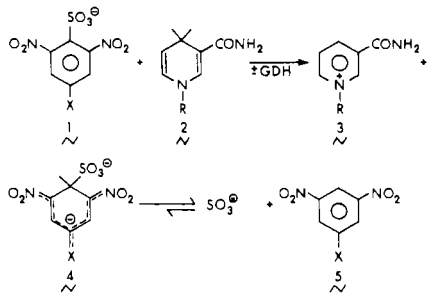
Comparison of the Structures of Enzymatic and Nonenzymatic Transition States. Reductive Desulfonation of 4-X-2,6-Dinitrobenzenesulfonates by Reduced Nicotinamide Adenine Dinucleotide†

Linda C. Kurz and Carl Frieden*

ABSTRACT: Transition state structures for enzymatic and nonenzymatic direct hydride transfer reductions of 4-X-2,6-dinitrobenzenesulfonates by NADH are compared using two experimental approaches. These are (1) electronic substituent effects, giving information about the transfer of charge, and (2) protium–deuterium isotope effects giving information about the transfer of the hydrogen nucleus. Hammett plots for the nonenzymatic reaction (ρ = 4.97 using σ constants) have been reported previously (Kurz, L. C., and Frieden, C. (1975), *J. Am. Chem. Soc. 97*, 677) and it was concluded that considerable negative charge has been transferred from reductant to oxidant in the transition state. A lower limit of 11 on the ρ value for the equilibrium constant is now reported. Thus the extent of charge transfer in the transition state is less than 0.5 and it is not likely that the electron is transferred in a prior equilibrium step. For the enzymatic reaction, no significant correlation between Michaelis constants and σ or σ^- is found while the substituent dependence of the maximal velocities, V_{max}, is precisely the same as that found for the nonenzymatic second-order rate constants, k_N. Log–log plots of V_{max} vs. k_N have a slope of 1.01 ± 0.06. The primary isotope effect on k_N is large, ~4.7, while the secondary effect is normal, ~1.2. It is concluded that the hydrogen nucleus is in flight in the transition state. Furthermore, with consideration of the electronic substituent effects, the electron and hydrogen nucleus transfers are closely coupled in these reactions. For the enzymatic reaction no isotope effects on Michaelis constants are found, while those on V_{max} are the same as those found for the nonenzymatic reaction. Thus the enzymatic reaction proceeds with a mechanism of prior equilibrium binding of both substrates followed by rate-determining hydride transfer. From these data, we conclude: (a) The structures of nonenzymatic and enzymatic transition states are quantitatively similar. (b) In these activated complexes the transfers of negative charge and the hydrogen nucleus are nearly synchronous.

L-Glutamate dehydrogenase (GDH[1]) catalyzes the reductive amination of α-ketoglutarate to glutamate using ammonia and either NADH or NADPH as coenzyme. The enzyme also catalyzes (Scheme I) the reductive desulfonation of 4-X-2,6-dinitrobenzenesulfonates, **1**, by NAD(P)H, **2**, to yield NAD^+, **3**, and the symmetrical sulfite Meisenheimer complexes, **4**, which rapidly dissociate to sulfite and the corresponding hydrocarbon, **5**. This reaction is stereospecific with respect to NAD(P)H, removing the same hydrogen from the 4 position of the coenzyme as in the normal reaction. Furthermore, the rate of the reaction is affected by the same purine nucleotides, such as GTP and ADP, which influence the rate of the normal reaction (Bates et al., 1970).

Scheme I



The reaction in Scheme I also occurs without enzymatic catalysis (Bates et al., 1970). The enzymatic catalysis of the reduction of the dinitrobenzenesulfonates by NADH makes valid comparisons with the nonenzymatic reaction more likely than several other dihydropyridine reductions which have been proposed as models for NAD^+-dependent dehydrogenases and for which several different chemical mechanisms have been postulated (Abeles et al., 1957; Suelter and Metzler, 1960; Steffens and Chipman, 1971; Creighton et al., 1973; Blankenhorn, 1976).

In a previous communication (Kurz and Frieden, 1975) the large effect of para substituents in the sulfonate on the second-order rate constant for the nonenzymatic reactions was described and we reported that electronic substituent effects on the maximal velocity of the enzymatic reactions were qualitatively similar. In addition both catalyzed and uncatalyzed reactions showed a large deuterium isotope effect on the rate of transfer of the hydrogen nucleus from the dihydropyridine to the sulfonate (Bates, 1972; Kurz and Frieden, 1975).

