not seem to play any role in the catalysis.

In all of our experiments a branched form of a poly(ethylenimine) was used. It is likely that this is essential. Past work[19] with a linear polyvinylpyridine and a neutral nitrophenyl ester substrate revealed no catalysis at all. However, this may not be relevant since the pK_a of the pyridine moiety in the latter polymer was very low and hence its nucleophilicity was poor.

The successful preparation of D,L-*N*-(4-pyridyl)proline, **22**, and of D,L-*N*-(4-pyridyl)phenylalanine[10] indicates that it should be feasible to synthesize a broad series of *N*-(4-pyridyl)amino acids through the reaction of specific amino acids with 4-phenoxypyridine. These can then be coupled to poly(ethylenimines) by the same procedure used to prepare the proline adduct I (Table II). Furthermore, the amino acid need not be converted to an *N*-alkyl derivative, for the catalytic effectiveness of the aminopyridine adduct to poly(ethylenimine) is not decreased if the exocyclic nitrogen is a secondary amine instead of a tertiary one (see derivatives K and L of Table II). Thus a general approach has been opened up for introducing chirality into these polymers.

It has been shown previously[20] that stereoselectivity in hydrolysis of amino acid nitrophenyl esters is manifested by poly(ethylenimines) containing covalently linked L-histidine. Other optically active amino acids could also be attached to poly(ethylenimine), but in general, they provide no effective nucleophile. With the aminopyridines, in contrast, essentially any amino acid could be coupled to the amine nitrogen to give a chiral entity with the desired side chain automatically linked to a pyridine nucleophile. Furthermore, the same procedure should work with peptides. Thus a wide range of specificities and stereoselectivity may be attainable. Thus these investigations demonstrate additional aspects of the versatility of poly(ethylenimines) as a macromolecular framework for the construction of synzymes.[1]

Acknowledgment. This investigation was supported in part by a grant (No. DMR 77-24152) from the Polymers Program, Division of Materials Research, National Science Foundation.

Registry No. 3, 80028-19-9; **4**, 80028-20-2; **5**, 80028-21-3; **6**, 64690-61-5; **7**, 80028-22-4; **8**, 80028-23-5; 9, 5747-92-2; **10**, 80028-24-6; **10**·HCl, 80028-25-7; **11**, 80028-26-8; **12**, 80028-27-9; **12**·HCl, 80028-28-0; **13**, 80028-29-1; **14**, 80028-30-4; **14**·HCl, 80028-31-5; **15**, 80028-32-6; **16**, 80028-33-7; **16**·HCl, 80028-34-8; **17**, 80028-35-9; **18**, 80028-36-0; **18**·HCl, 80028-37-1; **19**, 80028-38-2; **20**, 80028-39-3; **20**·HCl, 80028-40-6; **21**, 80028-41-7; DL-**22**, 80028-42-8; **23**, 80028-43-9; 4-aminopyridine, 504-24-5; methyl acrylate, 96-33-3; decanoyl chloride, 112-13-0; 1-benzyl-3-(ethoxycarbonyl)-5-pyrrolidinone, 5733-87-9; ethyl pyrrolidine-3-carboxylate HCl, 80028-44-0; 4-phenoxypyridine, 4783-86-2; ethyl nipecotate HCl, 65550-28-9; ethyl 6-aminohexanoate, 371-34-6; 4-(methylamino)pyridine, 1121-58-0; adipoyl chloride monoethyl ester, 1071-71-2; DL-proline, 609-36-9; poly(ethylenimine), 9002-98-6.

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The Pyridinium–Dihydropyridine System. Reduction Potentials and the Mechanism of Oxidation of 1,4-Dihydropyridines by a Schiff Base

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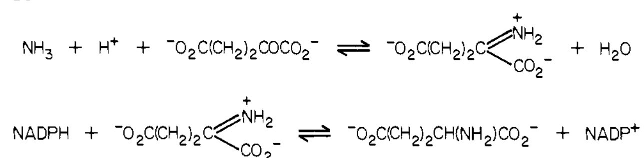
Contribution from the Laboratory of Molecular Biochemistry, Department of Biochemistry, University of Kansas School of Medicine and the Veterans Administration Medical Center, Kansas City, Missouri 64128, and the Department of Chemistry, University of British Columbia, Vancouver, B.C., Canada, V6T 1Y6. *Received June 8, 1981*

Abstract: As a model system for the glutamate dehydrogenase catalyzed reductive amination of α-ketoglutarate we have studied the reduction of a Schiff base, Δ^1-pyrroline-2-carboxylic acid, by a series of 14 N-1 and C-3 substituted 1,4-dihydropyridines, including NMNH, NADH, and NADPH. The reversible electrode potentials of eight of the dihydropyridines, all dihydronicotinamides, have also been determined. The reduction reaction has the following characteristics: (a) it is first order in protonated Schiff base (zwitterionic form) and first order in the dihydropyridine, (b) there is a small deuterium isotope effect when the C-4 position of the dihydropyridine is deuterated (1.20–1.57 at 25 °C), (c) there is a direct transfer of hydrogen from C-4 of the dihydropyridine to C-2 of the pyrroline, (d) the rates for seven N-1 substituted dihydronicotinamides are correlated satisfactorily with σ^* giving $\rho^* = -1.98$ (H_2O) and -1.78 (aqueous methanol), there being only a modest difference in rates in these two solvents, (e) there is a good correlation between the rates of reduction by the dihydronicotinamides and the E^0 values of the reversible two-electron dihydropyridine–pyridinium couple, the effect being 31.0 mV per logarithmic unit of rate, (f) there is a close correlation between the rates of reduction of pyrroline and of flavin by the dihydropyridines, and (g) the enthalpy and entropy of activation for the rate-controlling step in the reduction by 1-benzyl-1,4-dihydronicotinamide are 15.7 kcal mol^{-1} and -7.6 eu. We believe that direct hydride transfer has taken place to produce proline in a single step and it can be inferred that the transition state closely resembles products in structure. The similarity between pyrroline and flavin reduction suggests that the latter reaction may also be a direct hydride transfer.

