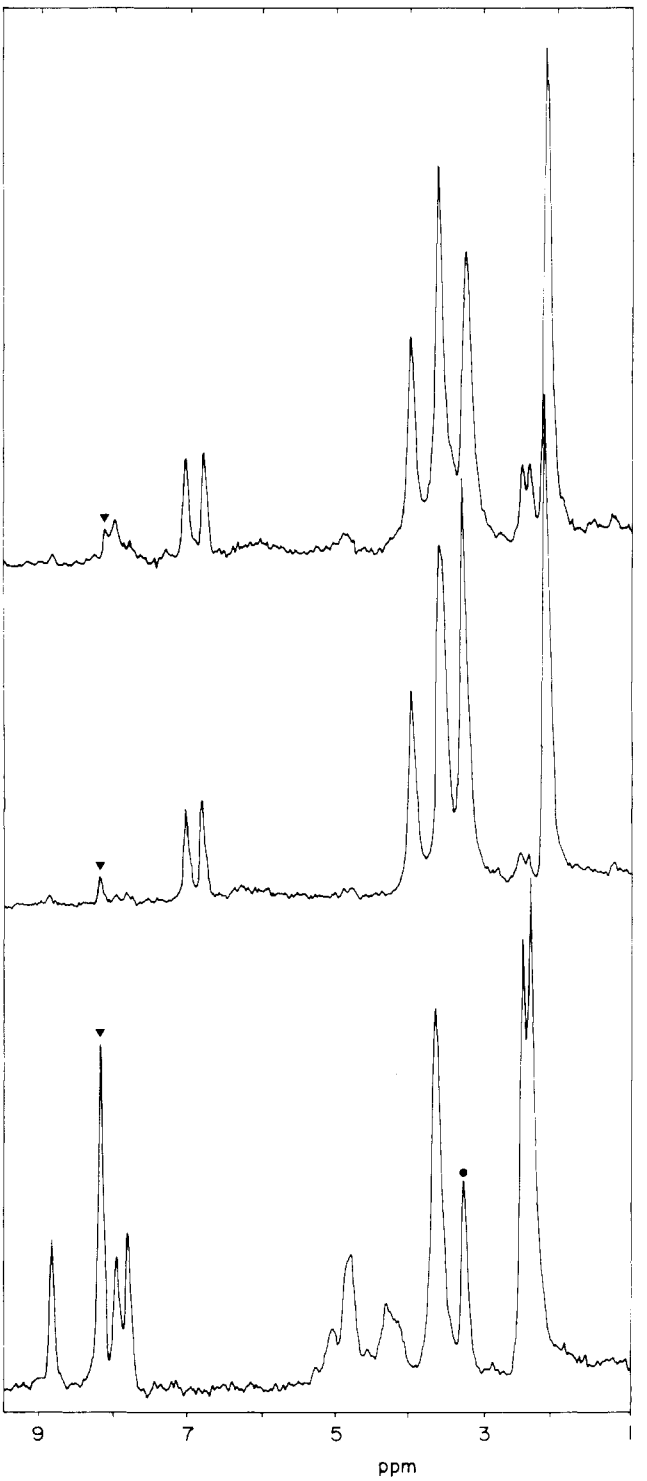
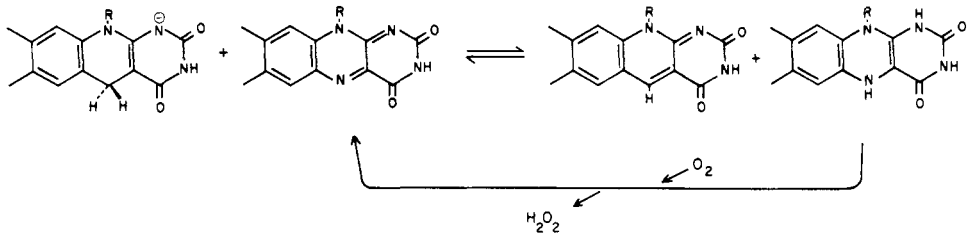


FIGURE 4: The proton magnetic resonance spectra of dihydrodeazariboflavin and deazariboflavin. (Upper) Dihydrodeazariboflavin, prepared with sodium borodeuteride; (center) dihydrodeazariboflavin, prepared with sodium borohydride; (lower) deazariboflavin. (▼) Chloroform peak; (●) the water peak. Refer to comments in the text with regard to the intensity of the water peak in the dihydrodeazariboflavin samples.

