of the analogous methanolysis product, on the absence of reactivity of the product with Ellman's reagent, and on the elucidation of the path of hydrolysis of **1** in alkaline solution.

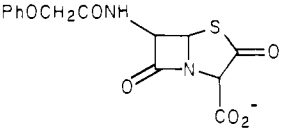
Particularly strong is the evidence from the Ellman's reagent and the spectra of the methanolysis product. Base-catalyzed methanolysis also produces two epimers, **10**, whose ^{13}C NMR spectrum shows clearly the continued existence of a thiol ester carbonyl group, and mass spectrum the absence of a β-lactam. Identification of the ultimate alkaline hydrolysis product as **12** is also important. The existence of a thiocarboxylate group in **12**, as indicated by the ^{13}C NMR spectrum and by the Ellman's reaction, demonstrates that cleavage of the thiol ester could not have occurred on conversion of **1** to **9** since **9** is shown to be an intermediate in the formation of **12**. In order to prove this point, we studied the rather complicated alkaline hydrolysis of **1** in some detail. Elucidation of the pathway was simplified considerably by the fact that intermediate **9** could be prepared in solution by hydrolysis at lower pH. The hydrolysis pathway of **1** in alkaline solution is given in Scheme III with the relevant rate and equilibrium constants.

The property of **9** whose observation originally led to most of this work, the absorption spectrum (Figure 1), was not a useful factor in the structure analysis because we were unable to find other examples in the literature of a 4-alkylidenethiazolidin-5-one ring system for comparison.

This work indicates that the 4-alkylidenethiazolidin-5-one ring is both stable and labile in aqueous solution; stable enough to not provide a concentration of the ring-opened isomer sufficient to react with Ellman's reagent at a significant rate but labile enough to allow rapid epimerization at C-5 of **9**—the half-time of epimerization can be estimated to be less than 5 min at pH 7–8.

We conclude, contrary to Bundgaard and Angelo,[7] that the initial site of nucleophilic attack of hydroxide ion and water (and phosphate and carbonate ions if they participate[7] as nucleophilic rather than general-base catalysts[37]) is at the β-lactam rather than the thiol ester carbonyl group, leading to β-lactam cleavage. The β-lactamases then do remain true to their calling.

The apparent failure of **1** to significantly inhibit β-lactamases may well be a result of the stability of the 4-alkylidenethiazolidin-5-one ring; **20**, containing a thiazolidin-5-one ring that is probably less stable to ring-opening after β-lactam hydrolysis, is reported to inhibit β-lactamases.[38]



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The effect of the structure of **1** vs. that of natural penicillins on the susceptibility of the β-lactam carbonyl group to nucleophilic attack can now be directly assessed by comparison of the second-order rate constants for hydroxide ion attack, 53 s^{-1} M^{-1} and 0.65 s^{-1} M^{-1}, respectively.[7] This considerable difference seems well in accord with the high β-lactam carbonyl stretching frequency and highly pyramidal β-lactam nitrogen atom in **1**.[6] Other nucleophiles such as amine nucleophiles may preferentially attack the thiol ester carbonyl group. It is likely that amine attack at these centers requires general-acid or general-base catalysis[35,36] and that the site of attack may vary with the mode of catalysis.[39] The thiol ester carbonyl group of the 4-alkylidenethiazolidin-5-one ring system does not seem readily susceptible to nucleophilic attack, presumably because of the conjugated double bond, such that in **9** the ring opens via an elimination pathway (**9** to **12**) rather than through thiol ester cleavage.

Acknowledgment. This work was supported by Wesleyan University and, in part, by the National Institutes of Health. We are grateful to Professor Philip Bolton for his assistance with the ^{13}C NMR spectra. The XL-200 NMR spectrometer at Wesleyan University was obtained with funds from the National Science Foundation and the Dreyfus Foundation.

Registry No. **1**, 47295-33-0; **9** (isomer 1), 84303-62-8; **9** (isomer 2), 84303-68-4; **10** (isomer 1), 84303-63-9; **10** (isomer 2), 84303-69-5; **12**, 84303-64-0; (*S*)-**13**, 84303-65-1; **16**, 84303-66-2; **19**, 84303-67-3; β-lactamase I, 9001-74-5; carbonate, 3812-32-6; hydroxide, 14280-30-9; DBU, 6674-22-2; hydroxylamine, 7803-49-8; Ellman's reagent, 69-78-3; glycinamide, 598-41-4.

(37) Bundgaard, H.; Hansen, J. *Int. J. Pharm.* **1981**, *9*, 273.

(38) Shimizu, B.; Saito, A.; Nishimura, T.; Nakahara, M. Jpn. Patent 79 103 885; *Chem. Abstr.* **1980**, *92*, 181171.

(39) Bruice, T. C.; McMahon, D. M.; *Biochemistry* **1972**, *11*, 1273.

Hydride vs. Electron Transfer in the Reduction of Flavin and Flavin Radical by 1,4-Dihydropyridines

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Abstract: Literature dealing with the existing controversy concerning the mechanism of hydride equivalent (H^- or e^-, H^+, e^-) transfer from *N*-alkyl-1,4-dihydronicotinamides is presented. Included are references to the dihydronicotinamide reduction of flavins. As a means of assessing the H^- and e^-, H^+, e^- reduction mechanism of flavins, we have studied the reaction of the *N*-alkyl[4,4-$^1H_2$]- and -[4,4-$^2H_2$]pyridines **3a–f** with the flavin **1** and flavin cation radical **2**. By electrochemical calculations, the $1e^-$ reductions of both **1** and **2** by **3a–f** are exothermic. The second-order rate constants and kinetic isotope effect for reactions of **3a–f** with **1** are reported. The disappearance of **2** in the presence of excess **3a–f** is zero order in [**2**]. Various arguments and experimental evidence have been combined to show that **2** does not react with the dihydropyridines **3a–f**. The reduction of **2** takes place by dihydronicotinamide reduction of flavin to yield dihydroflavin, which then reduces the flavin radical **2** by $1e^-$ transfer. It is concluded that since dihydronicotinamide does not reduce flavin radical cation by $1e^-$ transfer, it is unlikely to undergo stepwise $1e^-$, H^+, $1e^-$ reduction of flavin. These results are best interpreted to indicate that flavin reduction by dihydronicotinamides occurs by a hydride-transfer mechanism.

Despite the importance of the 1,4-dihydronicotinamides NADH and NADPH in biochemical redox reactions, the mechanisms for these reactions continue to be debated. It is yet to be settled as to whether the transfer of a hydride equivalent from dihydronicotinamide to substrate involves (i) transfer of a hydride ion in a single step; (ii) two-step transfer of an electron and a hydrogen atom; or (iii) overall transfer of two electrons and a proton in three separate steps. Abeles, Hutton, and Westheimer,[1] in a pioneering

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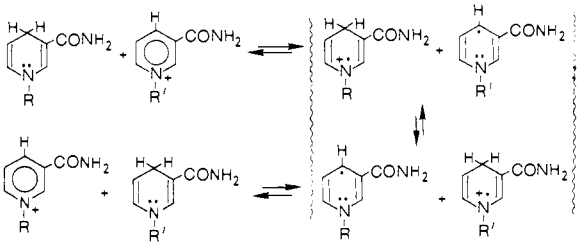
study of reduction of thiobenzophenone derivatives with *N*-benzyl-1,4-dihydronicotinamide, provided convincing evidence that radical species were not intermediates and proposed that a hydride ion was transferred directly from dihydronicotinamide to the hydride acceptor. Other studies that supported the concept of hydride transfer from dihydronicotinamide to substrate soon followed.[2]

The first strong evidence that net hydride transfer may have a multistep mechanism was provided by Steffens and Chipman.[3] Based upon discrepancies in kinetic and product isotope effects in the reduction of trifluoroacetophenone by 1,4-dihydronicotinamides, they postulated the formation of a noncovalent (charge-transfer-type) intermediate. Though the conclusions of this study have been retracted by Chipman,[4] it led to many investigations wherein kinetic and product isotope effects were compared for the reduction of various substrates by *N*-alkyl-[4,4-$^1H_2$]-, -[4-^{1}H,4-^{2}H]-, and -[4,4-$^2H_2$]dihydropyridines. A general pattern emerged in which the product isotope effect was often detectably greater than the kinetic isotope effect.[5-7]

