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A Comparison of the Glutamate Dehydrogenase Catalyzed Oxidation of NADPH by Trinitrobenzenesulfonate with the Uncatalyzed Reaction

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Abstract: We have measured the rates of hydrogen transfer between trinitrobenzenesulfonate (TNBS) and ten 1,4-dihydropyridines and found them to be first order in both reactants. A plot of log k_2, the second-order rate constant for hydrogen transfer, against log K_d, the dissociation constant for cyano-complex formation, is linear with slope 0.57 if the nicotinamide analogues are excluded. The kinetic parameters for the glutamate dehydrogenase catalyzed hydrogen transfer between TNBS and NADPH or 3-acetylpyridine adenine dinucleotide (3APADH) have been determined and shown to be consistent with a random order mechanism in which at least one pair of equilibria, either K_1 and K_2 or K_1' and K_2' (eq 11), are established rapidly. Based on a comparison of the kinetic deuterium isotope effects for the enzyme catalyzed and spontaneous reactions and on a comparison of rate ratios for two 1,4-dihydropyridines, we conclude that there is greater carbon–hydrogen bond breakage at the transition state in the catalyzed reaction than in the spontaneous reaction.

Introduction

Nicotinamide coenzymes are essential cofactors in many enzyme-catalyzed hydrogen transfer reactions. Unlike many other essential cofactors, there have been few studies where the nonenzyme-catalyzed reactions serve as suitable models for the enzyme-catalyzed reactions. Some studies of note include models for alcohol dehydrogenases,[1-5] transhydrogenases,[6-8] and flavin reductases.[9,10]

In a related study Bates et al.[11] report that bovine liver glutamate dehydrogenase catalyzes the oxidation of NADH by trinitrobenzenesulfonate. They concluded[11] that the reaction must occur at the active site because the hydrogen atom transferred from the reduced coenzyme originates on the B side of the nicotinamide ring, as it does in the reductive amination of α-ketoglutarate and because added mononucleotides produce kinetic effects similar to those reported for the steady state oxidative deamination of glutamate. Several other dehydrogenases do not catalyze this reaction. Recently, Kurz and Frieden[12] reported that a series of 4-substituted-2,6-dinitrobenzenesulfonates react with NADH, obeying the Hammett relationship. We report here, a study of the kinetics of the glutamate dehydrogenase catalyzed reaction and the spontaneous reaction between TNBS and a series of 1,4-dihydropyridines.

Experimental Section

Materials. The following reagents were purchased from Sigma Chemical Co. and used without further purification: trinitrobenzenesulfonic acid, oxidized (NAD) and reduced (NADH) nicotinamide adenine dinucleotide, oxidized (NADP) and reduced (NADPH) nicotinamide adenine dinucleotide phosphate, oxidized (NMN) and reduced (NMNH) nicotinamide mononucleotide, and oxidized (3APAD) and reduced (3APADH) 3-acetylpyridine adenine dinucleotide. Nicotinamide, 3-acetylpyridine, 3-formylpyridine, 3-methyloximepyridine, 3-cyanopyridine, and benzyl bromide were purchased from Aldrich Chemical Co. and used without purification. Hexadeuterioethanol, 99%, was purchased from Merck and Co. and L-glutamic acid from Calbiochem. We prepared L-glutamic-*2-d* acid, greater than 99% isotopic purity, as previously described.[13] Glutamate dehydrogenase was purchased from Sigma Chemical Co. as a suspension in ammonium sulfate. The enzyme was prepared for use in 0.1 M potassium phosphate buffer, pH 7.6, as described previously.[14] Yeast alcohol dehydrogenase was obtained from Sigma Chemical Co. as a lyophilized powder. This powder was dissolved in 0.1 M potassium phosphate buffer, pH 7.6 containing 3 mM EDTA and dialyzed for 5 h in 900 ml of the same buffer, followed by three changes of 0.1 M phosphate buffer for 36 h. The enzyme solution was filtered through a 0.45 μ Millipore filter, and the concentration was determined from the absorbance at 280 nm.[15]

All spectra were recorded in 1-cm cuvettes in aqueous solution using a Cary Model 14 spectrophotometer. Repetitive scans were collected and stored with the aid of a Varian 620i computer which was interfaced directly to the photomultiplier of the spectrophotometer. Kinetic experiments were conducted using a Gilford Model 2000 spectrophotometer fitted with thermostated cell compartment, with the temperature controlled to ±0.1 °C. The pH was measured with a Radiometer Model 26 pH meter. Melting points were determined on Thomas-Hoover capillary melting point apparatus and are corrected.

***N*-Benzyl-1,4-dihydronicotinamide.** The method of Mauzerall[16] was followed using 0.25 g of *N*-benzylnicotinamide bromide[17] and 0.82 g of sodium dithionite. The yellow crystals obtained after two recrystallizations from aqueous ethanol had mp 105–122 °C (lit. mp

120–122 °C).[16] The spectrum had λ_{max} at 360 nm and molar absorptivity of 7.11 mM^{-1}. The NMR spectrum in hexadeuterioethanol with external Me_4Si had the following resonances: C(4) protons, δ 2.78 (pair of doublets, 2 H); benzyl methylene protons, 3.98, (2 H); C(5) proton, 4.38 (pair of triplets, 1 H); exchangeable protons, 4.82; C(6) proton, 5.47 (pair of quartets, 1 H); C(2) proton, 6.78 (1 H); phenyl protons, 6.95 (s, 5 H). The spectrum reported by Caughey and Schellenberg[18] for this compound in deuteriochloroform is identical, except for a downfield shift of approximately 0.32 ppm.