However, we have observed that the extent of reoxidation by oxygen and the rate at which it proceeds are dependent on the methods used to reduce them. The 3 h half-time quoted by Edmondson et al. (1972) was for reoxidation of

Scheme I



deazaFMNH_2 prepared by dithionite titration. We find that dihydrodeazariboflavin prepared by hydrogen/platinum has a similar half-time (3–5 h) at 25 °C. On the other hand, 1,5-deazaRFH_2 generated either from sodium borohydride or by enzymatic reduction (NAD(P)H:flavin oxidoreductase) shows markedly slower kinetics, with observed half-times ranging between 30 and 40 h at 25 °C for several different samples. These preparations have additional disadvantages; deazariboflavin forms a sulfite adduct at basic pH (Brüstlein and Bruice, 1972) while prolonged catalytic hydrogenation results in further reduction of the dihydrodeazaflavin. Photoreduction by light and EDTA is completely unacceptable in that it yields a product, which though bleached in the 396-nm region, is no longer oxidizable by riboflavin (vide infra) or by the oxidoreductase and NAD^+.[3]

A qualitative study of the pH dependence of oxygen mediated reoxidation of dihydrodeazariboflavin indicates that the dihydrodeazaflavin N-1 anion ($pK_a \sim 7.2$) is the active species.[4]

For preparative purposes, degassed, slightly acidic solutions allow purification and storage (days) of reduced deazaflavins with no special difficulty. Reoxidation by oxygen exhibits a large kinetic isotope discrimination against tritium removal at C-5, indicating breakage of a C-5-^{1}H (or more correctly C-5^{3}H) bond is the slow step in reoxidation. Samples of [5-^{3}H]deazaRFH_2 release about 5% of the total tritium into water on reoxidation as opposed to the 50% expected for random removal of one of the two C-5 hydrogen species. This discrimination aids in the preparation of oxidized [5-^{3}H]deazariboflavin with high specific radioactivity.

The extremely slow rates of oxygen oxidation of dihydrodeazaflavins offer virtue and vice to enzymatic studies. While enzymatically reduced samples are isolable in the reduced form for characterization and further use, as we note in the accompanying paper (Fisher et al., 1976), the reduced deazaflavin coenzymes are not catalytically reoxidized by flavoenzyme oxidases. Reaction is blocked after stoichiometric reduction.

Reoxidation of 1,5-Dihydrodeazaflavins by Riboflavin and Other Oxidants. The kinetic sluggishness of dihydrodeazaflavin with molecular oxygen appears to be more the exception than the rule when other reoxidants are examined. Our attention has focused mainly on riboflavin catalysis of dihydrodeazaflavin oxidation.[3] Only catalytic amounts of the isoalloxazine are required because, unlike the deazaisoalloxazine molecules, dihydroriboflavin is rapidly oxidized by molecular oxygen and this regenerates oxidized riboflavin for another cycle. The presence of oxygen in air-equilibrated buffers drives the equilibrium toward oxidized deazariboflavin formation. In the absence of oxygen the action of riboflavin is stoichiometric, no longer catalytic. Under anaerobic conditions with equimolar riboflavin and dihydrodeazariboflavin, the reaction proceeds to essentially quantitative reduction of the riboflavin and oxidation of the deaza analogue. This is consistent with the more negative potential of the deazaisoalloxazine, −0.38 V by electrochemical measurements (Blankenhorn, 1975) and −0.31 V by enzymatic equilibrium (Fisher et al., 1976). Because the oxygen-mediated oxidation of reduced riboflavin is so rapid, in oxygenated buffers, the reaction between deazaRFH^- and RF exhibits pseudo-first-order kinetics. A plot of the observed rate constants against riboflavin concentration yields a bimolecular rate constant of 7.7×10^2 M^{-1} s^{-1} at 30 °C (pH 8.6).

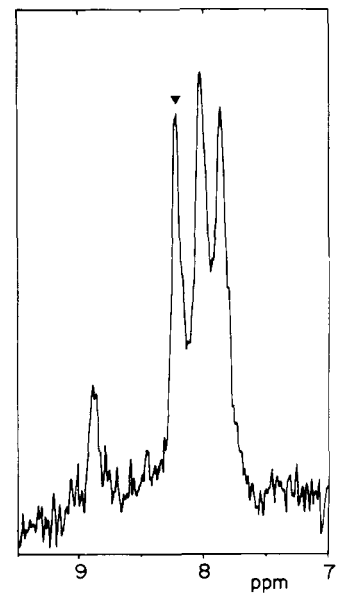


FIGURE 5: The proton magnetic resonance spectrum of the deshielded protons of [5-^{2}H]deazariboflavin, obtained from riboflavin oxidation of sodium borodeuteride prepared [5-^{2}H]deazaRFH_2. The C-5 hydrogen (δ 8.83) is greatly reduced in comparison to the one hydrogen signals of the C-7 and C-9 (δ 7.80 and 7.95) aromatic hydrogens. (▼) Chloroform peak.