Following the observations of Shinkai and Bruice[8] concerning metal ion catalysis of carbonyl group reduction by dihydronicotinamides, a number of investigators explored the effect of bivalent metal ions such as Mg(II) and Zn(II).[9-14] The effect has been interpreted in terms of the stability of the substrate–metal ion complex[15,16] and the assumption of an electron transfer to substrate, which is accelerated by certain metal ions. On the other hand, it has recently been suggested by van Eikeren et al.[17] that added magnesium salts serve not only to catalyze the reaction but to dry the aprotic solvents used. Traces of water were shown to yield hydrates of the 1,4-dihydronicotinamides. They demonstrated that formation of hydrates results in different kinetic and product isotope effects. Support for this comes from studies of other systems in which formation of hydrate adducts is unfavorable and for which there is not disparity between the kinetic (k_H/k_D) and product (Y_H/Y_D) isotope effects.[4,17-19] Shinkai and co-workers[20] have shown, however, that there still exists a discrepancy between the kinetic and product isotope effects for the reduction of *N*-methylacridinium ion by 3-carbamoyl-*N*-benzyl-1,4-dihydroquinoline. The latter compound cannot undergo hydration. Further support for a multistep mechanism was given by van Eikeren et al.[21] when they detected tritium isotope exchange with solvent (H_2O, pH 8.8) in the hydride equivalent transfer between *N*-benzyl-1,4-dihydronicotinamide and *N*-benzylnicotinamide cation. They interpreted their results in terms of a tight radical cation–radical pair intermediate since they were unable to detect nicotinamide dimer formation. In another study where the dihydronicotinamide and nicotinamide cation moieties are joined by a polymethylene chain, intramolecular "hydride-equivalent" transfer occurs without isotope exchange with the solvent protons.[22] Since intramolecular exchange between the 2- and 4-positions is impossible by the very nature of the geometry constraints of this system, any discrepancies between this study and the earlier work of van Eikeren et al.[21] might well be due to this complication involving the 1,2- and 1,6-dihydronicotinamide isomers.

On the basis of microscopic reversibility, any stepwise transfer of two electrons and a proton from 1,4-dihydronicotinamide to nicotinamide must be symmetrical. This requires the sequence to be e^-, H^+, e^- (eq 1). It has followed that the generally favored,



(1)

multistep mechanism is that of $1e^-$, H^+, $1e^-$. Ohno et al.[23-27] have recently (neglecting the importance of hydration) offered the following types of evidence in support of the mechanism of eq 1: (i) free energy relationships and kinetic isotope effects for the reaction of *N*-propyl-1,4-dihydronicotinamide with several aryl-substituted trifluoroacetophenones,[23] (ii) kinetic isotope effects for the reaction of *N*-aryldihydronicotinamides with *N*-methylacridinium ion,[24] (iii) optical yields for the reaction of substituted trifluoroacetophenones with a chiral NAD(P)H model,[25] (iv) spectroscopic detection of charge-transfer intermediates between 4,4-dimethyl-1,4-dihydropyridines and substituted trifluoroacetophenones,[26] and (v) variable amounts of solvent exchange in the reaction of NAD(P)H models with thiobenzophenone derivatives.[27]

Acceptance of the stepwise mechanism for hydride-equivalent transfer from *N*-alkyl-1,4-dihydronicotinamides is far from being universal. Substituent effect calculations of the charge of the migrating H atom in the reduction of isoquinolinium[28] and 3,4-dihydroisoquinolinium[29] cations by 1,4-dihydronicotinamides clearly indicate the hydridic nature of this reaction. Kreevoy and co-workers[30] support a direct hydride-transfer mechanism based on Marcus theory calculations of the relative reaction rates and equilibrium constants for hydride-equivalent reductions employing dihydropyridines and structurally related compounds.

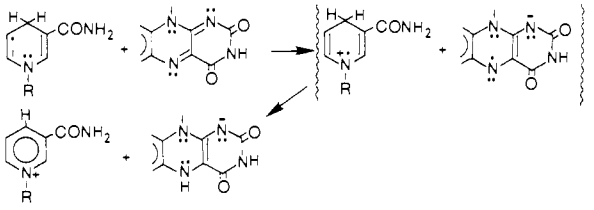
Searches for intermediates in dihydropyridine reduction reactions have also been extended to enzymatic reactions. Thus,

(1) Abeles R. H.; Hutton, R. F.; Westheimer, F. H. *J. Am. Chem. Soc.* **1957**, *79*, 712.
(2) See, for instance: (a) Norcross, B. E.; Klinedinst, P. E., Jr.; Westheimer, F. H. *J. Am. Chem. Soc.* **1962**, *84*, 797–802. (b) Dittmer, D. C.; Fouty, R. A. *Ibid.* **1964**, *86*, 91–95. (c) Schellenberg, K. A.; McLean, G. W. *Ibid.* **1966**, *88*, 1077–1078. (d) Schellenberg, K. A.; McLean, G. W.; Lipton, H. C.; Lietman, P. S. *Ibid.* **1967**, *89*, 1948–1950. (e) Huffman, R. W.; Bruice, T. C. *Ibid.* **1967**, *89*, 6243–6251. (f) Kurz, L. C.; Frieden, C. *Ibid.* **1975**, *97*, 677–679. (g) Kurz, L. C.; Frieden, C. *Biochemistry* **1977**, *16*, 5207–5216. (h) Kurz, L. C.; Frieden, C. *J. Am. Chem. Soc.* **1980**, *102*, 4198–4203.
(3) Steffens, J. J.; Chipman, D. M. *J. Am. Chem. Soc.* **1971**, *93*, 6694–6696.
(4) Chipman, D. M.; Yaniv, R.; van Eikeren, P. *J. Am. Chem. Soc.* **1980**, *102*, 3244–3246.
(5) Creighton, D. J.; Hajdu, J.; Mooser, G.; Sigman, D. S. *J. Am. Chem. Soc.* **1973**, *95*, 6855–6857.
(6) Ohno, A.; Ohnishi, Y. *Chem. Lett.* **1967**, 697.
(7) Ohno, A.; Kito, N. *Chem. Lett.* **1972**, 369.
(8) Shinkai, S.; Bruice, T. C. *Biochemistry* **1973**, *12*, 1750–1759.
(9) Ohno, A.; Yamamoto, H.; Okamoto, T.; Oka, S.; Ohnishi, Y. *Bull. Chem. Soc. Jpn.* **1977**, *50*, 2385–2386.
(10) Gase, R. A.; Boxhoorn, G.; Pandit, U. K. *Tetrahedron Lett.* **1976**, 2889–2892.
(11) Huges, M.; Prince, R. H. *J. Inorg. Nucl. Chem.* **1978**, *40*, 703–712.
(12) Ohno, A.; Yasui, S.; Gase, R. A.; Oka, S.; Pandit, U. K. *Bioorg. Chem.* **1980**, *9*, 199–211.
(13) Shinkai, S.; Ide, T.; Hamada, H.; Manabe, O.; Kunitake, T. *J. Chem. Soc., Chem. Commun.* **1977**, 848–849.
(14) Dittmer, D. C.; Lombardo, A.; Batzold, F. H.; Greene, C. S. *J. Org. Chem.* **1976**, *41*, 2976–2980.
(15) Ohno, A.; Yasui, S.; Yamamoto, H.; Oka, S.; Ohnishi, Y. *Bull. Chem. Soc. Jpn.* **1978**, *51*, 294–296.
(16) Ohno, A.; Kimura, T.; Yamamoto, H.; Kim, S. G.; Oka, S.; Ohnishi, Y. *Bull. Chem. Soc. Jpn.* **1977**, *50*, 1535.
(17) van Eikeren, P.; Grier, D. L.; Ellason, J. *J. Am. Chem. Soc.* **1979**, *101*, 7406–7409.
(18) Colter, A. K.; Saito, G.; Sharom, F. J. *Can. J. Chem.* **1977**, *55*, 2741–2751.
(19) Colter, A. K.; Saito, G.; Sharom, F. J.; Hong, A. P. *J. Am. Chem. Soc.* **1976**, *98*, 7833–7835.
(20) Shinkai, S.; Tsuno, T.; Manabe, O. *Chem. Lett.* **1981**, 1203.
(21) van Eikeren, P.; Kenney, P.; Tokmakian, R. *J. Am. Chem. Soc.* **1979**, *101*, 7402–7406.
(22) van Gerresheim, W.; Kruk, C.; Verhoeven, J. W. *Tetrahedron Lett.* **1982**, *23*, 565.
(23) Ohno, A.; Yamamoto, H.; Oka, S. *J. Am. Chem. Soc.* **1981**, *103*, 2041–2045.
(24) Ohno, A.; Shio, T.; Yamamoto, H.; Oka, S. *J. Am. Chem. Soc.* **1981**, *103*, 2045–2048.
(25) Ohno, A.; Nakai, J.; Nakamura, K.; Goto, T.; Oka, S. *Bull. Chem. Soc. Jpn.* **1981**, *54*, 3486–3488.
(26) Ohno, A.; Yamamoto, H.; Oka, S. *Bull. Chem. Soc. Jpn.* **1981**, *54*, 3489–3491.
(27) Yasui, S.; Nakamura, K.; Ohno, A.; Oka, S. *Bull. Chem. Soc. Jpn.* **1982**, *55*, 196–199.
(28) Bunting, J. W.; Chew, V. S. F.; Chu, G. *J. Org. Chem.* in press.
(29) Bunting, J. W.; Chew, V. S. F.; Chu, G. *J. Org. Chem.* in press.
(30) Roberts, R. M. G.; Stovic, D. O.; Kreevoy, M. M. *J. Am. Chem. Soc.* in press.