N-Benzyl-3-cyanopyridinium Bromide. To 4.0 g of 3-cyanopyridine in 50 ml of dioxane was added 6.6 g of benzyl bromide. After reacting for 2.5 days in the refrigerator, the crystals which had formed were filtered and washed repeatedly with benzene. This solid was recrystallized from ethanol and benzene (1:2) to yield a white solid, mp 147–149.5 °C. The NMR spectrum for this compound in D_2O had the following resonances: benzyl methylene protons, δ 5.85 (s, 2 H); phenyl protons, 7.13 (s, 5 H); C(5) proton, 8.27 (broad quartet, 1 H); C(4) and C(6) protons, 8.90, 9.17 (pair of broad doublets, 2 H); C(2) proton, 9.38 (broad singlet, 1 H). HDO occurred at δ 4.58 as a singlet.

Theor Anal. for $C_{13}H_{11}N_2Br$: C, 56.74; H, 4.03; N, 10.18; Br, 29.04. Found: C, 56.76; H, 3.97; N, 10.15; Br, 28.94.

N-Benzyl-1,4-dihydro-3-cyanopyridine. To 0.25 g of *N*-benzyl-3-cyanopyridinium bromide in 25 ml of water was added 25 ml of an aqueous solution of 0.50 g of Na_2CO_3 and 0.85 g of $Na_2S_2O_4$. A bright yellow solid slowly separated and was filtered and washed with an excess of water. This solid was recrystallized from approximately equal volumes of ethanol and water to give a solid, mp 56–58 °C, whose spectrum had λ_{max} at 345 nm. The NMR spectrum in hexadeuterioethanol (external Me_4Si) had the following resonances: C(4) protons, δ 2.75 (pair of doublets, 2 H); benzyl methylene protons, 4.02 (s, 2 H); C(5) proton, 4.33 (pair of triplets, 1 H); C(6) proton, 5.5 (pair of quartets, 1 H); C(2) proton, 6.47 (d, 1 H); phenyl protons, 7.00 (s, 5 H) with exchangeable protons from the solvent at δ 4.83. This compound is sensitive to light.

N-Benzyl-3-methyloximepyridinium Bromide. To a solution of 1.0 g of 3-methyloximepyridine in 25 ml of dioxane was added 1.42 g of benzyl bromide. The solid which separated was recrystallized from ethanol, mp 170–173 °C. The NMR spectrum in D_2O (external Me_4Si) had the following resonances: benzylmethylene protons, δ 5.77 (s, 2 H); phenyl protons, 7.35 (s, 5 H); C(5) proton, 7.98 (pair of doublets, 1 H); methyloxime proton, 8.22 (s, 1 H); C(4) and C(6) protons, 8.62, 8.82 (pair of broad doublets, 2 H); C(2) proton, 9.02 (broad s, 1 H). HDO resonance occurred as a singlet at δ 4.60.

Theor Anal. for $C_{13}H_{13}N_2OBr$: C, 53.26; H, 4.47; N, 9.56; Br, 27.26. Found: C, 53.02; H, 4.25; N, 9.38; Br, 27.04.

N-Benzyl-1,4-dihydro-3-methyloximepyridine. To 15 ml of an aqueous solution containing 0.52 g of Na_2CO_3 and 0.84 g of $Na_2S_2O_4$ was added 0.4 g of *N*-benzyl-3-methyloximepyridinium bromide. The solid which formed slowly was filtered and recrystallized from aqueous ethanol, mp 105–108 °C. The spectrum had λ_{max} at 348 and 298 nm. The NMR spectrum in hexadeuterioethanol (external Me_4Si) had the following resonances: C(4) protons, δ 2.68 (pair of doublets, 2 H); benzyl methylene protons, 3.95 (s, 2 H); C(5) proton, 4.33 (pair of triplets, 1 H); C(6) proton, 5.17 (pair of quartets, 1 H); C(2) proton, 5.92 (d, 1 H); phenyl protons, 6.77 (s, 5 H); methyloxime proton, 7.27 (s, 1 H). The rapidly exchangeable protons occurred as a singlet at δ 4.80.

N-Benzyl-3-acetylpyridinium Bromide. To a solution of 1.0 g of 3-acetylpyridine in 25 ml of dioxane was added 1.41 g of benzyl bromide. The solid which precipitated slowly was recrystallized from ethanol, mp 194.5–198.5 °C. The NMR spectrum in D_2O (external Me_4Si) had the following resonances: acetyl protons, δ 2.73 (s, 3 H); benzyl methylene protons, 5.90 (s, 2 H); phenyl protons, 7.32 (s, 5 H); C(5) proton, 8.23 (broad quartet, 1 H); C(4) and C(6) protons, 9.00 and 9.05 (pair of doublets, 2 H); C(2) proton, 9.40 (broad singlet). The HDO resonance occurred as a singlet at δ 4.63.

Theor Anal. for $C_{14}H_{14}NOBr$: C, 57.55; H, 4.83; N, 4.79; Br, 27.35. Found: C, 57.52; H, 5.00; N, 4.72; Br, 27.13.

N-Benzyl-1,4-dihydro-3-acetylpyridine. To an aqueous solution of 1.03 g of Na_2CO_3 and 1.69 g of $Na_2S_2O_4$, was added 0.8 g of *N*-benzyl-3-acetylpyridinium bromide. An oil separated from solution slowly and could not be induced to crystallize. The dark colored oil was separated into at least three components on thin-layer chromatography, using ethyl acetate as eluent. The fastest moving component was yellow and the spectrum had λ_{max} at 378 nm (lit. 371 nm).[19] The remaining components had λ_{max} at wavelengths shorter than 260 nm. The yellow component was isolated by thin-layer chromatography but could not be induced to crystallize. The NMR spectrum of this compound in hexadeuterioethanol (external Me_4Si) had the following resonances: acetyl protons, δ 1.83 (s, 3 H); C(4) protons, 2.68 (pair of doublets, 2 H); benzyl methylene protons, 4.12 (s, 2 H); C(5) proton, 4.58 (pair of triplets, 1 H); C(6) proton, 5.17 (pair of doublets); phenyl protons, 6.98 (s, 5 H); C(2) proton, 7.05 (d, 1 H). (Anderson and Berkelhammer[19] obtained crystals from ligroin, mp 61–67 °C.)