The pH–rate dependence of this reaction unambiguously indicates the dihydrodeazariboflavin anion as the reactive species, with the rate increasing through the pK_a to a plateau in the pH region 8.0–9.5. A similar situation is seen for dihydroisoalloxazines, where it is the dihydro anion also that is nonenzymatically oxidized by ethyl pyruvate (Shinkai and Bruice, 1973). The rate decrease at higher pH (>9.5) is most probably the result of increasing formation of the riboflavin anion (N-3) (pK_a = 9.9) which has been demonstrated to be inactive for the dehydrogenation of *N*-propyldihydronicotinamide (Suelter and Metzler, 1960).

[3] Similar observations have been made concurrently: V. Massey, H. Fenner, M. Duchstein, and P. Hemmerich, unpublished observations. These workers have identified the product of the light/EDTA photoreduction of deazaflavins as a C-5 adduct with EDTA.

[4] Exposure of dihydrodeazaflavins to light (Edmondson et al., 1972) accelerates autoxidation markedly ($t_{1/2}$ = 6 h, borohydride prepared deazaRFH^- under ambient fluorescent lighting).

Table I: Kinetics of Isoalloxazine-Catalyzed Oxidation of Dihydrodeazariboflavin.[a]

Oxidant	Rate Constant (M^{-1} s^{-1})
Riboflavin	770
FMN	560
FAD	360

[a] Assayed at 30 °C in 0.10 M sodium pyrophosphate (pH 8.65).

Table II: Kinetic Deuterium Isotope Effects of Isoalloxazine-Catalyzed Oxidation of Deuterated Dihydrodeazariboflavin.[a]

Oxidant	k_H/k_D DeazaRF-5-d_1[b]	DeazaRF-5-5-d_2[c]
Riboflavin	2.0 ± 0.1	6.7 ± 0.2
FMN	2.0 ± 0.1	8.9 ± 0.2
FAD	1.9 ± 0.1	8.0 ± 0.2

[a] Assayed at 30 °C in 0.10 M sodium pyrophosphate (pH 8.65). [b] Obtained by sodium borohydride reduction of [5-^{2}H]deazaRF, or sodium borodeuteride reduction of deazaRF. [c] Obtained by sodium borodeuteride reduction of [5-^{2}H]deazaRF.

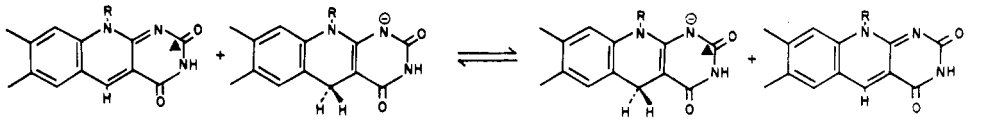
This reaction also allows us to establish further support for C-5 as the locus for hydrogen transfer in both chemical and enzyme catalyzed redox processes by use of deuterium kinetic isotope effects. Monodeuteriodihydrodeazariboflavin was synthesized by four methods: (1) nonenzymatic sodium borohydride reduction of [5-^{2}H]deazaRF; (2) sodium borodeuteride reduction of deazaRF; (3) enzymatically by oxidoreductase catalyzed 4(R)-[^{2}H]NADH reduction of deazaRF; and (4) NADH reduction of [5-^{2}H]deazaRF. At pH 8.6 with the same initial riboflavin concentration, each sample exhibited the identical kinetic isotope effect of 2.0 ± 0.1, indicating partially rate-limiting deuterium transfer from C-5 to riboflavin. That the same isotope effect is observed with the borohydride samples may be taken as evidence that reduction proceeds randomly to either deazaRF face. Since the enzymatic reduction proceeds far slower than nonenzymatic deazaflavin disproportionation (vide infra), that these isotope effects are equivalent is also consistent with this disproportionation process being random, occurring to distribute deuterium equally to each prochiral hydrogen position at the deazaRFH_2 C-5 position. As has been indicated above, the product kinetic isotope effect for riboflavin oxidation exceeds the kinetic isotope effect. This phenomenon has previously been observed for dihydronicotinamide model system studies (Steffens and Chipman, 1971; Creighton et al., 1973) and has been interpreted as requiring the partially rate-limiting formation of an intermediate prior to hydrogen transfer (Bruice et al., 1971; Main et al., 1972). Elimination of the option of protio transfer (by complete deuterium substitution at C-5 of deazaRFH_2) results in the manifestation of a near full kinetic isotope effect of 6.6 ± 0.1. The rate constant for deazaRFH^- oxidation, in the presence of an equimolar quantity of deazaRF, by a catalytic amount of riboflavin is the same as in the absence of the deazaRF. Thus, at 0.1 mM concentration any deazaRF–deazaRFH^- complex (a presumed intermediate in the disproportionation reaction) cannot be of sufficient stability as to effectively reduce the deazaRFH^- concentration for the redox reaction. Substitution of FMN or FAD for riboflavin decreases the rate slightly but causes little change on the isotope effects (Tables I and II). The sterically less hindered 10-methyl-1,5-dihydrodeazaisoalloxazine while exhibiting the same kinetic isotope effect (2.0) for oxidation of its monodeuterio species actually reacts overall some threefold slower than deazaRFH_2. This observation serves to emphasize the fact that little substantive information is known as to what factors accelerate, or deaccelerate, electron transfer in these intermediate complexes.