Thus, in both enzymatic and nonenzymatic reactions, the

† From the Department of Biological Chemistry, Division of Biology and Biomedical Sciences, Washington University School of Medicine, St. Louis, Missouri 63110. *Received May 7, 1977.* This work was supported in part by United States Public Health Service Grant AM 13332.

[1] Abbreviations used are: GDH, bovine liver glutamate dehydrogenase (EC 1.4.1.2); YADH, yeast alcohol dehydrogenase (EC 1.1.1.1); NADase, NAD nucleosidase (EC 3.2.2.5) from *N. crassa*; NAD^+, **3**, oxidized nicotinamide adenine dinucleotide; NADH, **2**, reduced nicotinamide adenine dinucleotide; NADPH, reduced nicotinamide adenine dinucleotide phosphate; [4,A-^{2}H]NADH, NADH stereospecifically labeled with deuterium in the A (*pro-R*) position; [4,B-^{2}H]NADH, NADH stereospecifically labeled with deuterium in the B (*pro-S*) position; [4,4-^{2}H]-NADD, NADH labeled with deuterium in both positions; 4-X-DNBS, 4-X-2,6-dinitrobenzenesulfonate where X = NO_2, CN, $CONH_2$, CF_3, Cl, COO^-, H; Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetic acid; IR, infrared.

step containing the chemical transformation, the hydride transfer, can be studied. It is our intention, insofar as possible, to determine the structures of the intermediates and transition states which lie along the reaction coordinates for these reactions. The response of several of the thermodynamic constants to changes in structure will be compared, using extrathermodynamic relationships, with the response of well understood rate processes and equilibria to analogous changes. With the addition of isotope effect data, we can obtain a quantitative comparison between the structures of the nonenzymatic and enzymatic transition states.

Experimental Section

Enzymes. GDH was purchased as a crystalline suspension in ammonium sulfate from Boehringer-Mannheim Corp. The crystals were dissolved in 0.1 M potassium phosphate buffer (K[PO_4]) (also containing 10^{-4} M EDTA) of the desired pH and dialyzed exhaustively to remove NH_4^+. After clarification, the A_{280}/A_{260} ratio was 1.9–2.0. The enzyme was assayed in a mixture containing 5 mM α-ketoglutarate, 20 mM NH_4Cl, 100 μM NADH and 50 mM potassium phosphate, 100 μM EDTA at pH 8.10. The specific activity of fresh preparations under these conditions was 2.25×10^4 μM min^{-1} mg^{-1} mL^{-1}. Enzyme solutions not used immediately were stored frozen at −20 °C and used only if their specific activity was >90% of the fresh preparation. YADH and NADase were used as obtained from Sigma Chemical.

Substrates. α-Ketoglutaric acid was recrystallized from acetone–benzene. Stock solutions (pH 8) were stored frozen at −20 °C. [4-^{1}H]NAD^+, [4,4-^{1}H]NADH, and [4,4-^{1}H]-NADPH (see footnote 1 for nomenclature) were used as obtained from Sigma.

[4,A-^{2}H]NADH was synthesized enzymatically in a reaction mixture (25 °C, pH 10.0) containing 14 mM [4-^{1}H]-NAD^+, 0.5 M ethyl-d_6 alcohol (99 atom % D, Merck), and 1 mg/mL YADH. The pH of the mixture was maintained by the addition of small amounts of a 1 N KOH solution. After the reaction had gone to completion (as determined from its 340 nm absorption), the pH was raised to 11.0 and the mixture was boiled 3 min to denature the enzyme. After centrifugation, the sample was diluted 90-fold and applied to a benzoylated-DEAE-cellulose column (Bio-Rad Cellex BD) in the HPO_4^{2-} form. Any NAD^+ present is slightly retarded by the column and elutes quantitatively with 1 mM K[PO_4], ~pH 8. The further development of the column with 2 mM K[PO_4], ~pH 8.2, elutes a 260-nm absorbing material (not reducible by alcohol dehydrogenase) not fully resolved from the 340-nm peak. After all the excess 260-nm absorbance was removed, the [4,A-^{2}H]NADH was eluted with 25 mM K[PO_4], pH 8.2. This material had an A_{260}/A_{340} ratio of ~2.3. Fractions were frozen and stored in liquid N_2. No changes in the absorbance spectrum of these samples were observed after storage for several months.