N-Benzyl-3-formylpyridinium Bromide. To a solution of 1.0 g of 3-formylpyridine in 25 ml of dioxane was added 1.60 g of benzyl bromide. An oil separated on standing over a 2-day period. The oil was collected, and a powder was obtained after extensive treatment with $CHCl_3$, mp 132–136 °C. Van Eys[20] reported that he was unable to crystallize the oil. This solid was used without further purification. The NMR spectrum in D_2O (external Me_4Si) had the following resonances: benzyl methylene protons, δ 4.80 (s, 2 H); hydrated aldehyde methine proton, 5.18 (s, 1 H); phenyl protons, 7.13 (s, 5 H); C(5) proton, 7.08 (broad quartet, 1 H); C(4) and C(6) protons, 7.67, 7.87 (pair of broad doublets, 2 H); C(2) proton, 8.00 (broad s, 1 H). The HDO resonance occurred as a singlet at δ 3.63.

N-Benzyl-1,4-dihydro-3-formylpyridine. To an aqueous solution of 0.76 g of Na_2CO_3 and 1.25 g of $Na_2S_2O_4$ was added 0.5 g of *N*-benzyl-3-formylpyridinium bromide. A dark oil separated slowly. The oil could not be induced to crystallize. When applied to a silica gel thin-layer plate, three components could be identified when eluted with ethyl acetate. The fastest moving of these was yellow and had a maximal absorbance at 370 nm. The compound was isolated from the silica gel using ethanol and was used without further purification. The NMR spectrum in hexadeuterioethanol (external Me_4Si) had the following resonances: C(4) protons, δ 2.70 (pair of doublets, 2 H); benzyl methylene protons, 3.82 (s, 2 H); C(5) proton, 4.67 (pair of triplets, 1 H); C(6) proton, 5.22 (pair of doublets, 1 H); C(2) proton, 6.83 (d, 1 H); phenyl protons, 7.00 (s, 5 H); formyl proton, 8.65 (s, 1 H).

1,4-Dideuterio-NADH. A solution of 0.418 g of NAD dissolved in Tris buffer was prepared, and 0.4 ml of hexadeuterioethanol and 0.2 ml of yeast alcohol dehydrogenase (31 mg/ml) were added. The progress of the reaction was monitored by periodically measuring the absorbance at 340 nm. When the reaction was complete, the solution was boiled for 5 min. The denatured protein was removed by filtration and the 4-monodeuterio-NADH was precipitated as the barium salt using the procedure of Rafter and Colowick.[21]

A solution of 0.403 g of monodeuterio-NADH was dissolved in 20 ml of 0.1 M potassium phosphate buffer, pH 7.6. A slight excess of sodium sulfate was added and the precipitate removed by filtration through a Millipore filter. A second solution, consisting of α-ketoglutarate (300 mM) and ammonium chloride (748 mM) in 4 ml of potassium phosphate buffer, pH 7.6 was prepared and mixed with the monodeuterio-NADH solution. Glutamate dehydrogenase (0.2 ml at 12 mg/ml) was added and the oxidation of coenzyme allowed to proceed to completion, the pH being maintained constant by addition of 0.1 M HCl. Monodeuterio-NAD was isolated using the final purification steps outlined by Kornberg.[22]

The monodeuterio-NAD was dissolved in 20 ml of 0.5 M Tris buffer and 0.2 ml of hexadeuterioethanol and 0.2 ml of yeast alcohol dehydrogenase (31 mg/ml) were added. The extent of reaction was monitored in the same way as described for monodeuterio-NADH. After boiling the solution on completion of the reaction and removing the denatured protein by filtration, the dideuterio-NADH was isolated by the method of Rafter and Colowick.[21] The precipitated barium salt of dideuterio-NADH was redissolved in 0.1 M Tris and reisolated by the above procedure. The white solid was used without further purification.

4,4-Dideuterionicotinamide Riboside. A solution of 0.1 g of dideuterio-NADH in 5.0 ml of water was prepared. A slight excess of sodium sulfate was added, and the precipitate was removed by centrifugation. The supernatant was added to 1.5 ml of 0.3 M sodium bicarbonate and 0.5 ml of 0.3 M magnesium chloride. Venom phosphodiesterase (3.9 units in 2 ml of water) from *Crotalus atorx* was added, and the pH of the solution was maintained at 8.2 by addition of 1 M potassium hydroxide. After approximately 30 min, 0.2 ml of an ammonium sulfate suspension of alkaline phosphatase was added, and the pH was adjusted to and maintained at 9.0 with 1.0 M potassium hydroxide. This mixture was left overnight. The precipitate which

Table I. Second-Order Rate Constants, k_2, for Hydrogen Transfer to TNBS for a Series of 1,4-Dihydropyridines and the Dissociation Constants, K_d, for Cyano-Complexes of the Corresponding Pyridinium Salts

R_1	R_2	k_2, M^{-1} min^{-1}	$10^5 \times K_d$, M
$CONH_2$	riboseP (NMNH)	8.36 ± 0.1	477.0 ± 150.0
$CONH_2$	RPPRA[a] (NADH)	7.15 ± 0.2	943.0 ± 28.0
$CONH_2$	RPPRPA[b] (NADPH)	9.10 ± 0.2	
$CONH_2$[c]	RPPRA	2.14 ± 0.2	
$CONH_2$[d]	RPPRA	4.53 ± 0.1	
$COCH_3$	RPPRA (3APADH)	1.55 ± 0.05	2.67 ± 0.08
$CONH_2$	CH_2Ph	96.3 ± 6.0	14 150.0 ± 150.0
$COCH_3$	CH_2Ph	11.8 ± 0.2	57.0 ± 6.0
CN	CH_2Ph	11.0 ± 0.3	24.7 ± 0.7
CHO	CH_2Ph	1.36 ± 0.05	1.07 ± 0.03
CH=NHOH	CH_2Ph	158.9 ± 12.0	6140.0 ± 150.0

[a] Ribosyl adenosine diphosphate. [b] Ribosyl adenosine-3′,5′-triphosphate. [c] 4,4-Dideuterio substitution. [d] 4-Monodeuterio substitution.

formed was removed by centrifugation, and the supernatant was boiled for 5 min. The denatured protein was removed by filtration through a Millipore filter. The filtrate was passed through an anion exchange column (AG1-X8, acetate form) and the fractions absorbing at 340 nm were collected and lyophilized. The isotopic composition of the white solid was determined by mass spectroscopy. The reduced 4,4-dideuterionicotinamide riboside contained less than 1.2% 4,4-dihydro-reduced-nicotinamide riboside and less than 4.3% 4-monodeuterio-reduced-nicotinamide riboside.