We have further examined the ability of several electron acceptors commonly used with flavoprotein dehydrogenases, to similarly oxidize deazaRFH^-. Thus, deazaRFH^- is oxidized by catalytic amounts of phenazine methosulfate (k = 5.7 (10^3) M^{-1} s^{-1}, 30 °C) and methylene blue (k = 5 (10^2) M^{-1} s^{-1}) in oxygenated buffer. The deazaRFH^- is rapidly oxidized stoichiometrically by dichlorophenolindophenol and ferricyanide (Fenner and Rössler, 1976) but only very slowly by nitro blue tetrazolium. On this basis, one might well expect that deazaflavin reconstituted flavoprotein dehydrogenases might be able to function catalytically as opposed to the reconstituted flavoprotein oxidases, which function only stoichiometrically with deazaflavins.

Disproportionation between Oxidized and Reduced Deazaflavins. The use of deazaflavin reconstituted enzymes has allowed the explicit demonstration of direct hydrogen transfer from substrate to the deazaflavin during enzymatic catalysis (Fisher and Walsh, 1974; Jorns and Hersh, 1975; Averill et al., 1975). Since the transferred hydrogen is incorporated at carbon 5 rather than nitrogen 5 of the normal coenzyme, exchange with solvent is prohibited and the hydrogen is retained on the deazaflavin through succeeding purification steps. Because this C-5 position is prochiral (in the same sense as the C-4 position of pyridine nucleotides), it was our hope that one could similarly establish for flavoenzymes, stereospecificity with respect to the flavin (deazaflavin) coenzyme. Thus, release of the [5-^{3}H]dihydrodeazaflavin from the active site of a given enzyme following tritium transfer from labeled substrate should yield the labeled deazaflavin, whose resulting chirality at C-5 would be dependent only on the inherent stereospecificity of the initial enzyme. Facilitating our efforts in this area was the availability of what might be the ideal assay system in the *B. harveyi* oxidoreductase (Fisher and Walsh, 1974). This enzyme is specific for 4(R)-NADH and since the reaction it catalyzes is reversible this enzyme will catalyze the oxidation of the tritiated dihydrodeazaflavin from the first enzyme by NAD^+. Tritium should either be retained in oxidized deazariboflavin or transferred to the 4(R) position of NADH. Thus, a direct correlation would then exist between the deazaflavin stereospecificity and the well-established pyridine nucleotide stereospecificity.

Unfortunately, experimental demonstration of this correlation proved to be far from straightforward. All our attempts to determine the stereospecificity of the dihydrodeazaflavin from reconstituted glucose oxidase and D-amino acid oxidase gave incomplete tritium transfer, with predominant retention of tritium in the oxidized deazaflavin and the remaining fraction reproducibly appearing in the NADH. Furthermore, [5-^{3}H]deazaRF prepared by oxidoreductase catalyzed reduction with 4(R)-[^{3}H]NADH when purified and reincubated with oxidoreductase and NAD^+ yielded identical results: partial transfer and partial retention. In searching for an explanation, we discovered that our failure to observe complete retention or transfer of tritium using the oxidoreductase was the result of a rapid intermolecular disproportionation (Scheme II). Disproportionation

Scheme II



involves no net oxidation or reduction of pairs of molecules, but the oxidized partner is reduced while the reduced partner is oxidized.

Since the oxidoreductase turns over deazaflavins catalytically, during oxidation of a chiral dihydrodeazaflavin by the enzyme, both the oxidized and reduced deazaflavins must necessarily coexist in solution. If chemical disproportionation were to occur then loss of stereochemical integrity at carbon 5 would ensue. To test for this reaction, we synthesized [2-^{14}C]deazariboflavin starting with [^{14}C]urea. As Scheme II indicates, if the ^{14}C species is initially oxidized, then disproportionation will generate radioactivity in the dihydro molecules. We have noted above that the dihydrodeazariboflavin is anionic above pH 7.2 while oxidized molecules are uncharged so the two are easily separable by DEAE-cellulose column chromatography. Part A of Figure 6 shows that disproportionation is rapid indeed at 0 °C. A replot of the data fits an exponential decay indicating a half-time of 6.1 min at 0 °C for equilibrium of the ^{14}C label. Oxidized and reduced forms are present (initially and at disproportionation equilibrium) at 84 μM concentrations. Part B of this figure is a log replot of data that indicates tritium transfer from a similar experiment where the oxidized deazaRF contained ^{14}C and the equimolar deazaRFH$^-$ contained ^{3}H equally at the *R* and *S* positions of carbon 5 (prepared from borotritiide). Now separation of oxidized and reduced forms and monitoring the ^{14}C/^{3}H ratio indicate that tritium equilibrates from reduced to oxidized deazaflavin with a half-time of 15 min. The slower rate of tritium transfer relative to ^{14}C indicates C–^{3}H breakage is a partially rate-determining step in the redox process.