Glutamate dehydrogenase (GDH) catalyzes the reductive amination of α-ketoglutarate, presumably through an imino intermediate (Scheme I).[3] As part of a study on GDH catalysis, we set out to determine the mechanism of the reduction step (the second step in Scheme I) in the absence of enzyme. Since the imino intermediate is very unstable in water, we used as a model the Schiff base, Δ^1-pyrroline-2-carboxylic acid, **1**, which is known to be reduced by NADH to proline.[4] We have studied the kinetics

Scheme I



(1) Veterans Administration Medical Center.
(2) University of British Columbia.
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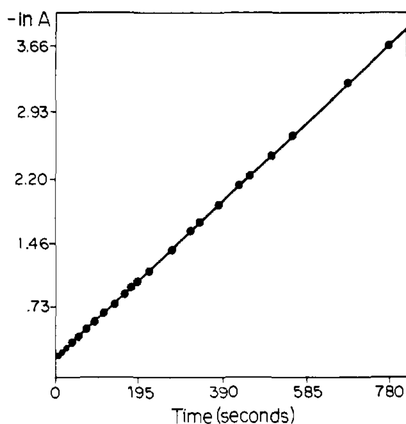
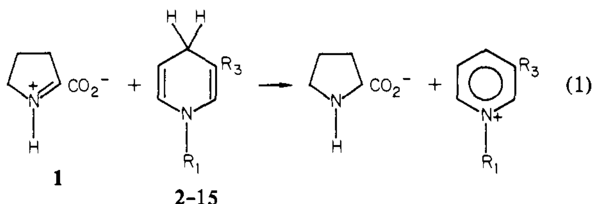


Figure 1. First-order plot for the reduction of **1** by **4** in water; pH 6.66; $T = 25$ °C; $\mu = 0.3$; $[\mathbf{1}]_{init} = 0.0796$ M.

of the oxidation–reduction shown in eq 1, besides determining the reversible two-electron reduction potentials of a number of the substituted pyridinium ions produced in the reaction. The rate and equilibrium data are combined to provide information regarding this and other oxidation–reductions of biochemical interest.



Experimental Section

Materials. NMNH, NADH, and NADPH obtained from Sigma Chemicals and NADH-d_1 and NADH-d_2 obtained from laboratory stock[5] were used without further purification. The synthesis of 1,3-disubstituted 1,4-dihydropyridines has been previously described.[6,7] The 4-deuterio compounds were prepared (>0.96 *d* at C-4 by NMR) by reduction of the pyridinium salt with sodium dithionite in D_2O (99.7 atom % D) using the general method of Caughey and Schellenberg.[8] Four and one-half cycles of oxidation by chloranil and reduction by dithionite in D_2O[9] gave the dideuterio compounds, which had no detectable signal at C-4 by NMR. In the case of 1-(carbamoylmethyl)-3-carbamoyl-1,4-dihydropyridine (**6**) the methylene protons of the carbamoylmethyl group were also found to have undergone exchange. It was assumed that this isotopic substitution had no effect on the oxidation reaction.

α-Keto-δ-aminovaleric acid **16** was synthesized as the hydrochloride salt by the method of Hasse and Wieland,[10] mp 113 °C dec, lit.[10] mp 113 °C dec.

Reaction Products. α-Keto-δ-aminovaleric acid hydrochloride (0.37 mmol) was dissolved in 4 mL of phosphate buffer (0.1 M), the pH adjusted to 6.40, and the volume made up to 10 mL with methanol to obtain a solution in 15.3 M methanol. Compound **4** (0.74 mmol) was added to this solution and the contents incubated at 40 °C for 24 h. The solution was evaporated to about 5 mL under reduced pressure, made basic to litmus, and filtered and the filtrate was run through a Sephadex G-10 column using water as eluant. The ninhydrin-active fraction was lyophilized, the resulting residue dissolved in D_2O in phosphate buffer (0.1 M, "pH" 7.00), and the NMR spectrum recorded. The spectrum was identical with that obtained for a mixture of 1-benzyl-3-carbamoylpyridinium chloride and proline recorded under identical conditions. The experiment was repeated with **4**-d_2, whereupon proline deuterated at C-2 and 1-benzyl-4-carbamoylpyridinium ion deuterated at C-4 were obtained.

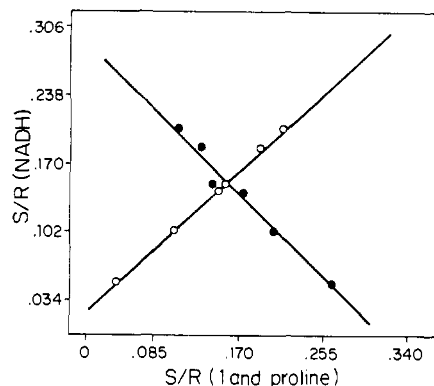


Figure 2. Correlation of rate of disappearance of NADH with disappearance of **1** (open circles) and with appearance of proline (filled circles).