Kinetics. All kinetic measurements were made in nonamine buffers. Stock solutions of TNBS and of the 1,4-dihydropyridines were prepared in 0.1 M potassium phosphate at pH 7.6. The reaction was started by adding an aliquot of the 1,4-dihydropyridine solution to a TNBS solution. The extent of reaction was determined by following the disappearance of the 1,4-dihydropyridine absorption. The first-order rate constant, k, was calculated from a linear least-squares fit to eq 1, where t is time and A_t is the absorbance at time t.

$$kt = \ln [(A_\infty - A_0)/(A_\infty - A_t)] \quad (1)$$

Stock solutions of TNBS and reduced coenzyme to be used for enzyme kinetics were prepared as described above. Stock solutions of glutamate dehydrogenase containing approximately 0.95 mg/ml protein for use with NADPH and approximately 4.8 mg/ml for use with 3APADH were prepared. The concentrations of the enzyme and coenzyme stock solutions were determined spectrophotometrically. The reactions were started by adding aliquots of stock enzyme and stock coenzyme solutions to the TNBS solution in the thermostated cell compartment of the spectrophotometer. The initial rate of change in absorbance, at the λ_{max} for the coenzyme, was recorded. When NADPH was used, the initial rates were converted directly into the change in concentration of coenzyme. When 3APADH was used, it was necessary to correct the measured absorbance change for the contribution of the nonenzyme-catalyzed oxidation of 3APADH. This correction was made as follows. Using the concentration-independent second-order rate constant, k_2, from Table I, a first-order rate constant for each TNBS concentration can be calculated. The time required, $t_{1/x}$, to consume $1/x$ of the 3APADH initially present is given by eq 2.

$$t_{1/x} = \ln (1 - 1/x)/k_{calcd} \quad (2)$$

If $1/x$ is chosen small enough, an estimate of the initial rate of oxidation of 3APADH by the nonenzyme catalyzed reaction will be given by:

$$\Delta[3APADH] = [3APADH]_0(1/x)/t_{1/x} \quad (3)$$

Using the appropriate molar absorptivity, the absorbance change contributed by the spontaneous reaction is calculated and subtracted from the observed absorbance change, giving the enzyme catalyzed initial rate.

Cyano-Complex Formation. All experiments were performed in 0.1 M glycine, pH 9.7, as buffer. Stock solutions of the pyridinium salt, approximately 0.2 mM, and of KCN at the desired concentration were prepared in this buffer. Aliquots of the KCN stock solution were added to the pyridinium salt, and the absorbance at equilibrium was determined. The dissociation constant for complex formation was calculated from the concentration dependence of the measured absorbances (eq 4),

$$A_{obsd} = (\epsilon/2)[K_d + P_t + C_t - (K_d + P_t + C_t)^2 - 4P_tC_t] \quad (4)$$

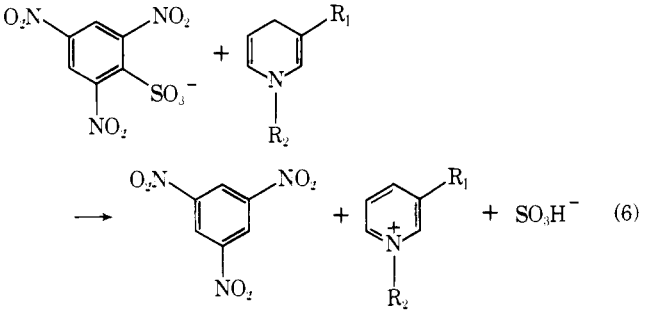
where ϵ is the molar absorptivity for the cyano-complex at λ_{max}, K_d is the dissociation constant, P_t is the total concentration of the pyridinium salt, C_t is the concentration of cyanide ion calculated from the concentration of KCN and the pK_a for cyanide (eq 5),

$$C_t = [KCN]_tK_a/(a_H + K_a) \quad (5)$$

a_H is the hydrogen ion activity determined at the glass electrode, and $[KCN]_t$ is the concentration of potassium cyanide added to the solution.

Results

We find that in addition to monitoring the decreasing absorbance of the 1,4-dihydropyridine, the reaction between TNBS and 1,4-dihydropyridines can be followed at 462 nm, the absorbance maximum for the trinitrobenzene-hydrogen sulfite complex characterized by Bernasconi et al.[23] When the concentration of TNBS is much larger than that of the 1,4-dihydropyridine, good first-order behavior is observed at both wavelengths giving the same rate constant. The rate constants for the reaction of TNBS with reduced nicotinamide adenine dinucleotide, NADH, are independent of pH when $5.0 \leq pH \leq 9.5$.[24] The rate constants for the reaction between TNBS and NADH increase as ionic strength and solvent polarity are increased. Added hydrogen sulfite has no effect on the observed rate constants at constant ionic strength. We found no kinetic solvent isotope effect for this reaction in D_2O, $k_{H_2O}/k_{D_2O} = 1.0$. These results indicate that 1,4-dihydropyridines are readily oxidized by 2,4,6-trinitrobenzenesulfonate anion to yield 1,3,5-trinitrobenzene, hydrogen sulfite, and the pyridinium salt (eq 6).