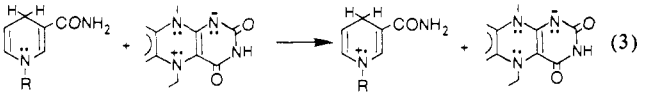
α-(hydroxyalkyl)cyclopropanes, when oxidized by horse liver alcohol dehydrogenase, do not undergo cleavage of the cyclopropane ring, implying that a radical intermediate is unlikely.[31]

The reduction of enzyme-bound flavin cofactors by reduced pyridine nucleotide analogues is an important reaction in biochemistry.[32] Gaining additional information on the mechanism of this reaction is the basic tenet of the present study. Suelter and Metzler studied the nonenzymatic reduction of several flavin analogues by 1-propyl-1,4-dihydronicotinamide and concluded that hydride transfer was occurring.[33] Their deduction was based on the observations that (i) electron-withdrawing substituents on the nicotinamide decreased the oxidation rate; (ii) the [4,4-$^2H_2$]dihydronicotinamide reacted 3.2 times slower than its protio analogue; and (iii) the oxidation rate increased with increasing solvent polarity. The reaction mechanism for net hydride transfer from dihydronicotinamides to flavin was later formulated to involve preequilibrium complex formation along the reaction path.[34] This was based on the lack of correlation of log k_{rate} for the redox reaction with $E_{1/2}$ potentials for a series of flavins and the good correlation between log k_{rate} with log K_e for complex formation of the flavins with tryptophan. Verification of the formation of flavin–dihydropyridine complexes was obtained by the direct observation of their long-wavelength absorption.[35-37] Although spectral observation of a complex does not preclude its existence on the reaction path, the correlation of rates and complex formation does. Reaction of several 1-substituted 1,4-dihydronicotinamides with flavin is characterized by a ρ^* of −1.91, which is in accord with the direct hydride-transfer mechanism.[38] Srinivasan et al.[39] provided further indirect evidence for direct H^- transfer by the good correlation of the rates of reduction of Δ^1-pyrrolidine-2-carboxylic acid (which is thought to react by a direct hydride-transfer mechanism) with dihydropyridines and the reduction of riboflavins by the same dihydropyridine reductants.

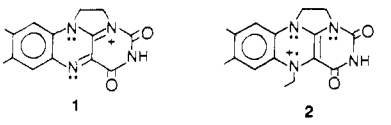
If the "hydride-equivalent" reduction of flavin by dihydronicotinamide, to yield 1,5-dihydroflavin, were to involve an initial one-electron transfer from dihydronicotinamide to flavin (eq 2),


(2)

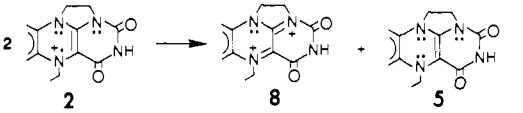
then it might be expected that dihydronicotinamides would reduce flavin radical to 1,5-dihydroflavin by one-electron transfer (eq 3) providing that the electron-transfer mechanism was thermo-


(3)

dynamically favored. In a previous investigation we examined the kinetics of reduction of N^5-ethyllumiflavinyl radical (FlEt·) by *N*-benzyl-1,4-dihydronicotinamide.[40] Though probably valid conclusions were drawn concerning the inability of 1,4-dihydronicotinamide to undergo one-electron oxidation by flavin radical, the overall kinetics of the reaction were complex due to the (endergonic) disproportionation of FlEt·, which provides traces (10^{-10} M) of oxidized flavin. In this manuscript, we describe our investigation of the *N*-benzyldihydronicotinamide reduction of 1,10-ethano-5-ethyllumiflavinylium radical cation (**2**), as well as


1 2

1,10-ethanolumiflavinium cation (**1**). The disproportionation of **2** (eq 4) is far less favorable than disproportionation of FlEt·


(4)

because of the dication nature of the oxidized species. Further, the reaction of **2** with dihydronicotinamide is a thermodynamically favored process.

Experimental Section

Materials. Acetonitrile was obtained from Burdick and Jackson Laboratories and was thoroughly deoxygenated before use. The *tert*-butyl alcohol was refluxed over CaH_2 for 24 h, distilled under nitrogen, and subjected to several freeze–thaw cycles. 1,10-Ethanolumiflavinium perchlorate (**1**) and 1,10-ethano-5-ethyllumiflavinylium perchlorate (**2**) were synthesized and purified in this laboratory by published procedures.[41] *N*-Benzyl-1,4-dihydronicotinamide (**3a**), 4,4-dideuterio-*N*-benzyl-1,4-dihydronicotinamide (**3b**), *N*-benzyl-3-acetyl-1,4-dihydropyridine (**3c**), *N*-benzyl-3-cyano-1,4-dihydropyridine (**3d**), 3-carbamoyl-*N*-benzyl-1,4-dihydroquinoline (**3e**), and *N*-methylacridan (**3f**) were prepared and purified as described elsewhere.[33,42,43] The isotopic purity of **3b** was assessed by mass spectral and NMR analysis and was found to be ~98.5% isotopically pure. 1,1′-Dibenzyl-1,1′,4,4′-tetrahydro-4,4-bipyridine-3,3′-dicarboxamide (**4**) was prepared by the electrochemical method of Moracci et al.[44]

Kinetic Measurements. All kinetic measurements were made at 30.0 ± 0.2 °C under strict anaerobic conditions. The slower runs were carried out in Thunberg cuvettes using a Cary Model 118C spectrophotometer. In a typical experiment, 1 mL of a solution ~10^{-4} M in **1** or **2** was placed in the upper bulb and 3 mL of a solution ~10^{-3}–10^{-2} M in **3a** to **3f** was placed in the cuvette. Prior to mixing, the cuvettes were equilibrated at 30 °C in the thermostated cell compartment of the spectrophotometer. After initiation of the reaction by mixing the contents of the cuvette, the disappearance of **1** (between 418 and 440 nm dependent upon reductant) and **2** (λ = 492 nm) was followed spectrally with time. For the more rapid reactions, a Durham Gibson Model 13001 stopped-flow spectrophotometer or an OLIS-Durham rapid scan stopped-flow spectrophotometer was used. The latter instrument was capable of scanning the UV and visible spectrum from λ = 200 to λ = 800 nm at 4-ms intervals. Stock solutions used in the stopped-flow experiments were prepared in the same glovebox that contained the stopped-flow instrument.