Kinetic Procedure. Kinetic runs were followed with a Hewlett Packard UV/vis 8450 A spectrophotometer that is capable of producing the entire spectrum at programmed time intervals of 1 s or greater. In a typical experiment, the hydrochloride salt of α-keto-δ-aminovaleric acid (**16**) was dissolved in 0.1 M buffer. The pH was adjusted to the desired value after the addition of potassium chloride to bring the ionic strength to 0.3. The solution (1 mL) was transferred to a 1-cm thermostated cell, the dihydropyridine solution (10 μL) added, and the contents mixed with a stirrer-adder. The absorbance loss of the dihydropyridine at its λ_{max} was recorded at time intervals. In all experiments **1** was present in large excess with the concentration of the dihydropyridine being such as to give an initial absorbance of 0.8–0.9 (7.0×10^{-5} to 1.5×10^{-4} M). The reaction followed a first-order course (Figure 1) over greater than 96% reaction, showing that the reaction is not reversible under the experimental conditions used.

The observed pseudo-first-order constants were corrected for the general acid catalyzed decomposition of dihydropyridines[6] from rates measured under identical conditions, where **1** is replaced by an equimolar amount of 4-methylpyridine, a compound whose pK_{SH^+} value (6.00) is comparable to that of **1** (5.99).[11]

The following experiment was conducted to show that the rate of disappearance of reactants coincided with the rate of appearance of proline. To a solution of D_3PO_4 in D_2O (0.2 M, "pH" 6.47) was added NADH (50 mM) and **1** (50 mM). The NMR spectrum was recorded as a function of time, using DSS as an internal standard, at about 37 °C. The absorption intensities of **1** at 3.20 ppm (multiplet, 2 H), of proline at 3.50 ppm (δ-CH_2 protons), and of NADH (the methylene protons of the dihydropyridine ring) were determined periodically up to about 50% reaction (about 35 min). With S the peak area for the sample at the desired chemical shift and R that for DSS, a plot of S/R for NADH vs. S/R for **1** generates a straight line (correlation coefficient 0.999) of nearly unit slope (0.93) (Figure 2). There is also a linear relation (correlation coefficient 0.985) between S/R for NADH and S/R for proline with a slope of −0.98.

Reduction Potentials. The reduction potentials of the dihydropyridine–pyridinium couples were determined potentiometrically essentially by the method of Rodkey.[12] Potentiometric measurements were made in a thermostated cell in a drybox under a helium atmosphere, with potentials recorded on a Beckmann Model G pH meter. The instrument was calibrated periodically with an unbuffered solution containing 0.1 M each of potassium ferrocyanide and potassium ferricyanide.[13] The cell potential was measured with a platinum electrode referred to a saturated potassium chloride–calomel electrode. The cell contents were assumed to have reached equilibrium when potentials recorded an hour apart agreed to within 1 mV. In general, the potentials were determined by the method of mixtures. The desired quantities of a substituted pyridinium salt and the corresponding dihydropyridine were weighed into a Metrohm polarography cell fitted with a water jacket. The weight of pyridinium salt and dihydropyridine were chosen to give a total concentration between 10^{-3} and 10^{-2} M. Two to four milligrams of mediator (riboflavin) and, when needed, approximately 0.5 mg of co-mediator (methyl viologen) were weighed into the cell giving a concentration of the mediator of 5–10% of the total pyridinium/dihydropyridine concen-

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tration. Twenty-five milliliters of 0.1 M Tris or glycine buffer solution were added to the cell and the crystals were dissolved by stirring the solution for 15–20 min. The pH of the solutions was varied between 8.80 and 10.00, in order to minimize decomposition, the dihydropyridines being acid labile and the pyridinium ions base labile, but to differing and variable extents.[6]

Equilibrium, as determined by a constant cell potential, was generally reached within 3–8 h. Those pyridinium ions with less negative reduction potentials tended to reach equilibrium the soonest. After the cell potential was recorded the solutions were analyzed to determine the final concentrations of pyridinium salt and dihydropyridine and the pH. A sample of the potentiometry solution was placed in a 0.1-cm absorbance cell in the drybox and sealed with a rubber septum.

As quickly as possible after removal from the drybox the absorbance of the solution was measured on a Cary 16 spectrophotometer at two wavelengths between 440 and 460 nm, and at three wavelengths between 300 and 400 nm. These wavelength regions include the absorbance maxima of the 1,4-dihydropyridines and the oxidized form of riboflavin. The concentrations of dihydropyridine and oxidized and reduced riboflavin were calculated by solving the systems of simultaneous linear equations containing the absorbance data. The concentration of pyridinium ion was determined by polarography and where applicable by spectrophotometry.

Mediators are required to obtain reproducible potentials with the dihydropyridine–pyridinium ion systems and so we tested xanthine oxidase and benzyl viologen for this purpose, two mediators Rodkey had used with success.[12] We found them to work well with NADH–NAD$^+$, but to give erratic results with other nicotinamide derivatives. We found, however, that reproducible results could be obtained using riboflavin as mediator. (In order to hasten equilibrium methyl viologen was sometimes used as co-mediator, though the final potential turned out to be independent of the presence of the viologen.) In order to ensure that riboflavin mediation was giving the correct potential a comparison was made with the xanthine oxidase/benzyl viologen system for NADH–NAD$^+$. After equilibration the two values agreed to within 5 mV. The value calculated for E^0 (pH 0) for the NADH–NAD$^+$ system in our work is −105 mV at 25 °C, very close to Rodkey's value of −102 mV at 25 °C.[12a] (Blankenhorn's more recent value of −320 mV at pH 8 and 20 °C[14] corresponds to a value of −83 mV at pH 0; however, this discrepancy is due chiefly to concentration[14] and temperature effects.[12a])

We were not able to obtain reproducible potentials for compounds which contain a cyanomethyl or a carbomethoxymethyl group, under the conditions used for the other compounds. These groups underwent partial hydrolysis in the time required to reach equilibrium.