(6)

When the first-order rate constants, k, determined above are plotted against TNBS concentration, linear plots which pass through the origin are obtained. The slope of these plots gives the concentration-independent second-order rate constant, k_2. These rate constants are collected in Table I for the

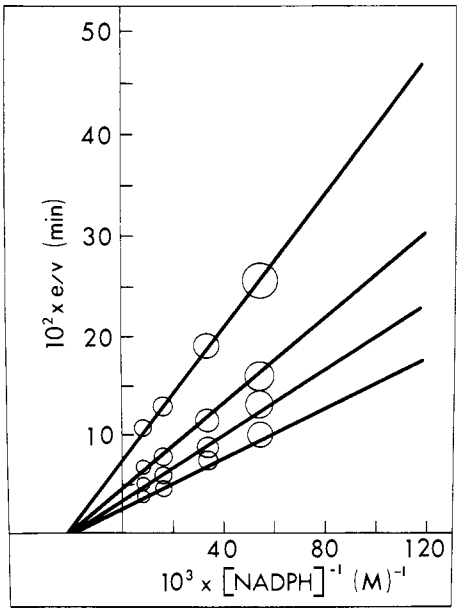


Figure 1. The double reciprocal plot of initial velocity vs. NADPH concentration for four concentrations of TNBS.

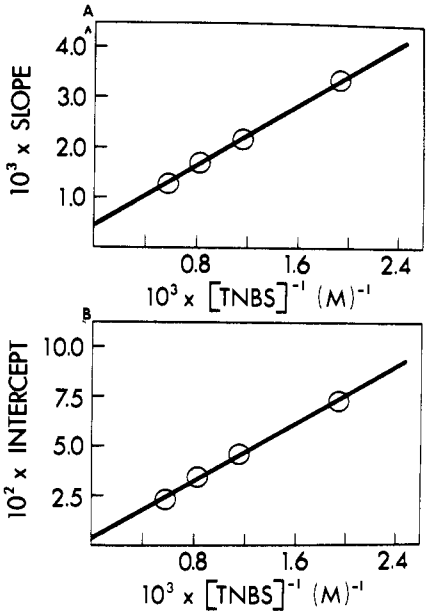


Figure 2. Replot of the slopes (A) and intercepts (B) from Figure 1 vs. the reciprocal of the TNBS concentration. The kinetic parameters, ϕ_1 and ϕ_{12}, are the intercept and slope, respectively, in A, and ϕ_0 and ϕ_2 are the intercept and slope, respectively, in B.

1,4-dihydropyridines studied. A significant kinetic deuterium isotope effect is observed when the C(4)-hydrogen atoms of NADH are replaced by deuterium, $k_2^H/k_2^D = 3.35$, representing the product of a primary and a secondary kinetic isotope effect.

The formation of a covalent complex between the oxidized pyridinium compounds and cyanide ion was also studied, and the pH independent dissociation constants determined are reported in Table I. Excellent agreement is obtained between the dissociation constants reported by Sund et al.[26] and those reported here for NAD, *N*-benzylnicotinamide, and 3APAD.

Glutamate dehydrogenase has been shown to catalyze the hydrogen transfer between NADH and TNBS.[11] We have extended the preliminary studies by examining the kinetic parameters of this reaction using NADPH and 3APADH as the reduced coenzymes.

It was necessary to establish that glutamate dehydrogenase does indeed catalyze the reaction between TNBS and these particular 1,4-dihydropyridines. This was done by varying enzyme concentration between 0.54 and 2.16×10^{-6} M, while the concentration of the substrates remained constant. Thus, when NADPH is 1.73×10^{-4} M and TNBS is 1.69×10^{-3} M, a plot of velocity vs. enzyme concentration has a slope of 0.0354 (mM/min)/(μM enzyme) and an intercept of 9.7×10^{-4} mM/min. The same plot for 3APADH at 2.14×10^{-4} M and TNBS 1.70×10^{-3} M had a slope of 3.22×10^{-4} (mM/min)/(μM enzyme) and an intercept of 4.24×10^{-4} mM/min. The intercepts on these plots correspond closely to the calculated initial rate of the spontaneous reaction. For 3APADH and TNBS at the concentrations above and with glutamate dehydrogenase at 2.06×10^{-6} M, the spontaneous reaction contributes more than 30% of the observed initial velocity. In contrast, when glutamate dehydrogenase is 0.63×10^{-6} M and NADPH and TNBS are at the above concentrations, the spontaneous reaction contributes approximately 4% to the observed velocity. Thus, at the enzyme concentrations used in this study, it was necessary to correct only the measured initial velocities for 3APADH (see Experimental Section for details).

The concentration dependence of the glutamate dehydrogenase catalyzed oxidation of NADPH by TNBS was investigated, and the double reciprocal plot of initial velocities vs. NADPH concentration is given in Figure 1. The best set of

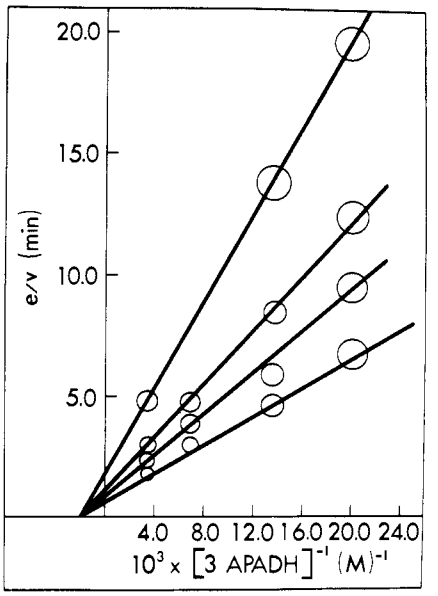


Figure 3. The double reciprocal plot of the corrected initial velocity vs. 3APADH concentration for four concentrations of TNBS.

straight lines was drawn through the data points, and the slopes and intercepts for these lines are plotted against the reciprocal of TNBS concentration in Figure 2. The corresponding double reciprocal plot for 3APADH, using the corrected initial velocities, is given in Figure 3, and the slopes and intercepts are plotted against the reciprocal of TNBS concentration in Figure 4.