In all experiments, the concentrations of **3a–f** were maintained significantly higher than the concentration of **1** or **2** ($2–6 \times 10^{-5}$ M). For the reaction of **1** with **3a–f**, 5% acetonitrile/95% *tert*-butyl alcohol (v/v) (due to solubility constraints) was employed as solvent, whereas reaction of **2** with **3a–f** was carried out in neat *tert*-butyl alcohol. For the reaction of **2** with **3a** and **3b**, 5% acetonitrile/95% *tert*-butyl alcohol was also used as solvent in order that a kinetic isotope effect could be obtained in the same solvent system as that used for reaction of **1** with **3a** and **3b**. First-order plots for reaction of **1** with **3a–f** were linear for more than 5 half-lives, and rate constants were calculated from least-square fits of

(31) MacInnes, I.; Nonhebel, D. C.; Orszulik, S. T.; Suckling, C. J. *J. Chem. Soc., Chem. Commun.* **1982**, 121.
(32) See: Bruice, T. C. *Prog. Bioorg. Chem.* **1976**, *4*, 1; *Enzymes 3rd Ed.* **1975**, *12A,B*.
(33) Suetler, G. H.; Metzler, D. B. *Biochim. Biophys. Acta.* **1960**, *44*, 23–33.
(34) Bruice, T. C.; Main, L.; Smith, S.; Bruice, P. Y. *J. Am. Chem. Soc.* **1971**, *93*, 7327–7328.
(35) Dolin, M. *Arch. Biochem. Biophys.* **1956**, *60*, 499–501.
(36) Blankenhorn, G. *Eur. J. Biochem.* **1975**, *50*, 351–356.
(37) Porter, D. J.; Bright, H. J. *J. Biol. Chem.* **1980**, *255*, 7362–7370.
(38) Stewart, R.; Norris, D. J. *J. Chem. Soc., Perkin Trans. 2* **1978**, 246–249.
(39) Srinivasan, R.; Medary, R. T.; Fisher, H. F.; Norris, D. J.; Stewart, R. *J. Am. Chem. Soc.* **1982**, *104*, 807–812. See also: Brown, A.; Fisher, H. F. *Ibid.* **1976**, *98*, 5682–5688.
(40) Powell, M. F.; Wong, W. H.; Bruice, T. C. *Proc. Natl. Acad. Sci. U.S.A.* **1982**, *79*, 4604–4608.
(41) Eberlein, G.; Bruice, T. C. *J. Am. Chem. Soc.* **1982**, *104*, 1449–1452.
(42) Anderson, A. G., Jr.; Berkelhammer, G. *J. Am. Chem. Soc.* **1958**, *80*, 992–999.
(43) Shinkai, S.; Hamada, H.; Kusano, Y.; Manabe, O. *J. Chem. Soc., Perkin Trans. 2* **1979**, 699–702.
(44) Moracci, F. M.; Liberatore, F.; Carelli, V.; Arnone, A.; Carelli, I.; Cardinali, M. E. *J. Org. Chem.* **1978**, *43*, 3420–3422.
(45) The redox potentials used herein are literature values determined in aqueous solution at pH 7. They may be considered good approximations for the actual potentials in *t*-BuOH solution.

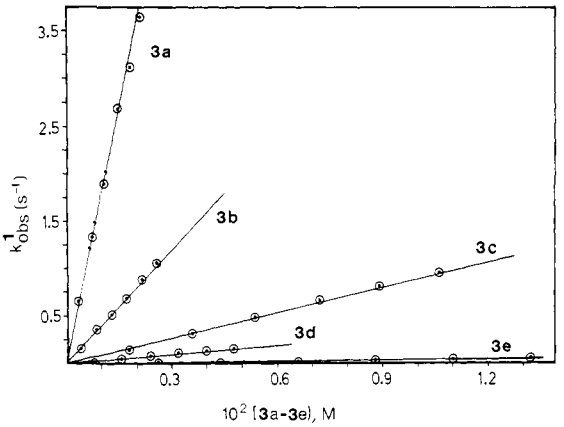


Figure 1. Observed pseudo-first-order rate constants vs. the concentration of **3a–e** for reaction of **1** in 5% acetonitrile/95% *t*-BuOH (v/v) at 30 °C. The data points for reaction of **1** and **3f** have been omitted for clarity.

Table I. Rate Constants for Reaction of 1 (M^{-1} s^{-1}) and 2 (s^{-1}) with 3a–f at 30 °C

	1, M^{-1} s^{-1} [a]	2, s^{-1} [b]
3a	1689 ± 32	2.30 (± 0.20) × 10^{-3}
3b	416 ± 3	5.88 (± 0.08) × 10^{-4}
3c	92 ± 1	2.09 (± 0.14) × 10^{-4}
3d	6.34 ± 0.09	1.67 (± 0.07) × 10^{-5}
3e	39.7 ± 0.7	9.60 (± 0.70) × 10^{-5}
3f	8.40 ± 0.1	1.94 (± 0.19) × 10^{-5}

[a] 5% Acetonitrile/95% *tert*-butanol solution. [b] *t*-BuOH solution, [2] = 3 × 10^{-5} M.

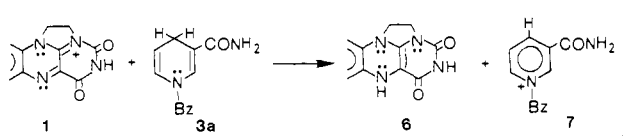
these plots. Zero-order rate constants obtained from the reaction of **2** with **3a–f** were expressed as d[**2**]/d*t*, whereby the change in concentration of **2** was obtained from either the known concentration of **2** in the reaction solution or from Beers law and the measured absorbance at *t* = 0 (**2**, ϵ = 6800 at λ = 492 nm).

Reaction Scheme Simulations. The time-course analogue-type simulations were performed on a department-built, microprocessor-based digital computer. All further calculations were performed on a Hewlett-Packard Model 9825A desk-top calculator attached to Hewlett-Packard's Model 9864A digitizer and Model 9867A plotter.

Product Studies. A typical experiment is described. The reaction of **2** (5 × 10^{-4} M) with **3a** (0.5–5 × 10^{-3} M) in 20 mL *tert*-butyl alcohol was allowed to proceed until the red color of **2** was replaced by a yellowish tinge. A fraction of the reaction solution was extracted with H_2O/ether (or benzene), and the aqueous phase was reduced in volume by high-vacuum rotary evaporation at room temperature to afford *N*-benzylnicotinamide as assayed for by NMR and UV spectroscopies. Another fraction was washed quickly with cold, neutral buffer solution; the organic layer was reduced in volume and crude 1,5-dihydro-1,10-ethano-5-ethyllumiflavin (**5**) was identified by TLC with three different solvent systems and by UV spectroscopy. A quantitative assay for the yield of **5** was obtained by addition of oxygen to the spent kinetic solution, which had been adjusted to pH 4; by this means, **5** is reconverted to **2**, which was determined by UV spectral analysis (λ = 492 nm).

Results

Reactions of **1** with **3a–f** were followed by monitoring the decrease in absorbance of **1** at 418–440 nm using stopped-flow spectrophotometry. The spectral changes from λ = 200 to 800 nm measured by conventional spectrophotometry were consistent with the formation of 1,5-dihydro-1,10-ethanolumiflavin (**6**) and *N*-benzylnicotinamide cation (**7**) (λ_{max} = 265 nm) (eq 5). The



(5)

disappearance of **1** in the presence of a large excess of **3a–f** followed the first-order rate law. The values of the pseudo first-order rate constants (k_{obsd}^1) were found to be proportional

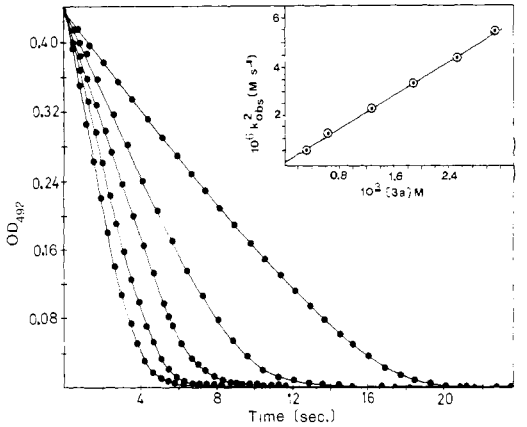


Figure 2. Time course for the reaction of **2** ([**2**] ≃ 2 × 10^{-5} M) with **3a** (0.06 –3.1 × 10^{-3}M) in *t*-BuOH at 30 °C; (inset) observed pseudo-zero-order rate constants vs. the concentration of **3a** for reaction of **2** in *t*-BuOH at 30 °C.