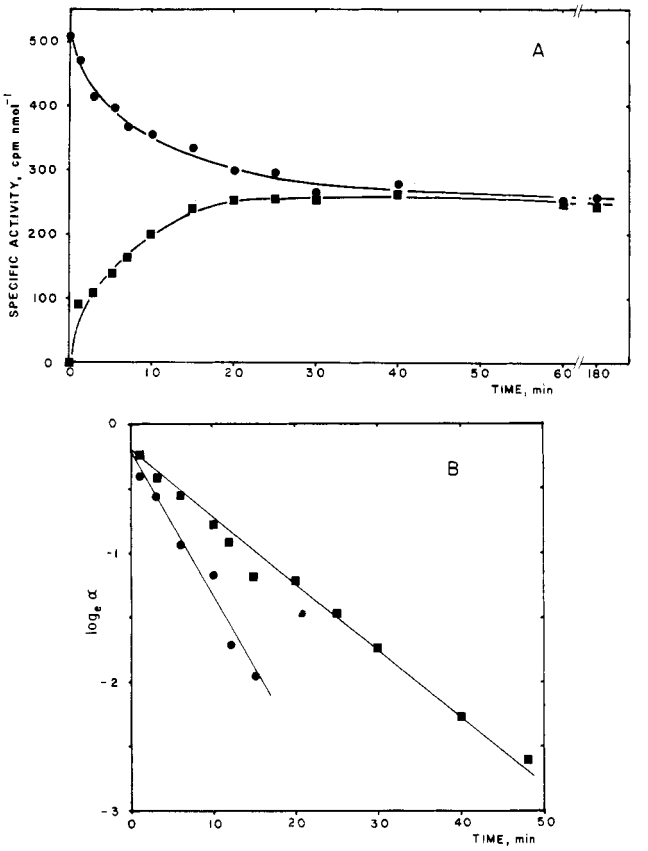


FIGURE 6: Disproportionation between the oxidized and reduced deazariboflavins. At time zero were mixed (A) [2-^{14}C]deazariboflavin (805 nmol, 506 cpm nmol^{-1}) and nonradioactive dihydrodeazariboflavin anion (878 nmol), each in 10 ml of 50 mM glycine buffer (pH 9.0) at 0 °C. At time points, 1-ml aliquots were withdrawn and forced through 0.7 × 1.5 cm DEAE-cellulose columns, and washed with 2 ml of water under syringe pressure. The specific radioactivity of the deazariboflavin was determined, and the column bound dihydrodeazariboflavin oxidized with trace riboflavin. This was then eluted with water and its specific activity determined. (●) Specific activity of deazariboflavin, and (■) of dihydrodeazariboflavin. (B) [2-^{14}C]Deazariboflavin (848 nmol, 506 cpm nmol^{-1}) and [5-^{3}H]dihydrodeazariboflavin anion (848 nmol, 23 600 cpm nmol^{-1}) assayed as above. Data are plotted logarithmically; (●) ^{14}C in deazariboflavin, $\alpha = (S/S_e) - 1$, where S = specific activity, S_e = equilibrium specific activity. (■) ^{3}H in dihydrodeazariboflavin, $\alpha = 1 - (S/S_e)$. Using as model the equation O* + R ⇄ O + R*, where O*, R*, O, and R represent ^{14}C-labeled oxidized and reduced, and nonradioactive oxidized and reduced species, respectively, the data fit the derived equation $k_{obsd} = k$(O + R + O* + R*) to yield a bimolecular rate constant of k = 22 M^{-1} s^{-1} at 0 °C.

Because of the selection against tritium, even the initial rate of tritium transfer clearly underestimates the rate at which chirality is lost from an initially chiral dihydrodeazaflavin. The initial rate of equilibration of the ^{14}C label should encompass the rate of chirality loss proceeding by complex formation, ^{3}H transfer, and complex dissociation. The rate of disproportionation itself should remain constant with time once an initial (fast) buildup of productive complex to equilibrium levels has occurred. The observed half-time for ^{14}C equilibration then must be longer than the half-time for chirality loss, since the radioactive assay does not detect disproportionation between two labeled or two unlabeled partners. Chirality will be lost whenever a hydrogen species is transferred to an oxidized deazaflavin molecule, assuming equal probability of transfer to the *si* or *re* face of the oxidized partner.