Polarographic Half-Wave Potentials and Determination of Pyridinium Ion Concentration. All polarograms were recorded on a Metrohm B 261 polarecord. The potentials were measured under nitrogen against a silver|silver chloride|saturated potassium chloride reference electrode, for which a value of 222 mV[15] was used to refer the observed polarographic potentials to the standard hydrogen electrode. The E 261 polarecord is fitted with a charging current compensation which was adjusted for each polarogram to obtain a base line as close to horizontal as possible.

In order to determine the concentration of pyridinium ion in the potentiometry solution, a calibration line was calculated by least squares from seven or more concentration–diffusion current pairs in acetate buffer. (The calibration lines were generally linear between 10^{-2} and 10^{-4} M, although the lines did not pass through the origin.) Deoxygenated solutions of acetic acid–sodium acetate buffer (total molarity 0.1) at a pH of 5.65 were used, the ionic strength being adjusted to 0.1 with sodium perchlorate. The latter is the preferred salt since more nucleophilic anions such as chloride formed addition products with some of the more easily reduced pyridinium salts. The potentiometer solution was removed from the drybox and sealed in an Erlenmeyer flask, and the polarogram recorded immediately upon pouring the contents into the polarography cell. The analysis must be carried out quickly and under nitrogen to minimize the riboflavin-catalyzed air oxidation of the dihydropyridines. After recording of the polarogram, the pH of the solution was determined and was found to be never more than 0.05 pH units from the original value.

Pyridinium salts have an absorbance maximum between 260 and 270 nm and attempts were made to use direct spectrophotometric measurements in a number of cases. Only **5** and **11** could be analyzed in this way. The results for these two compounds agreed with the polarographic assay to within 10%. The products of acid-catalyzed decomposition of 1,4-dihydropyridines exhibit large absorbance near 290 nm,[16] which interferes with direct spectrophotometric determinations of other pyridinium compounds. In any case polarographic determination of the pyridinium ion concentration has an advantage over spectrophotometry in that the latter is unable to detect decomposition that leaves the pyridinium ring intact. If the reduction potentials of the original pyridinium ion and its decomposition product are sufficiently different, as is often the case, then the two concentrations can be determined polarographically. A commonly met decomposition is the hydrolysis of the 3-carbamoyl group at pH >10. The 3-carboxy product has a half-wave potential between 200 and 300 mV more negative than the wave due to the 3-carbamoyl compound. The ester group of 1-(carbomethoxymethyl)-3-carbamoylpyridinium ion (also of 1-(carboisopropoxymethyl)-3-carbamoylpyridinium ion) undergoes significant base-catalyzed hydrolysis at a pH as low as 7 and the resulting 1-carboxymethyl ion is easily distinguished from the original ester on the polarograms.

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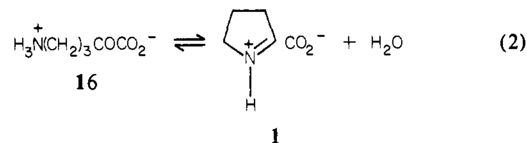
Table I. Rate Data for Reaction of 1 and 6[a]

pH[b]	$10^5 k_{obsd}$,[c] s^{-1}	pH[b]	$10^5 k_{obsd}$,[c] s^{-1}
4.00	315	6.76	55.2
4.43	306	6.86	41.8
4.96	275	7.24	22.5
5.02	273	7.50	12.9
5.43	242	7.73	7.68
5.76	193	7.89	5.17
6.08	152	8.20	2.63
6.51	81.5	8.58	1.07

[a] T = 25.0 °C; μ = 0.3; [buffer] = 0.1 M; $[1]_{init}$ = 0.0796 M. [b] pH 4.00-5.02 acetate buffer; pH 5.43-6.68 MES buffer; pH 7.24-8.58 Tris buffer. [c] Corrected for decomposition of dihydropyridine (less than 5% except at pH 4.00 (23%), 4.43 (16.1%), 4.96 (9.2%), and 5.02 (6.8%)).

Results and Discussion

Δ^1-Pyrroline-2-carboxylic acid (**1**) is derived from the spontaneous cyclization of α-keto-δ-aminovaleric acid (**16**) as shown in eq 2.[11,17] The equilibrium is known to favor the product unless



the carboxylate group is protonated[11,17] (pK_{HA} = 1.77).[11] Consequently, the rate measurements were made well above pH 2, in regions where almost all the substrate is present in the form of the cyclic imine. The stability of the dihydropyridines is also enhanced at higher pH values.

The pseudo-first-order rate constants for the oxidation–reduction (corrected for decomposition of dihydropyridine), k_{obsd}, varied directly with the concentration of **1**, showing the reaction to be first order in oxidant. The rates of disappearance of **1** and the appearance of proline are nearly identical (Figure 2); this and the product study confirm the finding of Meister et al.[4] that the stoichiometry of the reaction is represented by eq 1. Furthermore, oxidation of **4**-d_2 by **1** yielded proline-*2-d*. Therefore, the reduction involves the direct transfer of hydrogen from C-4 of the dihydropyridine to C-2 of the pyrroline.

pH Effect. Table I summarizes the response of the reaction rate of **6** to changes in the pH of the medium. A plot of log k_{obsd} vs. pH for this compound and for **4** is shown in Figure 3, from which pK_{SH^+} values of 6.04 and 6.07 are obtained for **1** from the reaction rates with **4** and **6**, respectively. These values correspond closely to the potentiometric pK_{SH^+} of **1** (6.05), determined under the same conditions. The reaction was carried out with **2** over the pH range 7.31–9.40 and a linear plot of log k_{obsd} against pH was obtained with a slope −1.0. These results clearly establish the protonated Schiff base, **1**, a zwitterion, as the active oxidant.