[4-^{2}H]NAD^+ was synthesized from [4,A-^{2}H]NADH as described above but without the isolation of the latter from the reaction mixture. After denaturation of YADH, the pH of the solution was lowered to 8 with 1 N HCl and appropriate amounts of stock solutions were added to give a final mixture 20 mM in α-ketoglutarate and NH_4Cl and 0.25 mg/mL GDH. After the oxidation had gone to completion, the mixture was boiled 3 min and centrifuged. After a 15-fold dilution the sample was applied to a DEAE (Whatman DE52)-cellulose column (0.3 mL column volume/mg of NAD^+) previously equilibrated with 0.05 M Tris-Cl, pH 8.0, 4 °C. NAD^+ and glutamate were eluted with 0.05 M Tris-Cl, pH 8.0. α-Ketoglutarate and NADH remained on the column. The NAD^+ containing fractions were combined and flash evaporated to a concentration of ~70 mM. For desalting the sample was applied to a G-10 Sephadex column (24 mL column volume/mL of NAD^+ solution) previously equilibrated with H_2O (pH 3–4). Fractions containing NAD^+ whose conductivity did not exceed background were combined and lyophilized.

[4-^{2}H]Nicotinamide was prepared from the [4-^{2}H]NAD^+ for the purpose of determining its isotopic purity. NAD^+ (1 mg/mL) was applied (3 mg NAD^+/mL column) to an AG50W-X8, Na^+ form, column (Bio-Rad) in order to exchange Na^+ for $Tris^+$. The NAD^+ was concentrated by evaporation and digested 24 h (0.03 mg of enzyme/mg of NAD^+) at 37 °C with NADase. After boiling the solution for 3 min to denature the enzyme, the pH was adjusted to 7 with 0.1 M NH_4OH. The sample was applied to an AG1-X8, Cl^- form, column (Bio-Rad). Nicotinamide was eluted with H_2O. Anionic species were retained on the column. The nicotinamide samples (monitored at 262 nm) were combined and evaporated carefully (nicotinamide sublimes) to the desired concentration. Samples (~10 μg) were placed in the direct probe sample holder of an LKB 9000 mass spectrometer and dried in the presence of desiccant at 1 atm pressure. Mass spectra were obtained in the region of the molecular ion. Sample and standards were prepared from the same NAD^+ lot. The protium content was calculated as suggested by Biemann (1962).

[4,4-^{2}H]NADD was prepared by reduction of [4-^{2}H]NAD^+ with YADH and deuterated ethanol in a manner similar to that described above for [4,A-^{2}H]NADH. Similarly [4,B-^{2}H]-NADH was prepared from [4-^{2}H]NAD^+ and unlabeled ethanol.

Based on the mole percent protium present at the C-4 position in the [4-^{2}H]NAD^+ starting material, the percent protium at the 4,B position on the [4,4-^{2}H]NADD and [4,B-^{2}H]NADH preparations was 2–3%. Calculated deuterium isotope effects were corrected for these isotopic impurities. This correction is a major concern in the accurate determination of large isotope effects. For example, with an isotope effect of 6 and a deuterated substrate containing 6% protium, a correction of over 15% must be applied to the experimentally observed H/D velocity ratio. Because of the complete stereospecificity for hydrogen transfer from one side of the dihydropyridine ring in the enzymatic reactions, and the partial specificity in the nonenzymatic reactions, calculations of the proper corrections requires knowledge of the isomer distribution of the protium contaminant as well as of the absolute amount present.[2]

4-X-2,6-Dinitrobenzenesulfonates (4-X-DNBS) were prepared (with the exception of X = NO_2 which was obtained commercially) by nucleophilic displacement of Cl^- by SO_3^{2-} from 4-X-2,6-dinitrochlorobenzenes. The prior equilibrium formation of a σ complex was suggested by the transient appearance of a red color (green for X = Cl) before development of the final color or precipitate. Because of the ready reversibility of the reaction and the limited solubility of reactants, the choice of solvent for these preparations is crucial. Condi-