As yet we have not examined the mechanism of hydrogen transfer in this deazaflavin disproportionation. Disproportionations of isoalloxazines are known to be exceedingly rapid and low concentrations of the semiquinone are found during steady-state measurements (Swinehart, 1966). A slower rate in the deazaisoalloxazine system might be expected since carbon–hydrogen bond breakage is required rather than nitrogen–hydrogen bond breakage in the isoalloxazines. The redox process appears not to proceed by proton transfer since on equilibration of [5-^{3}H]deazaRFH$^-$ with deazaRF no tritium ends up in the aqueous solvent, leaving hydride ion and hydrogen atom transfers as candidates. The corresponding disproportionation between NAD$^+$ and NADH, also involving C–H bond breakage, proceeds much more slowly than the deazaflavin reaction (Ludowieg and Levy, 1964).

Use of equimolar concentrations of oxidized and reduced deazaflavins raises the rate of chirality loss to its maximum but it seems clear that the oxidoreductase will be a poor choice to analyze chirality at C-5 of reduced deazaflavins unless conditions supressing the disproportionation are found (such as below the pK_a of the dihydro compound). This problem is likely to be endemic only to enzymes which

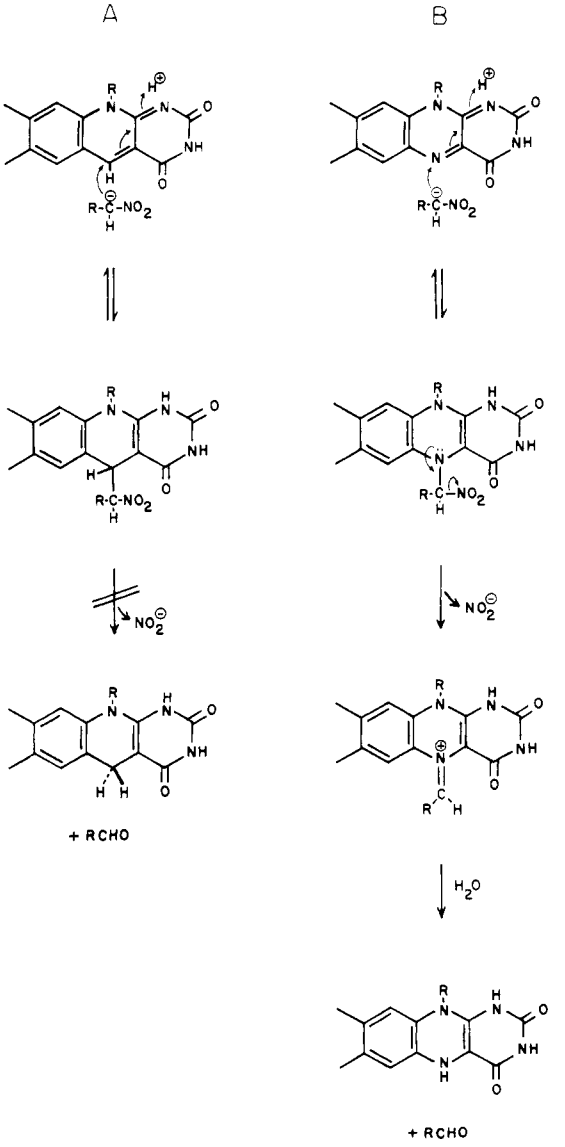
act catalytically rather than stoichiometrically on flavins and deazaflavins.

Reactivity of Deazaflavins with Nitroalkane Anions. Bright and his colleagues (Porter et al., 1973) have shown that D-amino acid oxidase (and glucose oxidase) can use nitroalkane anions as substrates, oxidizing them to a molecule of aldehyde and nitrite ion, during two-electron reduction of the flavin coenzyme which in its turn undergoes reoxidation by molecular oxygen. Of particular interest in this flavoenzyme redox catalysis is the trapping of N_5-substituted coenzyme–product adducts by added nucleophiles such as cyanide, lending credence to the idea of covalent adducts during flavoenzyme catalysis at least with these substrates. Most flavins do not undergo reduction by nitroalkane anions nonenzymatically although Yokoe and Bruice (1975) have shown that a particularly electron deficient isoalloxazine, 3,10-dimethyl-8-cyanoisoalloxazine, does undergo nonenzymatic reduction by anions of nitromethane and nitropropane.

Since the 5 position of the FAD is implicated as the site for entry of electrons during the D-amino acid oxidase reaction, we wished to examine the reaction of glucose oxidase reconstituted with deazaFAD (Fisher et al., 1976) with nitroalkanes. Although we find evidence of reaction with deazaFAD glucose oxidase (decrease in 396-nm absorption) controls show that the free deazaFAD also reacts rapidly with nitroethane anion and this chemical reaction has been examined in solution.