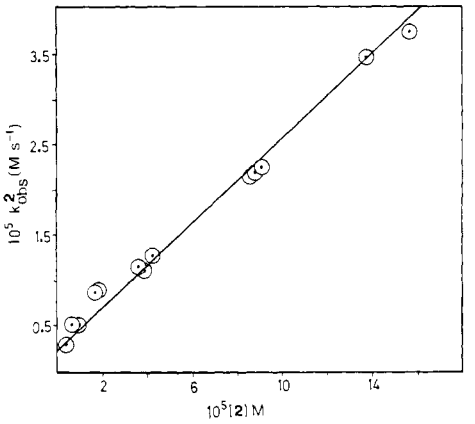
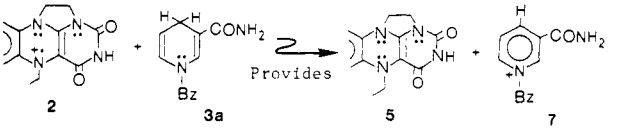


Figure 3. Dependence of observed pseudo-zero-order rate constant for reaction of **2** with **3a** ([**3a**] = 5 × 10^{-3} M) on the initial concentration of **2** used.

to the concentrations of **3a–f**, showing that the reactions are first order in both **1** and **3a–f** (Figure 1). The second-order rate constants in Table I were obtained from the least squares plots of Figure 1. The kinetic isotope effect for reactions of **1** with **3a** and **3b** was k_H/k_D = 4.06 ± 0.09.

Reaction of **2** with **3a** was followed conveniently at 492 nm by stopped-flow UV spectrophotometry; the spectral changes were consistent with the formation of **5** (λ_{max} = 245 nm) and *N*-benzylnicotinamide cation (**7**) (λ_{max} = 265 nm) (eq 6). The



(6)

products were verified by their isolation and were identified by NMR and UV spectral analysis (see Experimental Section). Shown in Figure 2 are plots of A_{492} vs. time. Examination of Figure 2 shows that the reaction is zero order in **2** for much of its time course, changing to first order in **2** near completion. The values of the apparent zero-order rate constants (k_{obsd}^2) were calculated from the slope of the zero-order plot, which may be expressed as d[**2**]/d*t*. The linear dependence of k_{obsd}^2 on [**3a**] can be seen in the inset of Figure 2. The pseudo-zero-order rate constants reported in Table I were linearly dependent on [**2**] used (Figure 3) and thus, for the plots of the time courses of Figure 2, a single concentration of flavin radical has been employed ([**2**] = 3 × 10^{-5} M). The kinetic isotope effect (k_H/k_D) for reaction of **2** with **3a** and **3b** is 3.9 ± 0.2 at 30 °C. Furthermore, log k_{rate} for reaction of **1** with **3a–f** in 5% acetonitrile/95% *tert*-butyl

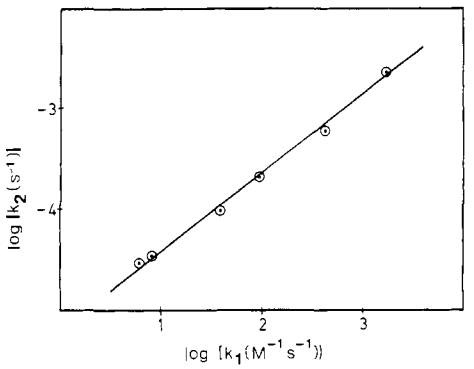


Figure 4. Correlation of the rates of reaction of **1** with **3a–f** in 5% acetonitrile/95% *t*-BuOH and the rates of reaction of **2** with **3a–f** in *t*-BuOH at 30 °C. The least-squares slope is 0.87 ± 0.03.

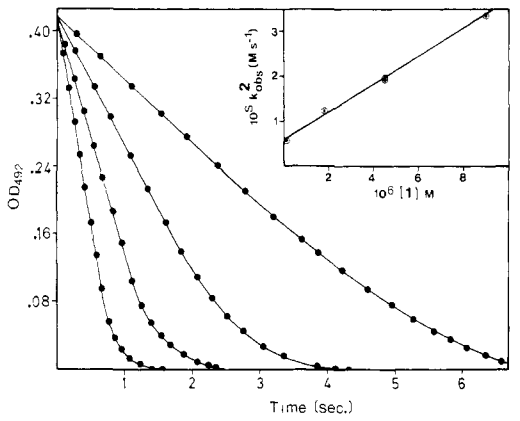


Figure 5. Dependence of observed pseudo-zero-order rate constant for the reaction of **2** ([**2**] = 3 × 10^{-5} M) and **3a** ([**3a**] = 2.4 × 10^{-3} M) on the concentration of added **1** to the reaction solution. The least-squares parameters derived from the plot of k_{obsd}^2 ($M\ s^{-1}$) vs. [**3a**] (see inset) are (3.12 ± 0.06) [**1**] + (5.17 ± 0.40) × 10^{-6}.

alcohol correlated well with the log (k_{rate}) for reaction of **2** and **3a–f** in *tert*-butyl alcohol. The least-squares slope was 0.87 ± 0.03 (Figure 4). Comment on this value is deferred to the Discussion section.

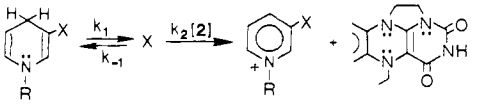
The addition of small concentrations of **1** to kinetic solutions containing initially **2** and **3a** increased the rate of decrease of **2**. The general appearance of the time dependence of [**2**] remained the same (Figure 5). Calculation of the first-order rate constants obtained from the zero-order components and plotting of these constants vs. the amount of **1** added provides a line of slope 3.12 ± 0.06 and intercept of 5.17 ± 0.40 × 10^{-6} (inset Figure 5). If it is assumed that the intercept is due to **1** as an impurity in **2**, then it is easily shown that the concentration of **1** in a 3 × 10^{-5} M stock solution of **2** must be 1.65 × 10^{-6} M, or approximately 5%. The reaction of **2** with **3a** was monitored from 220 to 800 nm by rapid-scan spectrophotometry; no evidence for an intermediate buildup was found.

Reaction of **2** with **3a** was carried out in anaerobic and oxygenated *tert*-butyl alcohol solutions. There could not be detected a change in the time course for disappearance of **2**. Similarly, addition of small concentrations of *N*-benzylnicotinamidium bromide gave no change in the observed rate of reaction. For example, reaction of **2** and **3a** (3.7 × 10^{-3} M) with variable amounts of **7** ([**7**] = 0.0–1.98 × 10^{-4} M) gave rate constants randomly scattered from 10.2 × 10^{-6} to 12.1 × 10^{-6} s^{-1}.

Discussion

The kinetics of the reduction of **1** by **3a–f** have been investigated under the pseudo-first-order conditions of [**3a–f**] ≫ [**1**] (*tert*-butyl alcohol, 30 °C, under N_2 atmosphere). The reactions are first order in both [**1**] and [**3a–f**] providing 1,5-reduced **1** (i.e., **6**) and the appropriate *N*-alkylpyridinium ion (as shown for **3a** in eq 2). The determined second-order rate constants are provided in Table I. The second-order rate constants for the reaction of [4-^{2}H,4-^{2}H] **3a** (i.e., **3b**) have also been determined, and these constants are also tabulated in Table I.