When **6** is oxidized by **1** at pH 6.50 ([**16**] = 0.0796 M, T = 25.0 °C, μ = 0.3) identical rates are observed, both in the presence and absence of added buffer (0.1 M 2-(N-morpholino)ethanesulfonic acid) (MES). This observation and the pH–rate data

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Table II. Rate Constants and Primary Deuterium Isotope Effects for the Reduction of 1 by Substituted Dihydropyridines[a]

compd	R_1	R_3	10^2k, M^{-1} s^{-1}	10^2k^{HD}, M^{-1} s^{-1}	primary isotope effect[b]	10^2k_{MeOH},[c] M^{-1} s^{-1}
2	CH_3	$CONH_2$	162	148	1.20	18.5
3	CH_2CH_2OH	$CONH_2$	49.8	45.5	1.21	15.7
4	$CH_2C_6H_5$	$CONH_2$	28.0	22.3	1.57[d]	4.37[e]
5	CH_2COCH_3	$CONH_2$	6.32	5.68	1.25	1.85
6	CH_2CONH_2	$CONH_2$	4.13	3.38	1.54[f]	1.70
7	$CH_2CO_2CH_3$	$CONH_2$	2.75	2.40	1.34	0.783
8	CH_2CN	$CONH_2$	0.327	0.273	1.48	0.0895
9	CH_2CO_2	$CONH_2$	36.8	30.3	1.55	
10	CH_2OCH_3	$CONH_2$	1.18	1.08	1.22	0.328
11	CH_2CONH_2	$COCH_3$	0.465	0.398	1.40	
12	CH_2CONH_2	CN	0.245	0.213	1.35	
13	NMNH		0.878			0.602
14	NADH		0.702	0.610	1.51[g]	0.560
15	NADPH		0.687			

[a] In water except where noted. $T = 25$ °C; $\mu = 0.3$. [b] Where K^{DD} was determined[d,f,g] an average was taken. [c] 15.3 M in methanol. [d] $k^{DD} = 0.180$ M^{-1} s^{-1}. [e] $k^{HD} = 0.0392$ M^{-1} s^{-1}; $k^{DD} = 0.0327$ M^{-1} s^{-1}; primary isotope effect = 1.33. [f] $k^{DD} = 0.0270$ M^{-1} s^{-1}. [g] $k^{DD} = 0.00458$ M^{-1} s^{-1}.

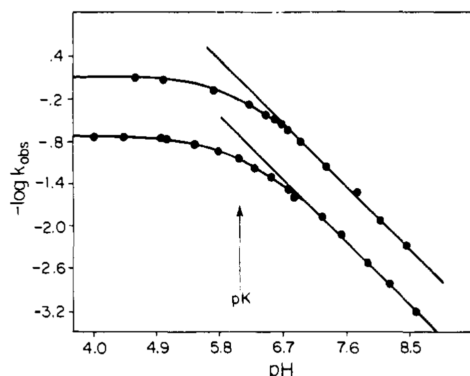


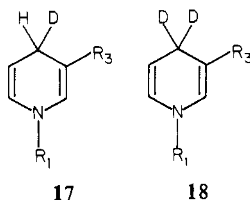
Figure 3. Variation of log k_{obsd} with pH for 4 (upper curve) and 6 (lower curve).

show that eq 1 represents the slow step of the reaction; i.e., the reaction is not subject to general acid–base catalysis. Consequently, the second-order constant, k, corrected for the decomposition of dihydropyridine is given by eq 3.

$$-d[\text{dihydropyridine}]/dt = k_{obsd}[\text{dihydropyridine}] = k[\mathbf{1}][\text{dihydropyridine}] \quad (3)$$

The concentration of 1 was calculated from the analytical concentration of α-keto-δ-aminovaleric acid hydrochloride and the pH of the medium. It is assumed that over the pH region studied (pH 4.00–9.45) the compound is entirely in the cyclic imino form.[11,17]

Isotope Effect. The effect of deuterium substitution at the 4 position was examined for a number of substituted 1,4-dihydropyridines. Table II gives values of k and k^{HD}, which are, respectively, the second-order rate constants for the reaction of 1 with the protio and monodeuterio dihydropyridines 17. The rate constants for the reaction of the dideuterio compound 18, k^{DD}, were also determined for three of the dihydropyridines.



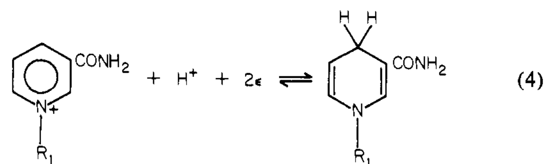
For reactions where all three rate constants are available it is possible in principle to evaluate the primary (p) and secondary (s) isotope effects.[9,18] However, the rate data are not sufficiently precise (±5%) to obtain values of both p and s. Consequently, we have assumed the value of s to be unity, a common practice, and calculated the p values.[9] When all three rate constants are measured average values of p are reported in Table II.

The values of p range from 1.20 to 1.57 and show no clear correlation with structure or reactivity. We conclude that for the Schiff base reduction the primary isotope effect is small and relatively insensitive to substituents in the dihydropyridine ring.