The slopes and intercepts of the plots in Figures 2 and 4 were used to calculate the kinetic parameters, ϕ_0, ϕ_1, ϕ_2, and ϕ_{12} of eq 7.[27] In this equation, S_1 represents the concentration of the coenzyme and

$$e/V = \phi_0 + \phi_1/S_1 + \phi_2/S_2 + \phi_{12}/S_1S_2 \qquad (7)$$

S_2 that of TNBS. Alternatively, all of the data points were fitted to eq 7, to give the best overall fit. The agreement between the two methods was good, and the values for the kinetic parameters listed in Table II are the average of the two

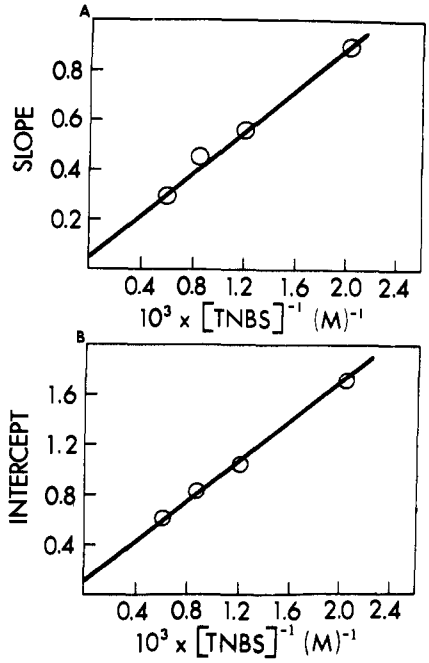


Figure 4. Replot of the slopes (A) and intercepts (B) from Figure 3 vs. the reciprocal of the TNBS concentration. The kinetic parameters, ϕ_1 and ϕ_{12}, are the intercept and slope, respectively, in A, and ϕ_0 and ϕ_2 are the intercept and slope respectively in B.

Table II. Kinetic Parameters for the Glutamate Dehydrogenase Catalyzed Reaction between TNBS and 1,4-Dihydropyridines

	NADPH	3APADH
ϕ_0 (min)	0.0036 ± 0.0013	0.24 ± 0.12
$10^3 \times \phi_1$ (M min)	0.000 375 ± 0.000 08	0.044 ± 0.005
$10^3 \times \phi_2$ (M min)	0.0352 ± 0.007	0.57 ± 0.05
$10^6 \times \phi_{12}$ (M^2 min)	0.001 52 ± 0.000 12	0.422 ± 0.001

methods along with the deviations from this average. Notably, the largest deviations are found for ϕ_0 and ϕ_1, the two parameters which make only small contributions to the observed velocity in the concentration ranges of this study.

We have measured the kinetic deuterium isotope effect for the glutamate dehydrogenase catalyzed hydrogen transfer between TNBS and 4,4-dideuterio-NADH. Under conditions where interference from the spontaneous reaction is minimal and at a single concentration of TNBS, the initial rates for the reaction with NADH and NADH-*4,4-d*$_2$, at several concentrations, were determined. The double reciprocal plot of velocity vs. NADH concentration (Figure 5) indicates that the kinetic isotope effect is reflected in V_{max} only, and is $V^H_{max}/V^D_{max} = 4.9 \pm 0.4$ at the concentrations used in this study.

Discussion

Bates et al.[11] have used spectral changes to describe the products and stoichiometry for the glutamate dehydrogenase catalyzed reaction between NADH and TNBS. We have confirmed that the same spectral changes occur when the enzyme is omitted from the reaction mixture. The rates of oxidation of NADH and the final equilibrium position for the uncatalyzed reaction are shown to be unaffected by the addition of products, indicating that the reaction is essentially irreversible. Thus, eq 6 accurately depicts the reaction under consideration.

The pH dependence of the oxidation of NADH by TNBS and the lack of a kinetic deuterium solvent isotope effect indicate that proton transfer to or from a component of the solvent preceding or during the rate-determining step of the reaction is unlikely. The reaction is favored by more polar solvents and by high ionic strength. Thus, it is unlikely that the transfer of hydrogen from 1,4-dihydropyridines to TNBS occurs by a mechanism involving hydrogen radicals, but rather by one involving polar transfer of hydrogen. Similar conclusions were reached by Kurz and Frieden[12] on the basis of substituent effects. Polar hydrogen transfer has been proposed previously as the preferred mechanism in enzymatic and nonenzymatic oxidations of 1,4-dihydropyridines.[4,8,28]

Several nicotinamide analogues were synthesized in which the carboxamide group is replaced by various substituents. We find that the rates of oxidation of the reduced analogues by TNBS are correlated very well with the dissociation constants for the covalent 4-cyanodihydropyridine complexes (Figure 6). It is apparent that most of the points fall on a single line of slope 0.57, the notable exceptions being NADH, NMNH, and

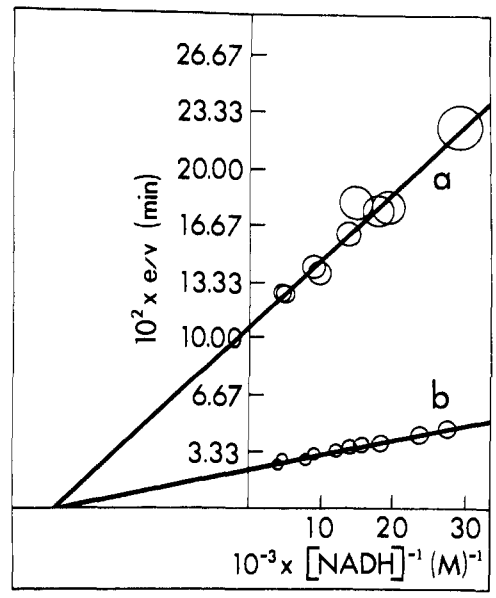


Figure 5. The double reciprocal plot of initial velocity vs. 4,4-dideuterio-NADH concentration (a), and NADH concentration (b), where TNBS concentration is 1.90×10^{-3} M.