The kinetics for the reaction of **2** with **3a–f** have been studied (30 °C, anaerobic conditions, *tert*-butyl alcohol) in the presence of a 10-fold or greater excess of the dihydro compound; the disappearance of **2** is zero order in this component throughout most of the time course, changing to first order toward completion of reaction. The most simplistic kinetic scheme that can be employed to explain (quantitatively) the kinetic results is that of eq 7. Indeed the plots of Figure 2 have been constructed by the



(7)

digital fitting of the line generated from the reactions of eq 7 to the experimental points. In the instance of the reaction of **3a** with **2**, the calculatable constants are k_1 = 2.03 × 10^{-2} s^{-1} and k_2/k_{-1} = 7.86 × 10^4 M. Since the value of k_1 cannot be reasonably associated with any chemical or conformational transformation, a sensible structure for X is not at all evident. For these sound reasons, the sequence of reactions of eq 7 must be abandoned. The reaction sequences of Schemes I–V are alternative explanations of the kinetics for the reactions of **2** with **3a**. Of these schemes, I, II, and V allow accurate simulation of the time course

Scheme I

$$PyrH_2 + O_2 \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} PyrH_2^{+}\cdot + O_2^{-}\cdot$$

$$\mathbf{2} + PyrH_2^{+}\cdot \xrightarrow{k_2} \rightarrow \mathbf{5} + PyrH^+$$

Scheme II

$$PyrH_2 + PyrH^+ \rightleftarrows PyrH_2^{+}\cdot + PyrH\cdot$$

$$\mathbf{2} + PyrH_2^{+}\cdot \rightarrow \mathbf{5} + H^+ + PyrH^+$$

$$\mathbf{2} + PyrH\cdot \rightarrow \mathbf{5} + PyrH^+$$

Scheme III

$$PyrH_2 + \mathbf{2} \rightarrow \mathbf{5} + PyrH\cdot$$

$$2PyrH\cdot \rightarrow (PyrH)_2$$

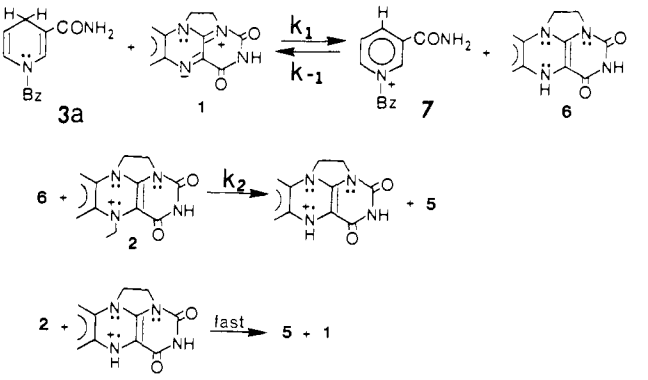
$$\mathbf{2} + {}^1/_2(PyrH)_2 \rightarrow \mathbf{5} + PyrH^+$$

Scheme IV

$$\mathbf{2} + \mathbf{2} \rightleftarrows \mathbf{5} + \mathbf{8}$$

$$PyrH_2 + {}^1/_2\mathbf{8} \rightarrow \mathbf{5} + PyrH^+$$

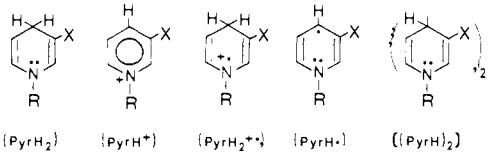
Scheme V



$k_1 = 1689\ M^{-1}\ s^{-1}$

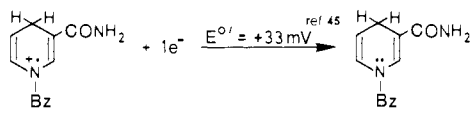
$k_2/k_{-1} = 7.8 \times 10^4$

for disappearance of **2**. Scheme V (provided with derived constants) will be shown in what follows as representing the correct mechanism. Reasons for discarding Schemes I–IV follow. The definition of the abreviations $PyrH_2$, $PyrH^+$, $PyrH_2^{+}\cdot$, $PyrH\cdot$, and $(PyrH)_2$ are as shown:



$(PyrH_2)$ $(PyrH^+)$ $(PyrH_2^{+}\cdot)$ $(PyrH\cdot)$ $((PyrH)_2)$

Reaction of O_2 with dihydronicotinamide is thermodynamically unfavorable, as calculated from the potentials of eq 8 and eq **9**,[45]



(8)

$$O_2 + 1e^- \xrightarrow{E^{\circ\prime} = -330\ mV} O_2^{-}\cdot \qquad (9)$$

by approximately 35 kJ M^{-1} but could be of kinetic importance if $PyrH_2^{+}\cdot$ (or $O_2^{-}\cdot$) were scavenged by rapid reaction of **2** as envisaged in Scheme I. A mechanism of this type requires that the observed rate be proportional to $[O_2]$, contrary to experimental observation. In control experiments in which the reaction of **2** with **3a** was carried out in the presence of variable concentrations of dissolved molecular oxygen, it was demonstrated that there was no appreciable change in observed rate from kinetics performed under anaerobic conditions, and thus Scheme I may be discarded.

Consumption of **2** in a reaction initiated by reaction between $PyrH_2$ and $PyrH^+$ (Scheme II) was ruled out by measuring the rate of reaction of **2** and **3a** with added concentrations of *N*-benzylnicotinamidium bromide (**7**). Although it has been suggested previously[21] that reaction of $PyrH_2$ and $PyrH^+$ gives radical and radical cation species, addition of **7** to reaction of **2** and **3a** did not affect the observed rates or kinetic order.

Reaction of **2** with $PyrH_2$ to yield $PyrH\cdot$ followed by rapid dimerization of the $PyrH\cdot$ radical could closely approximate the observed zero to first-order kinetics if a subsequent rapid reaction of **2** with $(PyrH)_2$ occurs (Scheme III). Although it is not immediately apparent by inspection that Scheme III is kinetically competent, we were able to show by digital simulation that such a mechanism is plausible. Further, the direct determination of the second-order rate constant for reaction of **2** with $(PyrH)_2$ = **4** demonstrates that the latter step of Scheme III is indeed rapid enough to accommodate such a mechanism ($k = 4.84 \pm 0.16 \times 10^5$ M^{-1} s^{-1}, *tert*-butyl alcohol, 30 °C).[46] However, digital simulation of the time dependence for disappearance of **2** by use of the reactions of Scheme III establishes that $(PyrH)_2$ should accumulate, as an intermediate, to ~50% of initial $[PyrH_2]$. The dimer **4** absorbs strongly at λ = 300 nm (ϵ = 4200) whereas neither the reactants nor the products do, and therefore a spectral trace of A_{300} vs. time should exhibit an increase in A_{300} (dimer formation) followed by a first-order decrease in A_{300} (reaction of **4** with **2**) if this mechanism is correct. In all experiments, however, A_{300} decreased smoothly with the same zero-order rate as that measured at 492 nm so that buildup of **4** does not occur and Scheme III is ruled out.

Disproportionation of **2** to give reduced flavin **5** and the oxidized flavin dication, 1,10-ethano-5-ethyllumiflavinium salt (**8**), deserves mention (Scheme IV). Although formation of **8** is extremely endergonic,[41] this could be compensated for by rapid reaction of **8** and $PyrH_2$. Reaction of another flavin radical and **3a** has been attributed to a mechanism of this type.[40] That this mechanism does not occur was demonstrated by the lack of dependence of the rate of reaction upon $[2]^2$.

The most reasonable explanation for the observation that the overall reductions of **2** by **3a–f** are zero order in [**2**] rests on the assumption that **2** carries **1** as an impurity. The oxidized flavin **1** undergoes two-electron ("hydride") reduction by **3a–f** to yield the 1,5-reduced flavin **6**, which then acts as a one-electron reductant for **2**. This is to say that the flavin radical, **2**, is not reducible by 1,4-dihydro-*N*-alkylnicotinamide (Scheme V). Justification for Scheme V will be considered first, prior to a consideration of the implications of this finding.

(46) Powell, M. F.; Bruice, T. C., results obtained in our laboratory.

Table II. Comparison of Observed and Calculated Rates of Reaction of 1 and 3a–f

	obsd, M^{-1} s^{-1}	calcd,[a] M^{-1} s^{-1}
3a	1689	1437
3b	416	367
3c	92	103
3d	6.3	10.4
3e	39.7	60.0
3f	8.4	12.3

[a] The calculated reaction rate of (1 + 3a-f) = rate (2 + 3a-f)(s^{-1})/1.6×10^{-6} M.

