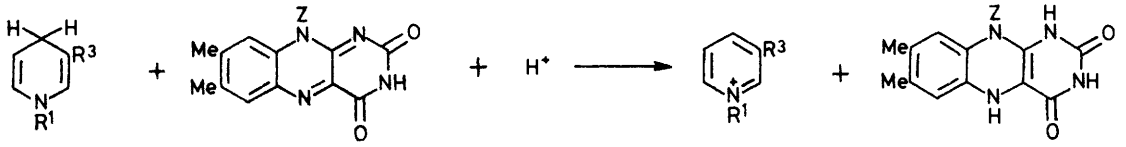
The Pyridinium–Dihydropyridine System. Part 2.[1] Substituent Effects on the Oxidation of 1,4-Dihydropyridines by Flavins

By **Ross Stewart** * and **Donald J. Norris**, Department of Chemistry, University of British Columbia, Vancouver, Canada V6T 1W5

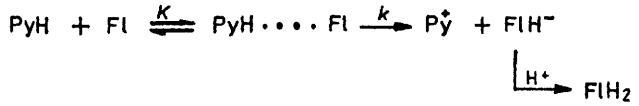
The oxidation of a series of 1,3-disubstituted 1,4-dihydropyridines by riboflavin or flavin mononucleotide has been studied in aqueous buffer at 25 °C. Substituents at the 3-position have a greater effect on the reaction rate than the same groups attached to 1-methyl. With carbamoyl at the 3-position, varying the 1-methyl substituent produces a linear relation with σ^*, except for the 1-methoxymethyl compound, which reacts more slowly than expected. The reaction of uncomplexed reactants has ρ^* −1.91, consistent with a hydride transfer path.

THE oxidation of the 1,4-dihydropyridine ring in NADH by flavin coenzymes is an important reaction in the respiratory chain.[2] The way in which the two substituents in the pyridine ring of the coenzyme, the carbamoyl group at C-3 and the ribosyladenosine diphosphate group at N-1, modify the redox properties of the ring is not well understood, so we examined this question by using a variety of substituents at these positions.



SCHEME 1

Previous work on NADH model compounds with alkyl groups at N-1 shows that the ribosyl unit at this position in the coenzyme considerably reduces the rate of oxidation of the dihydropyridine ring.[3-8] Other work with flavins and NADH or its derivatives indicates that transfer of hydrogen from C-4 of the dihydropyridine ring (PyH) to the flavin (Fl) occurs after a complex has been formed between the reactants,[7,9] as summarized in Scheme 2.



SCHEME 2

The synthesis of the dihydropyridine and pyridinium compounds and an examination of their reactions with aqueous buffer was the subject of an earlier paper.[1] Subsequent publications will deal with their electrochemical properties. The present paper deals with the non-enzymic oxidation of the dihydro-compounds by flavin coenzymes, in particular riboflavin (Rfl; Z = ribityl in Scheme 1) and flavin mononucleotide (FMN, Z = ribityl phosphate).

EXPERIMENTAL

Preparation of the 1,3-disubstituted 1,4-dihydropyridines has been described elsewhere.[1] 1,4-Dihydronicotinamide adenine dinucleotide (NADH), 1,4-dihydronicotinamide mononucleotide (NMNH), riboflavin, and flavin mononucleotide were obtained commercially and used without further purification.

The rate of the reaction between Rfl or FMN and 1,4-dihydropyridines was determined by following the loss of absorbance in the region between 430 and 470 nm due to the reduction of the flavin, with the dihydropyridine always being present in excess. A stock solution of each of the reactants was prepared and thermally equilibrated in an oxygen-free dry box and the reactants were mixed in either of the two following ways.

For reactions with half-lives >5 min the reactants were mixed in the dry box. The timer was started immediately

[1] Part 1, D. J. Norris and R. Stewart, *Canad. J. Chem.*, 1977, **55**, 1687.

[2] U. Eisner and J. Kuthan, *Chem. Rev.*, 1972, **72**, 1; H. Sund in 'Biological Oxidations', ed. T. P. Singer, Interscience, New York, 1968; R. Stewart, 'Oxidation Mechanisms', Benjamin, New York, 1964, ch. 11.

[3] C. H. Suelter and D. E. Metzler, *Biochim. Biophys. Acta*, 1960, **44**, 23.

[4] T. P. Singer and E. B. Kearney, *J. Biol. Chem.*, 1950, **183**, 409.

[5] J. B. Jones and K. B. Taylor, *Canad. J. Chem.*, 1976, **54**, 2974.

[6] F. Y-H. Wu, R. E. MacKenzie, and D. B. McCormick, *Biochemistry*, 1970, **9**, 2219.

[7] T. C. Bruice, L. Main, S. Smith, and P. Y. Bruice, *J. Amer. Chem. Soc.*, 1971, **93**, 7327.