Activation Parameters. The rates for the reaction of 1 and the N-benzyl compound, 4, were measured in Tris buffer (0.1 M; μ = 0.3; pH 8.05 at 25 °C and [16] = 0.0796 M) at various temperatures. The pH of the solution was also determined at the same temperature. The apparent $\Delta H^{\ddagger}$ and $\Delta S^{\ddagger}$ values obtained from an Arrhenius plot were then corrected for the temperature effect on the pK_{SH^+} of 1. The ΔH° value for the ionization of the latter was determined at 25.0 °C calorimetrically and found to be 9.38 kcal mole^{-1}; ΔS° = 4.1 eu. The activation parameters for the reaction in eq 1 ($R_1 = C_6H_5CH_2$, $R_3 = CONH_2$) are thus determined to be $\Delta H^{\ddagger}$ = 15.7 kcal mol^{-1} and $\Delta S^{\ddagger}$ = −7.6 eu.

Solvent Effects. We have also studied the kinetics of the reaction shown in eq 1 in aqueous methanol, the solvent employed in cryogenic experiments of the GDH reaction.[19] The rate data for aqueous methanol (15.3 M in CH_3OH) are collected in Table II. It is apparent that the reaction proceeds somewhat faster in water than in aqueous methanol, with the difference in rate becoming small with the least reactive compounds, the nucleotides. Hammett plots of log k against σ^{*}[20,21] are linear for compounds 2–8 in both water and aqueous methanol. Compounds 9 and 10, containing respectively a carboxymethyl and methoxymethyl substituents at N-1, are much less reactive than predicted by their σ^* constants and if they are excluded the ρ^* values are −1.98 (water) and −1.78 (15.3 M methanol). Such deviations and possible causes for them were reported before.[6,21] Based on similar ρ^* values and isotope effects (Table II) in these two solvents, we conclude that the mechanism of the redox reaction is not altered by the addition of methanol.

Reduction Potentials. The reduction potentials of the dihydropyridine–pyridinium ion couples (eq 4) measured for eight dihydronicotinamide analogues are listed in Table III. The



(18) R. P. Bell and D. M. Goodall, *Proc. R. Soc. London, Ser. A*, **294**, 273 (1966).

(19) (a) R. E. Johnson, P. J. Andree, and H. F. Fisher, *J. Biol. Chem.*, **256**, 3817 (1981); (b) *ibid.*, **256**, 6381 (1981).

(20) (a) M. Charton, *J. Am. Chem. Soc.*, **99**, 5687 (1977); (b) M. Charton, *J. Org. Chem.*, **29**, 1222 (1964); (c) R. W. Taft, in "Steric Effects in Organic Chemistry", M. S. Newman, Ed., Wiley, New York, 1956.

(21) R. Stewart and D. J. Norris, *J. Chem. Soc., Perkin Trans. 2*, 246 (1978).

Table III. Electrochemical Data for Substituted Nicotinamides (H_2O, 25 °C)

compd[a]	$E^{0'}_{pH}$, mV	pH	slope,[b] mV	E^0,[c] mV	E,[d] mV
2	−463	10.00	22.3	−167	−793
3	−441	9.70	29.7	−154	−758
5	−381	8.80	26.2	−121	−699
6	−385	9.20	29.2	−113	−682
7					−657
8					−554
9	−439	10.00	34.6	−143	−826
10	−357	8.80	39.5	−97	−657
13	−358	8.90	30.3	−95	−810
14	−365	8.80	*e*	−105	−683

[a] The numbering is that given in Table II. [b] Slope of plot of measured potential, E, against log $[Py^+]/[PyH]$. [c] Calculated potential at unit hydrogen ion activity using eq 7. [d] Polarographic half-wave reduction potential of pyridinium ions at 2.0 mM concentration, measured against Ag|AgCl|KCl(sat.) reference electrode. [e] Could not be determined because of decomposition of substrate. Polarographic assay showed that 15% of NAD^+ had been converted within 2 h to a second pyridinium compound, probably the mononucleotide. A correction was made to the measured potential but since only the total dihydropyridine concentration could be determined the correction can only be approximate. Nonetheless, the value of E^0 agrees well with that previously reported (ref 12a).

measured cell potential, E, is related to the standard reduction potential, E^0, and the concentrations of pyridinium ion, $[Py^+]$, and dihydropyridine, $[PyH]$, by the Nernst equation, which at 25 °C is given by eq 5.[12c] Equation 5 can be rewritten to give eq 6 and 7. Figure 4 shows a plot of measured potential against

$$E = E^0 - 29.6 \log \{[PyH]/[Py^+][H^+]\} \quad (5)$$

$$E = E^{0'}_{pH} + 29.6 \log \{[Py^+]/[PyH]\} \quad (6)$$

$$E^{0'}_{pH} = E^0 - 29.6(pH) \quad (7)$$

the logarithm of the ratio of oxidized and reduced forms for compound **10** in Tris buffer at pH 8.8. The slope, 29.5 mV, is very close to the theoretical value of 29.6 mV. The other compounds also gave good straight lines in such potentiometric titrations with, in most cases, near-theoretical slopes. The two exceptions are the compounds containing methyl or carboxymethyl at N-1. We cannot account for these anomalies, particularly since the linear free energy relations, discussed below, show no deviation for these compounds. We assume that some distortion occurs in the measured potentials for these two compounds when the ratio of oxidized and reduced forms departs appreciably from unity. In any case, the deviation from the listed value of E^0 in the worst case, **2**, is only 2 mV when the ratio of oxidized to reduced forms is as high as 2:1 or as low as 1:2.

The validity of eq 7 was tested by plotting $E^{0'}_{pH}$, the intercept of plots such as that in Figure 4, against pH for compound **6** (Figure 5). The observed slope of −30.5 mV per pH unit is in satisfactory agreement with the theoretical slope of −29.6 mV per pH unit (eq 7).