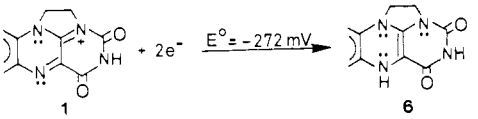
Steady-state assumption in [**6**] provides the rate expression of eq 10 for the reaction sequence of Scheme V. Since the reduction

$$\frac{-d[2]}{dt} = \frac{k_1 k_2 [PyrH_2][1][2]}{k_{-1}[PyrH^+] + k_2[2]} \qquad (10)$$

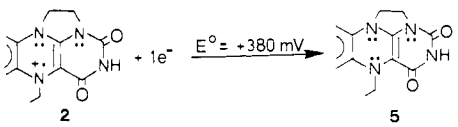
of flavins by $PyrH_2$ compounds is exergonic and kinetically facile and electron exchange between reduced flavin and flavin radical should be rapid, $k_2[2] >> k_{-1}[PyrH^+]$ and eq 10 reduces to eq 11, which is zero order in [**2**]. An estimation of the exergonicity

$$-d[2]/dt = k_1[1][PyrH_2] \qquad (11)$$

for the reaction of **6** and **2** (eq 14) may be obtained from the half-cell potentials of eq 12 and 13.[45] From the basic free-energy



(12)



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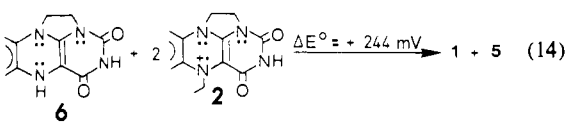
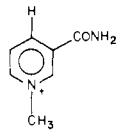
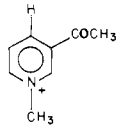
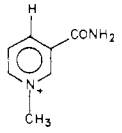


(14)

relation $\Delta G = -n\Delta EF$ (n = number of electrons transfered; F = Faraday constant) the reaction of **6** with **2** is calculated to be exergonic by 48 kJ mol^{-1}.

That oxidized flavin (**1**) catalyzes the reaction of **2** with **3a** was verified by experiments wherein **1** was added to reaction solutions containing **2** and **3a** (Figure 5). The concentration of **1** required as an impurity in **2** to provide the *observed kinetics* for disappearance of **2** in the presence of **3a** is calculated as 5% (see Results). The zero-order rate constants obtained for the disappearance of **2** in the presence of **3a**, when divided by 0.05[**2**], provide the independently determined second-order rate constant for reaction of **1** with **3a** (1689 M^{-1} s^{-1}) to within 15%. The true second-order rate constants for reaction of **1** with **3a–f** are compared to the calculated second-order rate constants employing the zero-order rates for disappearance of **1** in the presence of **3a–f** in Table II. The small discrepancy in observed and calculated values may be attributed in part to a small solvent effect. Thus, the reaction of **2** with **3a** was studied in *t*-BuOH while the reaction of **1** with **3a** was determined in 5% acetonitrile/95% *t*-BuOH (v/v). The similarity between the kinetic isotope effects for reaction of **1** with **3a** and **3b** ($k_H/k_D = 4.06 \pm 0.09$) and the like isotope effect for the zero-order disappearance of **2** in the presence

Table III. Redox Potentials for NAD^+ and Some Analogues

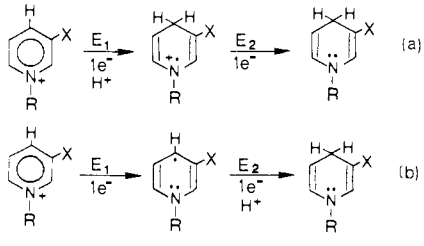
R	$E_1^{o\prime}$	$E_2^{o\prime}$	ox/red	ref
NAD^+	−850	+210	−320	*a*
NAD^+		+300		*b*
NAD^+	−730	+90	−320	*c*
	−845	+33	−406	*c*
	−640	−34	−337	*c*
	−900	+60	−420	*d*

[a] Blankenhorn, G. In "2nd Symposium Pyridine Nucleotide Dependent Dehydrogenases"; de Gruyter, W., Ed.; Verlag Tech: Berlin, 1977. [b] Farrington, J. A.; Land, E. J.; Swallow, A. J. *Biochim. Biophys. Acta.* 1980, *590*, 273. Anderson, R. F. *Biochim. Biophys. Acta.* 1980, *590*, 277. [c] Blankenhorn, G. *Eur. J. Biochem.* 1976, *67*, 67. [d] Brühlmann, U.; Haydon, E. *J. Am. Chem. Soc.* 1974, *96*, 6169.

of **3a** and **3b** ($k_H/k_D = 3.9 \pm 0.2$) strongly supports the mechanism proposed in Scheme V. A plot of the logarithms of the second-order rate constants for reaction of **1** with **3a–f** (Table I) vs. the logarithm of the zero-order rate constants for disappearance of **2** (at $[2] = 3 \times 10^{-5}$ M) in the presence of **3a–f** is shown in Figure 4. Inspection of Figure 4 shows the plot to be linear and of least-squares slope close to unity (0.9). This finding is as anticipated for Scheme V wherein the rate-determining step for the consumption of **2** is actually the reaction of **1** with **3a–f**.

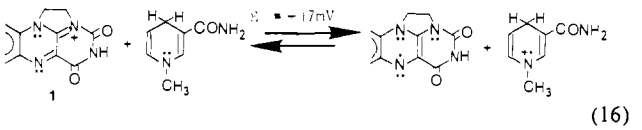
In this investigation, we set out to compare the rate constants for one-electron transfer from *N*-alkyl-1,4-dihydronicotinamides to the flavin radical **2** and hydride equivalent reduction of the flavin **1** by the same series of *N*-alkyl-1,4-dihydronicotinamides. What we have found (loc. cit.) is that the hydride reductions of **1** are very rapid and that one-electron transfer to **2** cannot be detected. Also noted is the fact that one-electron reduction of **2** by **1** is very rapid. These findings bear directly upon the question of whether *N*-alkyl-1,4-dihydronicotinamide reduction of flavins represent H^- transfer or the stoichiometrically equivalent mechanisms of $1e^- + H^+ + 1e^-$ stepwise "hydride equivalent" transfer.

In Table III are listed various reported one-electron redox potentials for nicotinamide and derivatives.[45] The potentials may apply to eq 15a or 15b. In what follows, we will presume eq 15a

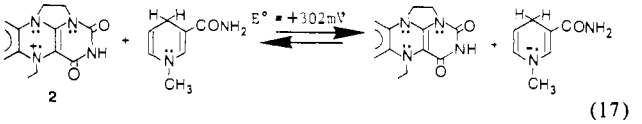


(15)

to be correct. Though the potentials listed in Table III differ from one another by 100 mV or so and are kinetic rather than thermodynamic,[47] they do indicate that the reactions of **1** and **2** (per se) with *N*-alkyl-1,4-dihydropyridines should be exergonic. Combining the half-cell potentials of eq 13 and 15 with the $1e^-$ redox potential of **1** ($E^\circ = +70$ mV)[41] provides the potentials of eq 16 and 17.



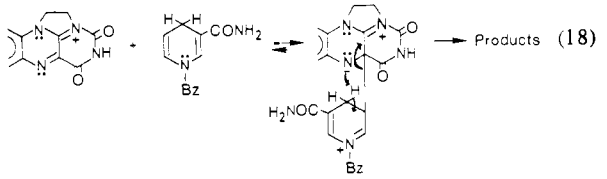
(16)



(17)

From eq 16 and 17, it is seen that one-electron transfer to **2** and to **1** is thermodynamically exergonic and that one-electron transfer to **2** is thermodynamically the much more favorable process (by 26 kJ mol^{-1}). The fact that the reduction of **2** by **3a–f** is not competitive with the reduction of **1** by **3a–f** does not support a radical mechanism for flavin reduction by dihydronicotinamides. Absence of a one-electron reaction between flavin or flavin radical and dihydronicotinamides is consistent with previous results. Thus, oxidants with similar or even slightly higher reduction potentials, such as ferric ion ($E^{\circ\prime} = +430$ mV), do not exhibit properties attributable to one-electron acceptance when reacted with dihydronicotinamides.[47] One-electron oxidants with potentials >600 mV, as spirocyclohexylporphyrexide ($E^{\circ\prime} = +690$ mV)[47] and ferricyanide ($E^{\circ\prime} = +690$ mV),[34,48] do undergo one-electron reduction by dihydronicotinamides as is shown by kinetic isotope and solvent effects. The reaction of flavin radical **2** with **3a** is thermodynamically much more favorable than is the reaction of the flavin **1** with **3a**, yet no significant reaction of **2** with **3a** occurs in the absence of **1** as a catalyst.