[8] J. L. Fox and G. Tollin, *Biochemistry*, 1966, **5**, 3865.

[9] D. J. T. Porter, G. Blankenhorn, and L. L. Ingraham, *Biochem. Biophys. Res. Comm.*, 1973, **52**, 447; G. Blankenhorn, *Biochemistry*, 1975, **14**, 3172.

and as quickly as possible, a 1.0 cm absorbance cell was filled to overflowing with the reaction mixture and sealed with a silicone rubber stopper and a rubber septum. The double seal was necessary to keep the cell free of oxygen for 2 h or more. The cell was removed from the dry box and placed in the thermostatted compartment of the spectrometer and absorbance readings taken until they remained unchanged for 30 min.

Too much time was required to fill the absorbance cell and remove it from the dry box for this method to be used with reactions having half-lives <5 min. For such reactions, the required volume of flavin stock solution was placed in the absorbance cell in the dry box and the cell was sealed with a silicone rubber stopper and rubber septum. A syringe needle was inserted into the space over the flavin solution in the cell, the top of this needle being isolated from the atmosphere by being itself in an inverted test tube sealed with a second rubber septum. This arrangement allowed the pressure to be relieved when the dihydropyridine solution was injected into the cell and, since the needle was never exposed to the atmosphere, it could not act as an avenue for oxygen to diffuse into the cell. The required volume of dihydropyridine stock solution was measured into a syringe in the dry box and a small volume of dihydropyridine stock solution was placed in a test tube to act as a back-up supply. The test tube was sealed with a rubber septum and the needle of the syringe containing the dihydropyridine solution was inserted through the septum. The syringe and absorbance cell apparatus were removed from the box and the absorbance cell placed in the thermostatted compartment of a recording spectrophotometer. The dihydropyridine solution was injected into the cell and the timer started as the cell contents were mixed. The recording of the absorbance was continued until no change was evident during 15 min. Reoxidation of flavin, caused by oxygen leaking into the cell, was noticeable within 1 h of mixing the solutions but this was longer than the reaction time for any of the systems prepared in this way.

Stock solutions of riboflavin and flavin mononucleotide were prepared by dissolving sample (*ca.* 7 mg) in buffer (75—100 ml) in a red coloured volumetric flask in the dry box. The riboflavin required 30 min or more of stirring to dissolve completely. The concentration of the stock solution was determined as soon as possible by measuring the absorbance of the solution at three or more wavelengths.

Stock solutions of 1,4-dihydropyridines were prepared by dissolving the required amount of dihydropyridine crystals in buffer (25 ml) in the dry box. The amount of dihydropyridine was chosen so that the reactions would have half-lives between 3 and 30 min. The concentration of the stock solution was determined by measuring the absorbance at three or more wavelengths.

High purity helium supplied the inert atmosphere in the dry box and great care was taken to ensure that air did not enter the chamber during the opening and closing operations.

* Riboflavin and FMN (which is much the more soluble and hence easier to use) oxidize (4) at identical rates and we have treated the two flavins as being interchangeable. Further support for this notion comes from the work of Jones and Taylor [5] who used FMN to oxidize 1-propyl- and 1-benzyl-dihydronicotinamide. Our results with riboflavin and the 1-methyl compound agree well with theirs when corrections are made (using the Hammett relation) for the difference in alkyl groups. Other reports indicate that FMN and riboflavin oxidation rate constants are roughly the same, although some have FMN reacting faster and others riboflavin.[3,4,11]

RESULTS AND DISCUSSION

The rate constants for the oxidation of most of the dihydropyridines were determined using equation (1), where X is the amount of reactant consumed at time t. The quantity on the left side of (1) was plotted against

$$\frac{1}{([\mathrm{PyH}]_o - [\mathrm{Fl}]_o)} \log \frac{([\mathrm{PyH}]_o - X)}{([\mathrm{Fl}]_o - X)} = \frac{k_2 t}{2.303} + \text{const} \quad (1)$$

time, as illustrated in Figure 1, and the second-order rate constant k_2 was calculated from the slope. (The curvature at longer times in Figure 1 is believed to be

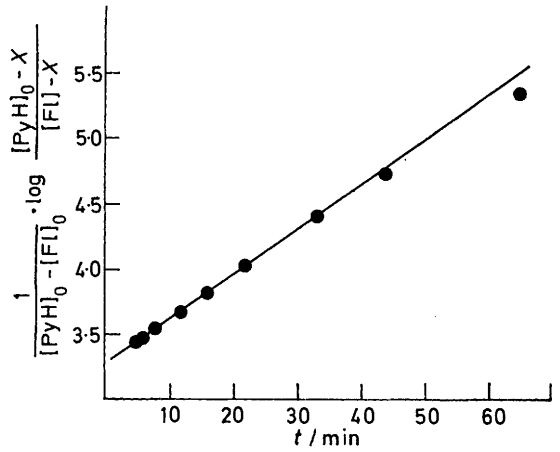


FIGURE 1 Rate plot for the oxidation of NADH (PyH) by riboflavin (Fl): 25°; pH 8.1; k_2 0.53×10^{-3} l mol^{-1} s^{-1}

caused by oxygen leaking into the cell.) The quantity X in (1) was calculated from the decrease in absorbance at 450 nm, a wavelength at which only the flavin absorbs.