[2] The first synthesis step is the production using YADH of [4,A-^{2}H]-NADH which contains no more (A position) protium than the 99%+ deuterated ethanol used in the preparation. However, for preparation of B and A,B labeled coenzyme, the YADH reaction mixture is reoxidized by the B-specific GDH to deuterated NAD^+ without removal of unreacted NAD^+ left over from the first step. The YADH-catalyzed reduction of this NAD^+ mixture with deuterated or unlabeled ethanol yields [4,4-^{2}H]NADD or [4,B-^{2}H]NADH with [4,A-^{2}H]NADH or NADH (diproteo) as the primary isotopic impurities, respectively. The mole percent protium present at the 4 position in the [4,4-^{2}H]NADD and [4-B-^{2}H]-NADH preparations then is given by that found at C-4 in the deuterated NAD^+ starting material (2–3%).

TABLE I: Preparative and Analytical Data for 4-X-2,6-Dinitrobenzenesulfonates.

X	Solvent	Elemental analyses[a] (Δ%) C	H	N	S
-CN[b]	CH_3CH_2OH	+0.21	−0.02	−0.11	−0.05
-$CONH_2$[c]	H_2O	−0.02	−0.10	+0.13	+0.13
-$COOCH_3$[d]	CH_3OH	+0.27	+0.04	+0.19	+0.10
-CF_3[e]	CF_3CH_2OH	−0.18	+0.06	+0.17	+0.27
-Cl[f]	$HOCH_2CH_2OH$	−0.01	−0.11	+0.01	−0.12
-COO^-	g	−0.21	+0.06	−0.13	−0.24
-H[h]	$HOCH_2CH_2OH$	+0.05	−0.21	−0.03	−0.06

[a] Δ% = absolute deviation from theoretical. Elemental analyses were carried out by Galbraith Laboratories, Inc. [b] SO_3^{2-} generated from HSO_3^- in situ. The method is similar to that used by Golumbic et al. (1946) for preparation of X = NO_2. Requires reflux with N_2 sweep for ~17 h. [c] Heated gently until all starting material went into solution. [d] Requires reflux for ~30 h. [e] Refluxed until a sample of the reaction mixture contained no water insoluble material. [f] The starting material, 2,6-dinitro-1,4-dichlorobenzene was prepared from 4-chloro-2,6-dinitrophenol as described by Matsumoto (1965). A suspension of K_2SO_3 and the chlorobenzene was heated gently until the color deepened to dark orange. The sulfonate was precipitated with isopropyl alcohol. [g] Prepared by acid hydrolysis of 4-$COOCH_3$-DNBS in 1 M trifluoroacetic acid. Hydrolysis continued until a sample evaporated to dryness and redissolved in D_2O showed no CH_3 protons in its NMR spectrum. [h] Preparation similar to 4-Cl-DNBS.

tions found acceptable for these syntheses along with analytical data are listed in Table I.

Products. 3,5-Dinitrochlorobenzene was synthesized from 3,5-dinitroaniline by the method described by Gunstone and Tucker (1963) but the work-up was that described by Welsh (1941). 3,5-Dinitro-α,α,α-trifluorotoluene was prepared from the 4-chloro compound by a method similar to that described by Newman and Blum (1964). These products had elemental composition and IR spectra consistent with their proposed structure. These, as well as the other hydrocarbon products obtained commercially, were recrystallized to constant extinction coefficient at 340 nm.

By-Products. ε-Mono-4-cyano-2,6-dinitrophenyl-L-lysine and ε-monotrinitrophenyl-L-lysine were prepared by the method of Okuyama and Satake (1960) except that the reaction mixture for the cyano adduct was kept at room temperature for 3 days. The absorption maximum for the cyano adduct was found at 430 nm with a molar extinction coefficient of 6.3×10^3. The ratio of absorbance at 430 relative to that at 340 was found to be 3.30. Elemental analyses for C, H, and N were within 0.1% of theoretical.