The mechanistic information gained from the kinetic isotope effects is 2-fold: (i) reactions of **1** and **2** with **3a–f** have similar rate-determining steps and (ii) hydrogen atom motion is, at least partially, rate determining. Two-electron transfer with rate-determining hydrogen atom motion can occur by two different mechanistic extremes, either by direct hydride transfer or by a $2e^-$ plus a proton transfer mechanism. The latter has been proposed to occur via 4a-adduct formation between flavin and dihydronicotinamide (eq 18).[49,50] Although it is possible to envisage



(18)

the proposed adduct between **1** and **3a** (eq 18), such an adduct cannot be formed in the reaction of **1** and *N*-methylacridan without loss of resonance of one of the aromatic ring systems. The rate constant for reaction of *N*-methylacridan and **1** ($k = 8.39 \pm 0.99$ M^{-1} s^{-1}) clearly shows this reaction does not have to surmount the >100 kJ mol^{-1} barrier associated with the loss of such resonance since such a barrier would result in a rate less than 10^{-9}. That the reaction of **1** and *N*-methylacridan is $\sim 10^2$ slower than reaction of **1** and **3a** can be explained solely on the basis of substituent effects. The reaction of dihydroquinoline[51] with **1** is approximately 10^2 faster ($k \simeq 7 \times 10^3$ M^{-1} s^{-1})[46] than is the reaction of **1** with **3e**. This provides an estimation of the rate retardation due to substitution of H for $CONH_2$. However, **3e** reacts approximately 10^2 slower than **3a** and *N*-methyl vs. *N*-benzyl substitution causes another factor of 2 rate retardation.[46] Combination of these "approximate" substituent effects gives a rate for reaction of **1** with *N*-methylacridan within an order of

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

(48) Okamoto, T.; Ohno, A.; Oka, S. *J. Chem. Soc., Chem. Commun.* **1977**, 784–786.

(49) Hamilton, G. A. *Prog. Bioorg. Chem.* **1971**, *1*, 83–157.

(50) Hemmerich, P.; Massey, V.; Michel, H.; Schug, C. *Struct. Bonding* **1982**, *48*.

(51) Birch, A. J.; Lehman, P. G. *J. Chem. Soc., Perkin Trans. 1* **1973**, 2754–2759.

magnitude of the experimental value; the agreement is good when considering the severe approximations made in a multiple substituent effect analysis of this type. This demonstrates that the rate of reaction of **1** and **3a** can be accounted for by the electronegativity of the substituent(s) without involving covalent adduct formation as proposed by Hemmerich[50] or Hamilton.[49] Most hydride transfers exhibit modest deuterium kinetic isotope effects in the 2-5 range, which is less than that calculated from the difference in zero-point stretching vibrations.[52,53] The isotope effect for the reaction of **1** and **3a** agrees well for a direct hydride transfer mechanism, especially when coupled with the knowledge that a $2e^-$ mechanism (without covalent adduct formation) is favored.

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Registry No. 1, 39092-09-6; **2**, 84193-79-3; **3a**, 952-92-1; **3c**, 19350-64-2; **3d**, 37589-77-8; **3e**, 17260-79-6; **3f**, 4217-54-3; **4**, 67146-57-0; deuterium, 7782-39-0.

(52) Stewart, R. *Isot. Org. Chem.* **1976**, *2*.
(53) Swain, C. G.; Wiles, R. A.; Bader, R. F. W. *J. Am. Chem. Soc.* **1961**, *83*, 1945-1950.

Nitrogen-15 Nuclear Magnetic Resonance Spectroscopy of Neomycin B and Related Aminoglycosides

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Abstract: Natural-abundance ^{15}N NMR spectra of four neomycin B derivatives and their structurally related components are reported. The data suggest that substituents at locations more remote than the γ position have little influence on the ^{15}N chemical shifts, and, therefore, a set of substituent effect parameters could be derived. Assignments for individual ^{15}N signals of the antibiotic derivatives are based on chemical shift comparisons with model aminosaccharides, where possible. Specific assignments of the N-1 and N-3 resonances are based on ^{15}N spin-relaxation experiments in which Gd[2.2.1] cryptate was employed as a spin-labeling reagent for neomycin B in aqueous and dimethyl sulfoxide solutions. For all derivatives of neomycin B studied, the ^{15}N chemical shift data suggest that the 2,6-diamino-2,6-dideoxy-L-idopyranosyl portion (ring D) adopts the $^4C_1$ conformation (**1b**). Complete ^{15}N chemical shift titration data for the antibiotic are used to compute pK_a values for the individual nitrogen functions to within ± 0.04 pK_a unit and also to determine the extent and sites of protonation in commercial neomycin sulfate preparations. In general, ^{15}N protonation shifts are found to be downfield (6.4-14.2 ppm) and have been correlated with nitrogen structural types.

Nuclear magnetic resonance (NMR) spectroscopy has enjoyed an increasingly important role in the structural elucidation of aminoglycoside antibiotics during the past two decades. Initially, ^1H NMR studies[1] at low field provided a direct method for assignment of the configuration of anomeric linkages between specific sugar residues in a number of antibiotic substances. Since the advent of pulse Fourier transform methods, ^{13}C NMR spectroscopy has been applied successfully in studies of the gross structures of many antibiotics.[2]

More recently, natural-abundance, ^{15}N NMR spectroscopy has proved to be a promising method for the structural, conformational, and quantitative analysis of a wide variety of nitrogen containing compounds,[3] including amino sugar derivatives[4-7] and the aminoglycoside antibiotics.[8,9] However, full realization of the potential of this technique must depend on the development of a wider range of spectral assignment methods and correlations of ^{15}N NMR parameters with structure and conformation than is available at the present time. The parameters of particular interest are ^{15}N chemical shifts, coupling constants, spin-lattice relaxation times (T_1), nuclear Overhauser effects (NOE), protonation shifts, substituent effects, and pK_a values. Measurement of ^{15}N relaxation times at natural abundance and on reasonably small samples has become feasible only recently, owing to the development of high-field, superconducting spectrometers having improved sensitivity.

The use of ^{15}N NMR rather than ^{13}C NMR to probe the structures of complex aminoglycosides offers advantages because of the generally simpler and more readily interpretable ^{15}N spectra, the greater sensitivity (by a factor of 2^{3b}) of ^{15}N chemical shifts to structure, and the opportunity to examine pendant amino groups directly, for which conformational effects would be expected to be maximal. However, the ^{15}N method also presents some hazards because of the possibility of nulled resonances due to a partial negative NOE, the absence of well-defined substituent and geometric shift parameters in systems as complex as aminosaccharides, and the uncertain effects of solvents on chemical shifts and of trace paramagnetic metal ions on ^{15}N relaxation phenomena. Also, in contrast to most ^{13}C-^1H coupling constants, the measurement of ^{15}N-^1H coupling constants is usually complicated by the presence of rapid chemical exchange of NH protons.

In the hope of gaining additional insight into correlation of ^{15}N NMR parameters with the molecular structure and stereochem-

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(1) (a) Rinehart, K. L., Jr.; Chilton, W. S.; Hichens, M.; von Philipsborn, W. *J. Am. Chem. Soc.* **1962**, *84*, 3216-3218. (b) McGilveray, I. J.; Rinehart, K. L. Jr.; *Ibid.* **1965**, *87*, 4003-4004.
(2) For a recent review, see: Naito, T.; Toda, S.; Nakagawa, S.; Kawaguchi, H. *ACS Symp. Ser.* **1980**, *No. 125*, 257-294.
(3) (a) Levy, G. C.; Lichter, R. L. "Nitrogen-15 Nuclear Magnetic Resonance Spectroscopy"; Wiley Interscience: New York, 1979. (b) Roberts, J. D. *Rice Univ. Stud.* **1980**, *66(3)*, 147-178.
(4) Coxon, B. *Carbohydr. Res.* **1974**, *35*, C1-C3.
(5) Botto, R. E.; Roberts, J. D. *J. Org. Chem.* **1977**, *42*, 2247-2249.
(6) Coxon, B. *Pure Appl. Chem.* **1977**, *49*, 1151-1168.
(7) (a) Coxon, B.; Hough, L. *Carbohydr. Res.* **1979**, *73*, 47-57. (b) El Khadem, H. S.; Coxon, B. *Ibid.* **1981**, *89*, 321-325.
(8) Dorman, D. E.; Paschal, J. W.; Merkel, K. E. *J. Am. Chem. Soc.* **1976**, *98*, 6885-6888.
(9) Paschal, J. W.; Dorman, D. E. *Org. Magn. Reson.* **1978**, *11*, 632-634.