Listed in Table III are the potentials $E^{0'}_{pH}$, the reduction potentials E^0 (pH 0), and the polarographic half-wave potentials $E_{1/2}$ determined at 2.0 mM concentration of substrate. The $E_{1/2}$ values were found to be markedly concentration dependent particularly in the case of **9**. Thus, although the use of polarography as an analytical tool in the present work seems perfectly satisfactory, the $E_{1/2}$ values should not be taken as absolute values for the one-electron reduction potentials of the pyridinium ions.

Mechanism. The rate-controlling step of the reaction between Δ^1-pyrroline-2-carboxylic acid and the dihydropyridines has the following characteristics: (a) protonated Schiff base (zwitterion **1**) is the active oxidant, (b) there is a samll deuterium isotope effect when deuteriodihydropyridines are the reductants, (c) there is a negative entropy of activation, (d) there is a small reduction in rate when methanol is added, (e) electron-withdrawing groups at N-1 in the dihydronicotinamides (and also at C-3 in the other dihydropyridines) lower the rate significantly, and (f) the hydrogen atom at C-2 of the proline comes from the C-4 methylene group of the dihydropyridine.

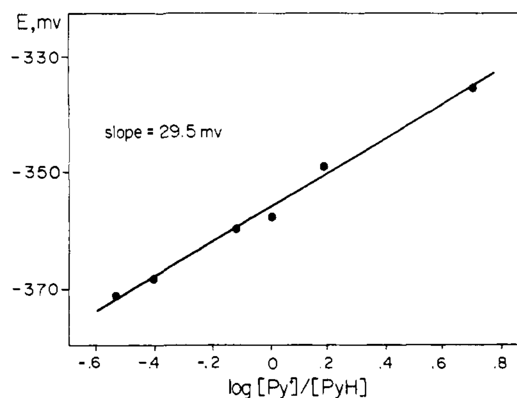


Figure 4. Plot of measured cell potential against logarithm of ratio of oxidized and reduced form of **10**; pH 8.80, 0.1 M Tris buffer; $E^{0'}_{8.80}$ = −357 mV.

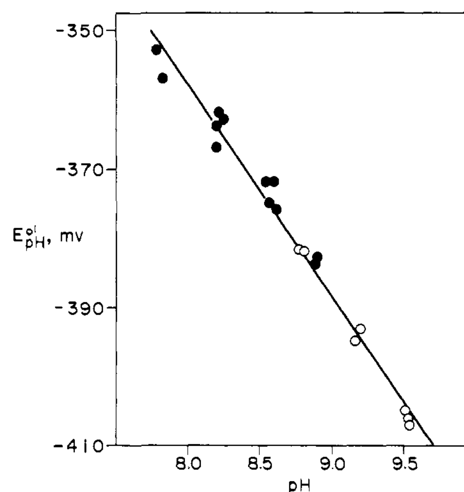


Figure 5. Plot of $E^{0'}_{pH}$ against pH for compound **6** in 0.1 M glycine buffer (open circles) and in 0.1 M Tris buffer (filled circles). Slope = −30.5 mV per pH unit.

In view of the structures of the oxidant and reductant in the present case, the most attractive mechanism is direct hydride transfer from C-4 of the dihydropyridine to C-2 of the pyrroline. The reaction characteristics listed above are compatible with such a route and two of them, the isotope and substituent effects, provide a considerable amount of information about the transition state, particularly when the electrochemical characteristics of the dihydronicotinamides are also considered.

Blankenhorn[14,22] has drawn attention to the significance of plots of log k against E^0 for reactions in which a series of reducing agents react (a) with an oxidant (rate constant k) and (b) reversibly in a two-electron manner at an electrode (E^0).[23] Kurz and Kurz[24] have pointed out that, regardless of whether k represents a one- or two-electron step, a slope of −30 mV per logarithmic unit of rate will result if there is no effect of substituents on the rate of the reverse reaction, that is, in the present case if the effect of substituents on the pyridinium ions is the same as on the transition state of the oxidation–reduction.

Figure 6 shows a plot of log k for the rate-controlling step of the present reaction against E^0 for all the dihydronicotinamides

(22) G. Blankenhorn, in "Pyridine Nucleotide-Dependent Dehydrogenases", Proceeding of 2nd International Symposium, H. Sund, Ed., W. de Gruyter, Berlin, 1977, p 185.

(23) Although the dihydropyridine–pyridinium system does not itself react reversibly at the electrode, it is in equilibrium with the mediator which does so.

(24) L. C. Kurz and J. L. Kurz, *Eur. J. Biochem.*, **90**, 283 (1978).

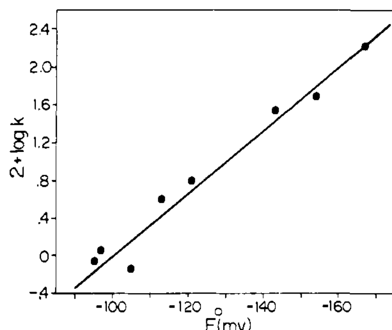


Figure 6. Rate of reduction of **1** by dihydronicotinamides as a function of the latter's half-cell potentials. Slope = −0.0323 logarithmic units per millivolt or −31.0 mV per logarithmic unit.

for which we have been able to obtain data. There is a good correlation (correlation coefficient 0.982) with the slope being −0.0323 logarithmic units per millivolt. This is equivalent to a change of 31.0 mV per logarithmic unit, very close to the value required for the substituent effect to be entirely in the forward step (conversion of reactants to transition state) and completely absent in the reverse step (conversion of products to transition state). This, in turn, is tantamount to a unit Brønsted slope and suggests that the transition state is highly productlike, that is the transferred unit (almost certainly a hydride ion) is much more strongly bound in the transition state to the carbon atom of the proline than to the carbon atom of the nicotinamide.