Kinetic Measurements. The reaction shown in Scheme I was studied in 50 mM potassium phosphate buffers, 100 μM EDTA, pH 8.10, I = 0.12. Exceptions to these conditions are noted in the text. Stock solutions of sulfonate salts for kinetic runs were prepared in H_2O. (It should be noted that neutralization of a solution of the free acid gives some hydrolysis to the highly absorbing phenol. In addition, sulfonates are considerably less soluble in the final reaction mixture containing buffer salts than in water.) Runs were initiated by addition of a solution of one component (usually the sulfonate) using a Hamilton syringe to a cuvette containing a thermally equilibrated (25.0 ± 0.1 °C) reaction mixture. Mixing was accomplished via a neoprene disc stirrer attached to the needle of the syringe which was raised and lowered under the surface of the solution prior to starting absorbance measurements. The delivery syringe was thermostated whenever addition volumes exceeded 10% of the volume of the reaction mixture. Solutions not containing enzyme were passed through a Millipore filter. Initial velocities were measured by following the disappearance of the dihydropyridine and sulfonate absorbances at 340 nm. The latter contributed 4–6% to the total extinction coefficient difference. Most enzyme rates were obtained using a Gilford 240 spectrophotometer equipped with an expanded scale recorder (0–0.1 absorbance) and automatic cell changer. Runs requiring more sensitivity and stability were made using Cary 16K or Cary 118 spectrophotometers. The latter was used with a dual wavelength monitor when measurements at two wavelengths were required for the same reaction mixture.

Kinetic Constants: Nonenzymatic Reaction. The low reactivity of most of the compounds and their high absorbance severely limit the range of concentrations for studying the reaction. With the exception of the p-NO_2 compound (with which experiments over a wide concentration range are feasible), second-order rate constants were calculated from initial velocities obtained from <5% of the expected change. Corrections for degradation of the coenzyme and sulfonate were applied when necessary.

Kinetic Constants: Enzymatic Reaction. Initial velocities were obtained over as wide a concentration range (of both substrates) as feasible. The absorbance changes at 340 nm were corrected for the velocity of the nonenzymatic reaction (as measured under the same conditions in the absence of the enzyme) and, when necessary, the absorbance change resulting from modification of lysine groups on the enzyme (vide infra). Velocities were further adjusted for differences in enzymatic specific activity (monitored frequently in long experiments).

The more reactive p-NO_2 and p-CN sulfonates undergo a nucleophilic displacement of sulfite by unprotonated amino groups on the enzyme to yield the highly absorbing nitrophenylated amino acids.[3] Because of the greatly decreased velocity of the enzymatic reduction rate which results when coenzyme labeled with deuterium at C_4, B is used as substrate, the modification reaction makes a significant contribution to the change in absorbance during the reduction reaction (but not when [4,B-^{1}H]NADH is substrate) even though its actual extent is small. As is the case with many enzymes, the rates of chemical modification are significantly changed in the presence of substrates or effectors.[4] We are thus required to measure the rate of modification by the sulfonate substrate simultaneously with our measurement of the rate of its enzyme catalyzed reduction. Fortunately, it is only the absorbance contribution from the chemical modification which causes difficulty. The modification with the p-NO_2 compound has been thoroughly studied (Coffee et al., 1971; Goldin and Frieden, 1971) and under our conditions does not alter the kinetic properties of the enzyme.

We have prepared the ε-mono-4-X-2,6-dinitrophenyl-L-lysine for X = NO_2 and CN and studied their spectra. The absorbance maximum (430 nm) for the p-CN (but not for the p-NO_2) modification product was found to be well resolved from the wavelength (NADH, 340 nm) used to follow the reduction. We monitored each reaction mixture at both wavelengths and, using the 430/340 nm extinction coefficient ratio obtained from the spectra of the modified lysine, we obtained the corrected 340-nm rate.

[3] The modification causes no difficulty with the less reactive sulfonates. Apparently the substituent dependence of the rate of the modification reaction is considerably greater than that of the rate of the reduction. These sulfonates may be of interest to protein chemists as they would provide a series of lysine modification reagents of a wide range of reactivity.

[4] In addition, the time course of the modification reaction is complex. During the time interval of interest, the modification by 4-NO_2-DNBS (in the absence of coenzyme) shows two phases.

As a result of the overlap in the spectra of the products of the modification and reduction reactions, only an approximate correction can be obtained for the *p*-NO_2 compound. Our data for the modification by the *p*-CN compound and previous experience in this laboratory with that by the *p*-NO_2 compound suggest that, since it appears that the chemical modification rate is slower in the presence of NADH than in its absence (unpublished), the true rate would lie between that actually observed and that corrected assuming the modification rate observed in the absence of coenzyme. This range of values will be given where appropriate.