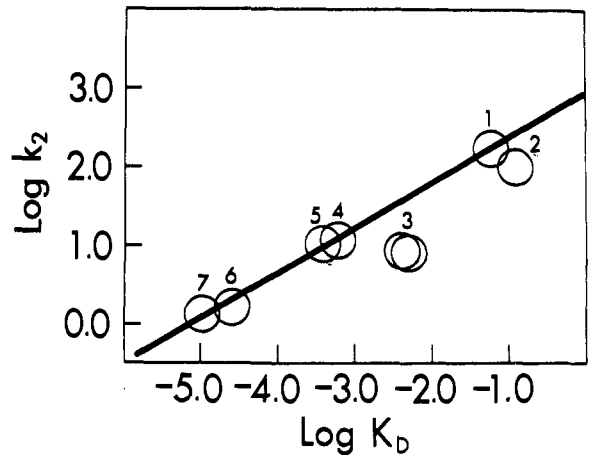


Figure 6. The plot of log k_2, the second-order rate constant for TNBS oxidation of 1,4-dihydropyridines, vs. log K_d, the dissociation constant for cyano-complex formation of the corresponding pyridinium salts. The points correspond to: (1) *N*-benzyl-3-methyloxime-1,4-dihydropyridine, (2) *N*-benzyl-1,4-dihydronicotinamide, (3) reduced nicotinamide adenine dinucleotide and reduced nicotinamide mononucleotide, (4) *N*-benzyl-3-acetyl-1,4-dihydropyridine, (5) *N*-benzyl-3-cyano-1,4-dihydropyridine, (6) reduced 3-acetylpyridine adenine dinucleotide, and (7) *N*-benzyl-3-formyl-1,4-dihydropyridine. The least-squares line through points 1, 4, 5, 6, and 7 has slope 0.57 and intercept 2.908. The correlation coefficient is 0.996.

N-benzyl-1,4-dihydronicotinamide, which all react significantly more slowly with TNBS than indicated by their cyano-complex dissociation constants. The slopes of plots of this nature are often correlated with the extent of bond making and bond breaking in the transition state.[29] The slope of the plot in Figure 6 indicates that there is considerable breaking of the C(4)–H bond in the transition state. The large kinetic deuterium isotope effect observed in the oxidation of NADH by TNBS also indicates that there is considerable stretching of the C(4)–H bond.[29,30]

Kaplan[31,32] has previously noted a preferred order of reactivity of nicotinamide analogues with nucleophiles and postulated that the oxidation potentials of the analogues are proportional to this reactivity. The correlation of reactivity with oxidation potential has been noted for several enzyme-catalyzed reactions where hydrogen transfer is thought to be the slow step.[33-35] Examples of a similar correlation of reactivity in nonenzyme-catalyzed reactions has been reported.[9,10] Thus, if cyano-complex formation is proportional to the oxidation potential of 1,4-dihydropyridines, the correlation of the rate constants for reactions of nicotinamide analogues with the dissociation constants for the cyano-complexes establishes a correlation of the oxidation potential with the reactivity of the nicotinamide analogue.

In a recent study of the reduction of isoalloxazines by 1,4-dihydropyridines, Bruice et al.[10] indicated that complex formation between the isoalloxazine and the adenine rings may be an additional factor affecting the rates. The significance of preequilibrium complex formation, as in eq 8,

$$S_1 + S_2 \overset{K_1}{\rightleftharpoons} S_1S_2 \overset{k_2}{\rightarrow} \text{products} \qquad (8)$$

in the reactions of 1,4-dihydropyridines has received much attention recently.[3,4,10] We have sought evidence, both kinetic and nonkinetic, in support of complex formation in the reaction of TNBS with 1,4-dihydropyridines. The unexpectedly low reactivity of NADH indicated in Figure 6 might appear to be evidence in support of nonproductive complex formation with the adenine ring, as suggested by Bruice et al.[10] However, both NMNH and *N*-benzyl-1,4-dihydronicotinamide are also less reactive than expected (Figure 6), and neither of these latter analogues possess an adenine ring with which to form a nonproductive complex. Thus, the lower rate of reaction of NADH with TNBS cannot be caused by noncovalent complex formation at the adenine ring. If preequilibrium complex formation with the pyridine ring occurs in the TNBS mediated oxidation of NADH, the complex cannot be detected spectrally or kinetically. Such a complex, if it exists, must be quite unstable and have a transient existence only.

The reaction between NADPH and TNBS is unique in that it is the first hydrogen transfer reaction of dihydropyridines where the rates of both the enzyme-catalyzed and spontaneous reactions are kinetically accessible. The kinetic ϕ parameters listed in Table II have been dissected into rate constants and equilibrium constants for several possible mechanisms.[27] Ordered mechanisms of the type shown in eq 9a and 9b

$$E + S_1 + S_2 \overset{k_1}{\underset{k_{-1}}{\rightleftharpoons}} ES_1 + S_2 \overset{k_2}{\underset{k_{-2}}{\rightleftharpoons}} ES_1S_2 \overset{k_3}{\rightarrow} \text{products} \qquad (9a)$$

$$E + S_1 + S_2 \overset{k_1'}{\underset{k_{-1}'}{\rightleftharpoons}} ES_2 + S_1 \overset{k_2'}{\underset{k_{-2}'}{\rightleftharpoons}} ES_1S_2 \overset{k_3}{\rightarrow} \text{products} \qquad (9b)$$

can be ruled out. For glutamate dehydrogenase, the formation of an enzyme-reduced coenzyme complex, ES_1, is known to be rapid (see below). Thus mechanism 9b can be eliminated, and preequilibrium formation of ES_1 in the ordered mechanism 9a reduces the expression for velocity to a three parameter equation. When the data were fitted with three kinetic parameters using a linear least-squares analysis of all of the data points, a notably poorer fit was obtained than when four kinetic parameters were used to fit the data in the same way. We have also considered Theorell-Chance[36] mechanisms, such as illustrated by eq 10.