The magnitude of the isotope effect is relevant here. Though there are significant exceptions, hydride transfers often exhibit modest deuterium isotope effects, many in the range of 2–5, significantly less than that calculated from the difference in energy of the zeropoint stretching vibrations.[25] Even for the common run of hydride transfers the isotope effects we observe are very low, though they are consistent with the values previously found for the reduction of a series of trifluoroacetophenones by compound **6**, a reaction also presumed to be a hydride transfer.[9] Isotope effect maxima are expected to appear with symmetrical transition states, that is, when the hydrogen being transferred is roughly midway between its bonding partners. Such maxima have indeed been observed in a number of cases of proton transfer and in at least one case of hydride transfer.[26] It follows that a transition state that closely resembles reactants or products should have a low isotope effect and, in our case, because of the unit Brønsted slope one can conclude that the transition state closely resembles products.

There is growing skepticism about the value of assigning structure to transition states on the basis of kinetic and energetic parameters (see, for example, ref 27 and references therein). We use the phrase "the transition state closely resembles products" partly for the benefit of those who find the concept useful and partly to encapsulate a number of experimental phenomena in a single digestible phrase.

Implications for Enzyme Catalysis. The cyclization reaction of α-keto-δ-aminovaleric acid to form **1** (eq 2) followed by the reduction of **1** by NADPH (eq 1) constitutes an overall reaction whose stoichiometry is closely analogous to that of the GDH catalyzed reaction (Scheme I). The difference between the systems lies in the use of a covalently bound amino group in the model reaction in lieu of the binding of free ammonia in the enzyme-catalyzed reaction. We have shown here that the formation of a protonated α-iminocarboxylate permits a facile reduction by NADPH leading to the formation of an α-amino acid. Thus, we have demonstrated that the reaction course indicated in Scheme I constitutes at least a possible mechanism for the enzyme-catalyzed reaction. Since no other pathway of carrying out a reaction with a stoichiometry resembling that of Scheme I has ever been demonstrated, the mechanism described in that scheme must be considered as a very likely one. The ability to carry out the overall reaction will now permit the study of each of the two constituting steps.

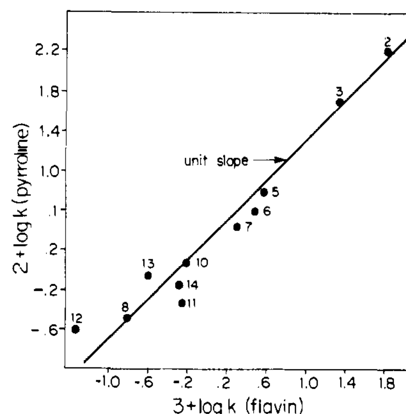


Figure 7. Correlation between the rates of reduction of **1** and of flavin by dihydropyridines. The numbering is that given in Table II. Rate data for the flavins from ref 21.

Relationship to Flavin Reductions. It seems clear that dihydronicotinamides follow a two-electron (two-equivalent) redox pathway with most compounds that have two-electron capability. Even dithionite, which can react by either one- or two-electron pathways, appears to react with NAD analogues by hydride transfer.[28] The mechanism with the nicotinamide–flavin reaction is, however, less clear.[29] Hemmerich,[29] while conceding that Blankenhorn has satisfactorily demonstrated that hydrogen transfer from dihydronicotinamide to a flavin proceeds by a two-electron transfer, has pointed out that the identity of the atom which carries the two electrons remains an unresolved question. Blankenhorn[22] argues that it is a hydride ion while Hemmerich[29] favors a proton transfer plus two-electron σ transfer. In this connection it may be significant that there is a close correlation between the rates of reduction of the pyrroline **1** by the series of dihydropyridines and the rates of reduction of riboflavin (or FMN) by the same reagents.[21] In Figure 7 the logarithms of the respective rate constants are plotted against each other with the straight line being drawn with unit slope. There is a good correlation between the two reactions, the largest deviations occurring with the only two dihydropyridines that are not dihydronicotinamides. There seems little doubt that the pyrroline reduction takes place by hyride transfer and we believe the close rate correlation with the flavin reduction lends support to the suggestion that the dihydronicotinamide–flavin reaction is also a hydride transfer reaction.

Acknowledgment. This work was supported by the National Science Foundation (PCM-7826256), the National Institutes of Health (GM-15188) and the Natural Sciences and Engineering Research Council of Canada. We acknowledge helpful discussions with Drs. L. C. Kurz and J. L. Kurz.

Registry No. 1, 2139-03-9; **2**, 17750-23-1; **3**, 7145-37-1; **4**, 952-92-1; **5**, 64881-17-0; **6**, 64881-21-6; **7**, 64881-18-1; **8**, 64881-20-5; 9, 64881-16-9; **10**, 53164-23-1; **11**, 64881-22-7; **12**, 64881-23-8; NMNH, 4229-56-5; NADH, 58-68-4; NADPH, 53-57-6; **16**·HCl, 80028-67-7.

(25) R. Stewart, in "Isotopes in Organic Chemistry", Vol. 2., E. Buncel and C. C. Lee, Eds., Elsevier, Amsterdam, 1976.
(26) R. Stewart and T. W. Toone, *J. Chem. Soc., Perkin Trans. 2*, 1243 (1978).
(27) E. M. Arnett and R. Reich, *J. Am. Chem. Soc.*, **102**, 5892 (1980).
(28) G. Blankenhorn and E. G. Moore, *J. Am. Chem. Soc.*, **102**, 1092 (1980).
(29) P. Hemmerich, comments following paper listed in ref 22.