This situation is eased by lowering the pH from 8 to 7 where the fraction of unprotonated amino groups and therefore the modification rate are smaller. In addition (vide infra) the sulfonate Michaelis constant is about threefold smaller and the maximal velocity about threefold greater at pH 7 than at 8, both serving to diminish the importance of the modification reaction. Nevertheless the problem remains significant.

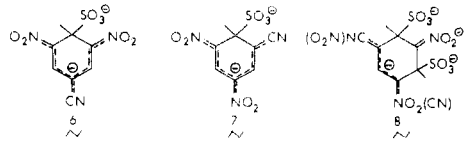
The corrected initial velocity data, v_i, were fitted by the method of least squares (nonlinear) to eq 1 or 2 (Busing and Levy, 1962)

$$\frac{v_i}{[E]} = \frac{V_M}{1 + \frac{K_A}{[A]} + \frac{K_B}{[B]} + \frac{K_A K_B}{[A][B]}} \quad (1)$$

$$\frac{v_i}{[E]} = \frac{V_M}{1 + \frac{\alpha K_A}{[A]} + \frac{\alpha K_B}{[B]} + \frac{A K_A K_B}{[A][B]}} \quad (2)$$

where $v_i/[E]$ is the observed velocity divided by the enzyme concentration; V_M is the maximal velocity; K_A and K_B are the Michaelis constants for substrate A (NADH) and B (sulfonate), respectively, and α is the interaction factor.

Apparent Dissociation Constant for Sulfite Complexes of 4-CN-2,6-Dinitrobenzene. Visible spectra of dilute aqueous solutions of sodium sulfite (~3 mM) and 4-CN-2,6-dinitrobenzene (~75 μM) were recorded. While neither reactant alone absorbs at these concentrations, mixtures showed absorption maxima at 400 and 510 nm. This absorbance spectrum, which is very similar to those obtained for a variety of nucleophile-nitrobenzene solutions (Strauss, 1970), results from formation of anionic cyclohexadienate σ complexes. In analogy with other 1-substituted 3,5-dinitrobenzenes (Foreman and Foster, 1969; Crampton and Khan, 1972), a mixture of symmetric, **6**, and asymmetric, **7**, complexes results with a great preponderance of the latter. As with trinitrobenzene (Crampton, 1967) 1:1 complexes (**6** and **7**) are formed in dilute solutions but increases in sulfite concentration (or ionic strength) result in the formation of 1:2 complexes, **8**, before



conversion into **6, 7** is complete. In 1 M $NaSO_3$, 1 M Na_2SO_4 solutions all absorbance from the mono complexes has disappeared. Absorbances[5] were obtained for a series of solutions, all 75 μM in hydrocarbon, with sulfite concentrations from 3 to 50 mM. The data at the higher sulfite concentrations required correction for the presence of the 1:2 complex (which only makes a significant contribution to the 400 nm absorbance, $\epsilon = 5.91 \times 10^3\ M^{-1}$). The data were fit by the method of least squares (Busing and Levy, 1962) to eq 3

$$a/A = (1/K\epsilon)(1/b) + 1/\epsilon \quad (3)$$

where a is the constant hydrocarbon concentration (75 μM); A is the absorbance at 510 nm, b is the varied sulfite concentration; K is the association constant; and ϵ is the molar extinction coefficient for the complexes (at 510 nm). K was found to be 23.16 ± 0.50 M^{-1} and $\epsilon_{510} = 1.178 \pm 0.025 \times 10^4\ M^{-1}$.

Results

Kinetic Constants: Nonenzymatic Reaction. The second-order rate constants for the nonenzymatic reaction (pH 8.10, 0.05 M potassium phosphate buffer, 10^{-4} M EDTA, 25.0 °C) are given in Table II. Within experimental error, the same values are obtained at pH 7.00 as at pH 8.10.