$$E + S_1 \overset{k_1}{\underset{k_{-1}}{\rightleftharpoons}} ES_1 + S_2 \overset{k_2}{\underset{k_{-2}}{\rightleftharpoons}} ES_1' + S_2' \rightarrow \text{products} \qquad (10)$$

Assuming that S_1 corresponds to NADPH, then $k_1 = 4.4 \times 10^4\ M^{-1}\ s^{-1}$ and $k_{-1} = 1.9\ s^{-1}$. These values are significantly smaller than those reported by Di Franco and Iwatsubo,[37] $k_1 = 2.0 \times 10^6\ M^{-1}\ s^{-1}$ and $k_{-1} = 30\ s^{-1}$, and by Malcolm,[38] $k_1 = 7 \times 10^5\ M^{-1}\ s^{-1}$ and $k_{-1} = 150\ s^{-1}$. In addition, the dissociation of the weakly bound products from the enzyme surface is expected to be much faster than hydrogen transfer to the pseudosubstrate, TNBS. Thus, the Theorell-Chance[36] mechanism where NADPH is bound first is unlikely. The alternative order, where TNBS is bound first, is even less likely. When this reagent is incubated with glutamate dehydrogenase, it reacts rapidly with two lysine residues in the absence of NAD(P)H.[39] These two residues are thought to be near the active site. If TNBS were the first substrate bound, the competing amination reaction would prevent complete oxidation of NADPH, contrary to observation.

The only mechanism consistent with this set of kinetic parameters is a random order mechanism where at least one pair of equilibria, either K_1 and K_2 or K_1' and K_2' are established rapidly (eq 11). If S_1 represents NADPH, and the equilibria

$$E + S_1 + S_2 \overset{K_1}{\rightleftharpoons} ES_1 \overset{K_2'}{\rightleftharpoons} ES_1S_2 \overset{k_3}{\rightarrow} \text{products} \qquad (11)$$
$$\qquad\qquad\quad K_2 \rightleftharpoons ES_2 \rightleftharpoons K_1'$$

for K_1 and K_2 are rapidly established, we find $K_1 = 4.3 \pm 1.1 \times 10^{-5}$ M and $K_2 = 4.1 \pm 1.0 \times 10^{-3}$ M. The dissociation constant, K_1, shows good agreement with the 2.6×10^{-5} M obtained by Frieden[40] for NADPH during the reductive amination of α-ketoglutarate. In addition, the dissociation constants for TNBS, K_2, agree reasonably well when the coenzyme is changed. This identity in K_2 is required for the mechanism assumed. Due to the limited concentration range accessible, we were unable to obtain data where the kinetic parameters ϕ_0 and ϕ_1 could be determined more precisely. Despite this limitation, the large kinetic deuterium isotope effect for this reaction requires that hydrogen transfer occur during or prior to the slow step. Since the isotope effect is reflected only in the V_{max} parameter[41] (see Figure 5) it can be expressed by the following equation:

$$\frac{V^{H}_{max}}{V^{D}_{max}} = \frac{k_3{}^{H}}{k_3{}^{D}}\left(\frac{1 + \dfrac{K_1{}'^{(D)}K_2}{K_1{}^{(D)}[S_2]}}{1 + \dfrac{K_1{}'^{(H)}K_2}{K_1{}^{(H)}[S_2]}}\right) \approx \frac{k_3{}^{H}}{k_3{}^{D}} \qquad (12)$$

where the constants are those of eq 11. Replacement of the C(4) hydrogens of NADH with deuterium should have little or no effect on K_1 and K_1' and an even smaller effect on the ratio K_1'/K_1, allowing the simplification noted. Thus the slow step for the enzyme-catalyzed oxidation of coenzyme by TNBS must be hydrogen transfer.

Rate-limiting hydrogen transfer in both the spontaneous and enzyme-catalyzed reactions provides a unique opportunity to compare the transition state structure for the two reactions. The kinetic deuterium isotope effect for the enzyme-catalyzed reaction is considerably larger than that for the spontaneous reaction, indicating that the carbon-hydrogen bond breaking process in the enzyme-catalyzed reaction is more complete than in the spontaneous reaction. Further evidence in support of this comes from a comparison of relative rate constants for NADPH and 3APADH in the two processes, viz., $k_3{}^{NADPH}/$

$k_3^{3APADH} = 70$ and $k_2^{NADPH}/k_2^{3APADH} = 7.9$. If the rate constants for hydrogen transfer in the enzyme-catalyzed reaction are proportional to the dissociation constants of the cyano-complexes, this rate ratio corresponds to a slope of 0.72 in the plot of log k_3 vs. log K_D. Thus for this reaction, carbon–hydrogen bond breakage at the transition state is more complete on the enzyme surface than in solution.

An estimate of the small rate acceleration provided by glutamate dehydrogenase is obtained by comparing the rate constants for the enzyme-catalyzed and uncatalyzed reactions; for NADPH, $k_{enz}/k_2 = 30$ M and for 3APADH, $k_{enz}/k_2 = 3$ M. It must be kept in mind, however, that TNBS bears no structural resemblance to the natural substrates, α-ketoglutarate and glutamate. In fact, one might wonder why catalysis is observed at all, since other dehydrogenases do not catalyze this reaction.[11] Caughey et al.[42] have reported that substrates and inhibitors with electronegative substituents separated by approximately 7.4 Å are readily bound to glutamate dehydrogenase. If TNBS binds to glutamate dehydrogenase at the same site as these inhibitors, it is possible that catalytically productive binding occurs. Since catalysis of this reaction is observed, some assistance from the enzyme residues catalyzing the reductive amination of α-ketoglutarate may be evident. Alternatively, the accelerating effect of glutamate dehydrogenase for the coenzymes studied thus far is small enough that the enzyme may be acting only to increase the effective concentrations of the reactants. At the present time, we have no indication which residues could be involved in this reaction, or what function they would perform.

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