The values of k_2 for the oxidation of the 1,3-disubstituted 1,4-dihydropyridines (1)—(9) by riboflavin or flavin mononucleotide at pH 7.5 to 10.5 are given in the Table. At higher pH ionization occurs at N-3, greatly reducing the oxidative strength of the flavin.[3,10] Reactions at pH < 7 can be complicated by insolubility of the dihydroflavin produced. With these exceptions the oxidation does not appear to be greatly affected by changes in acidity (Table 1) and so the pH selected for each compound was that which would minimize the extent of decomposition of the particular dihydropyridine and its pyridinium oxidation product by aqueous buffer.[1]

The oxidation of 1-substituted 3-carbamoyl-1,4-dihydropyridines by Rfl or FMN * shows a good linear free energy relationship when log k_2 is plotted against the appropriate σ^* substituent constants (Figure 2).†

† σ^* Values [12] were calculated from Charton's σ^I values [13] by using the equation $\sigma^*_{CH_2X} = \sigma^I_X/0.45$

[10] G. Blankenhorn, *Eur. J. Biochem.*, 1975, **50**, 351.
[11] I. M. Gascoigne and G. K. Radda, *Biochim. Biophys. Acta*, 1967, **131**, 498.
[12] R. W. Taft, *J. Amer. Chem. Soc.*, 1957, **79**, 1045; 'Steric Effects in Organic Chemistry', ed. M. S. Newman, Wiley, New York, 1956, ch. 13.
[13] M. Charton, *J. Org. Chem.*, 1964, **29**, 1222.

The reaction constant ρ^* is -1.91 when the point for 1-methoxymethyl is excluded, its oxidation rate being only one-seventh that expected. The reasons for this unusually slow rate, which is comparable to those for NADH and NMNH, are not understood. A feature shared by these three dihydropyridines and by none of the others is the presence of an oxygen atom on the α-carbon atom of the 1-substituent. Lindquist and Cordes [14] have previously pointed out that NAD^+ has an abormally high affinity for cyanide ion, a result that is consistent with the greater stability we observe for (3), and for NADH and NMNH, in comparison to related compounds having no oxygen function at the α-carbon atom.

Second-order rate constants for the oxidation of 1,4-dihydropyridines by flavin (Fl) at 25°

Dihydropyridine	R^1	R^3	Flavin [a]	pH [b]	10^3k_2/l mol^{-1} s^{-1} [c]
(1)	CH_3	$CONH_2$	Rfl	9.8—10.3	64
(2)	CH_2CH_2OH	$CONH_2$	Rfl	9.8	21
(3)	CH_2OCH_3	$CONH_2$	Rfl	8.1—8.7	0.61
(4)	CH_2COCH_3	$CONH_2$	Rfl	8.1	3.7
			FMN	8.1	3.7
(5)	CH_2COOCH_3	$CONH_2$	FMN	7.8	2.0
(6)	CH_2CN	$CONH_2$	FMN	8.2	0.15
(7)	CH_2CONH_2	$CONH_2$	FMN	7.8	3.0
				9.4	3.0
(8)	CH_2CONH_2	$COCH_3$	Rfl	8.1	0.55
(9)	CH_2CONH_2	CN	FMN	6.9	0.045
Special compounds					
NADH			Rfl	8.1	0.53
NMNH			FMN	8.1	0.25

[a] Rfl = riboflavin; FMN = flavin mononucleotide. [b] Buffer, 0.1M-tris(hydroxymethyl)aminomethane. [c] Estimated uncertainty (standard deviation) is ±7% or less for all compounds except (1), (2), and (6), whose uncertainties are approximately twice as great.