Addition of the performed nitroalkane anions to deazariboflavin at neutral pH apparently gave no reaction as evidenced by no decrease in the 396-nm absorption. However, lowering the pH to below 5 resulted in the rapid but incomplete bleaching in this band followed by a slower return of this absorbance. This process could be repeated several times although with a progressively decreasing amount of initial 396-nm absorption loss concomitant with an increasing rate of 396 nm return. Several pieces of experimental evidence suggest that this phenomenon is the result of covalent addition of the nitroalkane anion to the oxidized deazaflavin to yield an acid-stabilized 5-nitroethyldihydrodeazaflavin adduct, which then decomposes at a slower, pH-dependent rate back to nitroethane and deazaflavin (Scheme III). We may eliminate the possibility of a redox process operating to produce free 1,5-dihydrodeazaRF which then reoxidizes, since 1,5-dihydrodeazariboflavin is completely stable to oxygen oxidation on the time scale of these experiments and similar kinetics were seen anaerobically. Furthermore, addition of nitroethane anion to an acidic solution of 1,5-dihydrodeazariboflavin did not result in oxidation to deazariboflavin. The spectrum obtained following the 396 nm loss and return is identical with the starting spectrum, and deazaflavin may be recovered quantitatively from the reaction mixture by P2 chromatography. However, the difference spectrum obtained at the point of minimum 396-nm absorbance following nitroethane anion addition showed formation of a new absorbance peak centered at 311 nm, ϵ approximately 8000–10 000 M^{-1} cm^{-1}, distinct from the λ_{max} of 1,5-dihydrodeazariboflavin (λ_{max} 320 nm [ϵ 12 000 M^{-1} cm^{-1}]) but very similar to the reported maximum for the 5-sulfitodihydrodeazaisoalloxazine (10% CH_3CN, λ_{max} 307 nm (ϵ 12 500 M^{-1} cm^{-1}]). That the anion of nitroethane is directly responsible (i.e., the nucleophile) for formation of this adduct is strongly suggested by the observation that preincubation of the anion in the acetate buffer for several minutes followed by initiation with the deazariboflavin gave much lower adduct yields. Adjusting the pH upward by addition of base at the point of maximum adduct formation greatly increased the rate of decomposition. The faster rates of decomposition observed with successive pulsing of the deazariboflavin with the nitroalkane anion thus correspond to the instantaneous pH adjustment upward with each addition. The partial loss of the 396-nm absorbance is the result of competition between the deazaflavin and the buffer for the nitroalkane anion; once this is quenched no more adduct is formed and the decomposition pathway predominates. Although these reactions are done at a pH well below that of the pK_a (= 7.2) for the dihydrodeazariboflavin, it is possible that the nitroalkane acts as a specific base catalyst for the adduct decomposition.

Scheme III



While the evidence for a 5-nitrodeazaflavin adduct is so far preliminary, the data point out an exclusively distinct fate for the 5-nitroethyl adduct of deazariboflavin and of riboflavin. The latter undergoes smooth reduction enzymatically and in one nonenzymatic case, presumably by using the lone electron pair on nitrogen 5 to assist expulsion of the nitro group as nitrite. The resultant imine is attacked by water to yield aldehyde and dihydroflavin. In the deaza series with carbon at position 5, there is no lone pair to facili-

tate expulsion and net reduction, and the favored mode of breakdown is simply back reaction to oxidized deazaflavin and nitroalkane anion. Whether the covalent nitroalkane deazaflavin adduct could be stabilized on a flavoenzyme long enough to inactivate it effectively is currently unknown.

The data presented in this manuscript add to evidence demonstrating the qualitative similarity between isoalloxazines and deazaisoalloxazines (Jorns and Hersh, 1975). Nevertheless, the differences that do exist allow the deazaflavins to serve as a unique probe of the flavoenzyme active site and the factors determining flavin redox catalysis. The substitution of carbon for nitrogen has allowed explicit demonstration of hydrogen transfer to and from the substrate by the deazaflavin. Our observation that dihydrodeazaflavins are oxidized by flavins suggests a lower redox potential for deazaflavins, which has been confirmed by our enzymatic studies (Fisher et al., 1976). The ability of several electron acceptors, but not oxygen, to oxidize dihydrodeazaflavins may imply the capacity of deazaflavin reconstituted dehydrogenases to turn over catalytically as opposed to the deazaflavin reconstituted oxidases. One may infer from the behavior of deazaflavins with nitroalkane anions that while deazaflavins have a higher propensity as nucleophilic acceptors relative to flavins, the adduct derived from such addition may not be nearly as stable as has previously been presumed. Certainly further nonenzymatic experiments with nucleophiles derived from flavoprotein substrates (e.g., α-hydroxy acid carbanions) are required to substantiate this statement. Finally, we would again like to emphasize the general applicability of the enzymatic syntheses of FMN and FAD. These have provided an access to a large number of isotopically labeled flavins and deazaflavins at these coenzyme levels on a scale (micromole) and specific radioactivity that would not be possible with published chemical syntheses. The techniques described here should be valuable in establishing the enzymatic competence of future flavin coenzyme analogues.