Using this reaction we determined the difference in reactivity of the A and B hydrogens of the coenzyme[6] (where R in **2** is adenine diphosphoribose) by measuring, with 4-CN-DNBS as substrate, the apparent rate constants for hydride transfer from [4,4-^{1}H]NADH, [4,B-^{2}H]NADH, [4,A-^{2}H]NADH, and [4,4-^{2}H]NADD.[7]

Assuming that the secondary isotope effect is the same for both A and B transfer, we obtained a value of 1.18 ± 0.03 (five determinations). Values as high as 2.3 have been obtained for other reactions (San Pietro et al., 1955).

In order to correct apparent primary isotope rate effects for the secondary ones, values for both this reactivity ratio and for the secondary isotope effect are required (see below). The latter is given by the same experiment and was found to be 1.207 ±

[5] The absorbance of these solutions may undergo a biphasic decrease with time. The first phase can be eliminated (presumably by exclusion of O_2) by purging all solutions with N_2 gas (<5 ppm O_2). The second phase (first order in both SO_3^{2-} and hydrocarbon) could not be eliminated but is sufficiently slow to allow extrapolation of the absorbance to zero time.

[6] The conformation of the coenzyme in solution is responsible for this difference. Physical and chemical evidence (San Pietro et al., 1955; Velick, 1958; Oppenheimer et al., 1971; and others) supports the proposal that open and folded forms of the coenzyme are in rapid equilibrium in aqueous solutions. In the folded form, the pyridine ring lies against the adenine which sterically hinders approach of an oxidant to the B side.

[7] Apparent rate constants (*C, D, E, F* in eq 4) for hydride transfer from [4,4-^{1}H]NADH, [4,B-^{2}H]NADH, [4,A-^{2}H]NADH, and [4,4-^{2}H]NADD are the sum of the two different rate constants for A and B hydrogens.

$$k_{H,H}^{A} + k_{H,H}^{B} = C;\ k_{H,D}^{A} + k_{D,H}^{B} = D;$$
$$k_{D,H}^{A} + k_{H,D}^{B} = E;\ k_{D,D}^{A} + k_{D,D}^{B} = F \quad (4)$$

In each rate constant, $k_{m,n}^{i}$, i indicates the side (A or B) from which the hydrogen nucleus m (H or D) is transferred with the isotope n (H or D) occupying the other position. Assuming that the secondary isotope effect on deuterium transfer is the same as that on protium transfer, then the rate constants can be factored (eq 5)

$$k_{D,D}^{A} = k_{D,H}^{A}(k_{H,D}^{A}/k_{H,H}^{A});\ k_{D,D}^{B} = k_{D,H}^{B}(k_{H,D}^{B}/k_{H,H}^{B}) \quad (5)$$

and that the secondary isotope effect is the same for both A and B transfer (eq 6)

$$k_{H,D}^{B}/k_{H,H}^{B} = k_{H,D}^{A}/k_{H,H}^{A} \quad (6)$$

then the reactivity ratio, r (eq 7)

$$r = k_{H,H}^{A}/k_{H,H}^{B} = k_{H,D}^{A}/k_{H,D}^{B} = k_{D,H}^{A}/k_{D,H}^{B} = k_{D,D}^{A}/k_{D,D}^{B} \quad (7)$$

can be obtained as one of the roots of the polynomial (eq 8).

$$(FC - ED)r^4 + (E - D)^2 r^3 + 2(E^2 + D^2 - ED - FC)r^2 + (E - D)^2 r + FC - ED = 0 \quad (8)$$

With these same assumptions the rate of the secondary isotope effect, SIE, is given by eq 9.

$$\mathrm{SIE} = [(E + D)/2F] - \{[(E + D^2)/2F] - (C/F)\}^{1/2} \quad (9)$$

TABLE II: Kinetic Constants for the Enzymatic and Nonenzymatic Reactions[a] in 0.050 M K[PO_4] buffer, 10^{-4} M EDTA, pH 8.10, I = 0.12, 25 ± 0.1 °C.