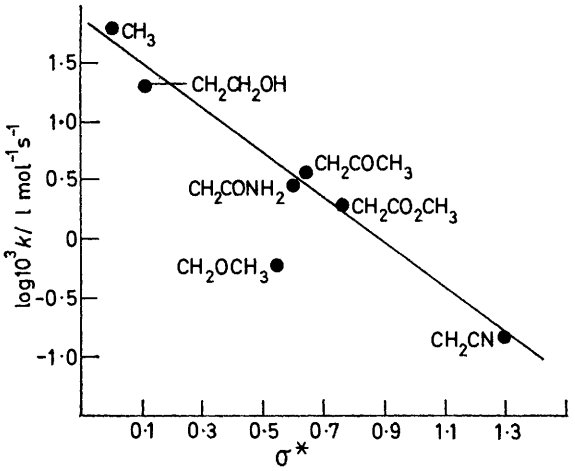
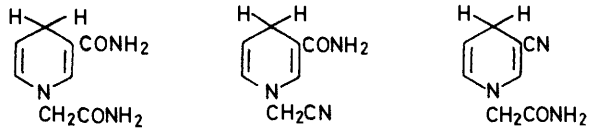


FIGURE 2 Hammett plot for the oxidation of a series of 1-substituted 3-carbamoyl-1,4-dihydropyridines by flavins in aqueous buffer. The σ* values were calculated using Charton's σ^I values and the relation $\sigma^*_{(CH_2X)} = \sigma^I_X/0.45$; [12,13] $\rho^* = -1.91$, correlation coefficient 0.994 excluding the point for methoxymethyl

The effect of 3-substituents on the rate of oxidation of 1,4-dihydropyridines is significantly greater than the effect of the same substituents on the 1-methyl group. There are insufficient data to compare Hammett plots directly but the magnitude of the effect is evident from a few comparisons. The rate of oxidation of (9) by FMN is roughly 1/70 of that of (7), whereas (6), which is isomeric with (9), is oxidized at 1/20 of the rate of (7). Replacing carbamoyl by cyano at the 3-position of the ring has thus 3.5 times the effect of the same change at the 1-methyl group. Similarly, the oxidation rates of

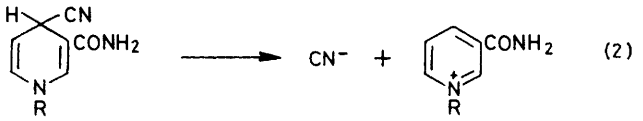


(7) (6) (9)

k_2/l mol^{-1}s^{-1} = 3·0 0·15 0·045

the isomeric compounds (4) and (8) show that the 3-position is the more sensitive site with regard to substituent effects (despite the nitrogen atom, which bears most of the developing charge in the transition state, being nearer to the 1-methyl substituent than to the 3-substituent).

Cyanide addition has been suggested as a model for hydride reduction of pyridinium ion [15] and it is significant that very similar values of ρ have been reported [16] for both rates and equilibria of cyanide addition to benzaldehydes [17] and sodium borohydride reduction of acetophenones,[18,19] indicating a close analogy between cyanide and hydride addition. From the results of Lindquist and Cordes [14] for cyanide loss from 4-cyano-1,4-dihydropyridines one can calculate $\rho^* = -1.5$ for the forward reaction in equation (2). This value is reasonably



(2)

near the ρ^* value we observe for the rate of oxidation of

[14] R. N. Lindquist and E. M. Cordes, *J. Amer. Chem. Soc.*, 1968, **90**, 1269.
[15] K. Wallenfels and H. Dieckmann, *Annalen*, 1959, **621**, 166.
[16] J. P. Klinman, *J. Biol. Chem.*, 1972, **247**, 7977.
[17] J. W. Baker and H. B. Hopkins, *J. Chem. Soc.*, 1949, 1089.
[18] H. Kwart and T. Takeshita, *J. Amer. Chem. Soc.*, 1962, **84**, 2833.
[19] H. Adkins, R. M. Elofson, A. G. Rossow, and C. C. Robinson, *J. Amer. Chem. Soc.*, 1949, **71**, 3622.

1.4-dihydropyridines by flavins, −1.91. The latter value is thus not unreasonable for a reaction in which hydride removal from C-4 is the rate-controlling step.*

Since there is good evidence from other systems for complex formation preceding the hydrogen transfer step,[7,9,10] as shown in Scheme 2, the question arises as to what extent the measured ρ^* describes the rate-controlling step. Under the conditions of our work (PyH concentrations from 10^{-3} to 10^{-5}M) complex formation could not be observed and given the size of K in related systems, this was to be expected. Accordingly, the experimental ρ^* value measures the effect of substituents on the conversion of the isolated reactant molecules, PyH and Fl in Scheme 2, into the transition state. That is, even though complex formation probably occurs and is followed by hydrogen transfer within the complex in the reactions being studied, the effect of substituents that we observe tells us nothing about either step. There is general agreement that the NADH–flavin reaction is a two-equivalent process [2] and, therefore, the reaction whose ρ^* has been measured can appropriately be described as hydride transfer from dihydropyridine to flavin.

Financial support of the National Research Council of Canada is gratefully acknowledged.

[7/1017 *Received*, 14*th June*, 1977]

* The acid-catalysed hydration of these compounds, which is believed to proceed by rate-controlling protonation at C-5, has an almost identical value of ρ^*, −2.0,[1] presumably because both reactions generate a positive charge at N-1.