Acknowledgments

We would like to thank Mr. Richard Laura for his synthesis of deazariboflavin and Professor Vincent Massey for informing us of the several experiments alluded to in the text. Finally, special thanks to Dr. James Beevar for his suggestion to us of adsorptive chromatography as a unique means of desalting and purifying flavin compounds.

References

Averill, B., Schonbrunn, A., Abeles, R., Weinstock, L., Cheng, C., Fisher, J., Spencer, R., and Walsh, C. (1975), *J. Biol. Chem. 250,* 1603–1605.

Blankenhorn, G. (1975), *Biochemistry 14,* 3172–3176.

Bruice, T., Main, L., Smith, S., and Bruice, P. (1971), *J. Am. Chem. Soc. 93,* 7526–7528.

Brüstlein, M., and Bruice, T. (1972), *J. Am. Chem. Soc. 94,* 6548–6549.

Creighton, D., Hajdu, J., Mooser, G., and Sigman, D. (1973), *J. Am. Chem. Soc. 95,* 6855–6857.

Cromartie, T., and Walsh, C. (1976), *J. Biol. Chem.* (in press).

Edmondson, D., Barman, B., and Tollin, G. (1972), *Biochemistry 11,* 1133–1138.

Fenner, H., and Michaelis, P. (1972), *Arch. Pharm. (Weinheim, Ger.) 305,* 751–757.

Fenner, H., and Rössler, H. (1976), in Flavins and Flavoproteins, T. Singer, Ed., Amsterdam, Elsevier (in press).

Fisher, J., Spencer, R., and Walsh, C. (1976), *Biochemistry,* following paper in this issue.

Fisher, J., and Walsh, C. (1974), *J. Am. Chem. Soc. 96,* 4345–4346.

Flexser, L., and Farkas, W. (1952), *Chem. Abstr. 47,* 8781g.

Fowler, F. (1972), *J. Org. Chem. 37,* 1321–1323.

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

Haley, E., and Lambooy, J. (1954), *J. Am. Chem. Soc. 76,* 2926–2929.

Hersh, L., and Jorns, M. (1976), in Flavins and Flavoproteins; T, Singer, Ed., Amsterdam, Elsevier (in press).

Honjo, M., Imai, K., Honda, H., and Yoshioka, Y. (1971), *Chem. Abstr. 74,* 126010z.

Jorns, M., and Hersh, L. (1975), *J. Biol. Chem. 250,* 3620–3628.

Lee, J., and Murphy, C. (1975), *Biochemistry 14,* 2259–2268.

Lown, J., Akhtar, M., and McDaniel, R. (1974), *J. Org. Chem. 39,* 1998–2006.

Lowry, O., Rosebrough, N., Farr, A., and Randall, R. (1951), *J Biol. Chem. 193,* 265.

Ludowieg, J., and Levy, A. (1964), *Biochemistry 3,* 373.

Main, L., Kasperek, G., and Bruice, T. (1972), *Biochemistry 11,* 3991–4000.

Mayhew, S., van Erem, E., Strating, M., and Wassink, J. (1976), in Flavins and Flavoproteins, T. Singer, Ed., Amsterdam, Elsevier (in press).

McCormick, D. (1971), *Methods Enzymol. 18B,* 544–548.

Moffat, J., and Khorana, H. (1958), *J. Am. Chem. Soc. 80,* 3756–3761.

Nakamura, N., and Tanaka, M. (1968), *Nippon Nogei Kagaku Kaishi 42,* 281–287.

O'Brien, D., Weinstock, L., and Cheng, C. (1970), *J. Heterocycl. Chem. 7,* 99–105.

Porter, D., Voet, J., and Bright, H. (1973), *J. Biol. Chem. 248,* 4400–4416.

Rivlin, R., and Langdon, R. (1966), *Adv. Enzyme Regul. 4,* 45–58.

Shinkai, S., and Bruice, T. (1973), *J. Am. Chem. Soc. 95,* 7526–7528.

Spencer, R., Fisher, J., Laura, R., and Walsh, C. (1976), in Flavins and Flavoproteins, T. Singer, Ed., Amsterdam, Elsevier (in press).

Steffens, J., and Chipman, D. (1971), *J. Am. Chem. Soc. 93,* 6694–6696.

Suelter, C., and Metzler, D. (1960), *Biochim. Biophys. Acta 44,* 23–33.

Swinehart, J. (1966), *J. Am. Chem. Soc. 88,* 1056–1058.

Tanaka, M., and Nakamura, N. (1971), *Chem. Abstr. 74,* 110407y.

Tauscher, L., Ghisla, S., and Hemmerich, P. (1973), *Helv. Chim. Acta 56,* 630–644.

Ukita, T., and Nagasawa, K. (1959), *Chem. Pharm. Bull. 7,* 465–468.

Weber, G. (1950), *Biochem. J. 47,* 114–121.

Yokoe, I., and Bruice, T. (1975), *J. Am. Chem. Soc. 97,* 450–451.