X[b]	$10^3 \times k_N$ (M^{-1} s^{-1})	$10^2 \times V_m$ (s^{-1})	$10^5 \times K_A$ (M)[c]	$10^3 \times K_B$ (M)[d]	N[a,e]
$-NO_2$	164.2 ± 5.7	237 ± 80	3.14 ± 0.20	4.43 ± 0.21	88
-CN	31.07 ± 0.27	30.3 ± 1.0	5.72 ± 0.33	7.44 ± 0.28	52
$-CONH_2$	3.745 ± 0.045	3.35 ± 0.54	4.94 ± 0.36	4.51 ± 0.26	54
$-CF_3$	1.672 ± 0.010	1.57 ± 0.14	4.72 ± 0.23	1.00 ± 0.10	42
$-COO^-$	0.3210 ± 0.0082	0.462 ± 0.041	4.44 ± 0.53	9.0 ± 1.1	47
-Cl	0.180 ± 0.011				
-H	0.0132 ± 0.0022				

[a] Parameters for enzymatic reaction from least squares fit of the data to eq 1. [b] X is the substituent in the 4 position of the sulfonate (1 of Scheme I). [c] A = NADH. [d] B = sulfonate with X in para position. [e] Number of observations on which least-squares fits to eq 1 were determined.

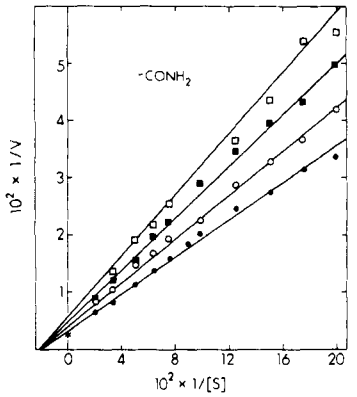


FIGURE 1: Lineweaver-Burk plot for the reduction of 4-carboxamido-2,6-dinitrobenzenesulfonate by NADH, catalyzed by glutamate dehydrogenase, where the sulfonate is designated as S. [NADH] = 195 μM (●), 100 μM (○), 65 μM (■), 45 μM (□). [GDH] ≅ 0.04 mg/mL, 0.05 M K[PO_4], 10^{-4} M EDTA, pH 8.10, 25 °C. Solid lines were calculated from the least-squares parameters given in Table II. * indicates the maximal velocity.

0.020. Although only determined for the 4-CN-DNBS, these values were assumed to be the same for the other sulfonates.

Kinetic Constants: Enzymatic Reaction. The corrected initial velocity data were fit to eq 1 and 2 using a nonlinear least-squares program (Busing and Levy, 1962). With the exception of the 4-NO_2-DNBS and 4-CN-DNBS, the kinetic parameters are essentially the same using either equation. For the 4-CN and 4-NO_2 compounds the values of the Michaelis constants, but not the maximal velocities, are somewhat different. However, the standard error of estimate (goodness of fit) was not improved by the addition of the fourth parameter. Thus the quality of the data for these two substrates does not allow an unambiguous choice between the two equations (eq 1 and 2). For convenience we have assumed that the three parameter equation is appropriate for all the sulfonates and the kinetic constants so obtained are given in Table II. It should be noted that our interpretations of these data are independent of which equation is used to fit the data. Although catalysis of the reduction of 4-Cl- and 4-H-DNBS occurs, it is not possible for technical reasons to obtain kinetic constants for these substrates.

A double-reciprocal plot of the data using 4-$CONH_2$-DNBS is shown in Figure 1. The solid lines were calculated from the three least-squares parameters resulting from the fit of the data to eq 1. The asterisk on the ordinate shows the extrapolated maximal velocity.

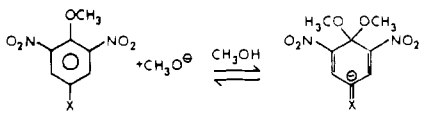
Electronic Substituent Effects: Nonenzymatic Reaction. We have previously reported (Kurz and Frieden, 1975) that the second-order rate constants for the nonenzymatic reaction obey a linear free-energy correlation with σ and σ^- substituent constants. The correlation with σ yields a ρ of 4.97 ± 0.31 and that with σ^- a ρ of 3.24 ± 0.31 both with correlation coefficients of 0.982.

Electronic Substituent Effects: Enzymatic Reaction. The effects of para substituents on the kinetic constants for the enzymatic reaction have been determined and are compared with those for the nonenzymatic reaction in Figure 2 which shows a plot of the log of the enzymatic maximal velocity (obtained from the three-parameter (eq 1) least-squares analyses) vs. the log of the nonenzymatic rate constant. The plot is linear with a slope of 1.012 ± 0.055 and the correlation coefficient is 0.996. A plot using maximal velocities from fits to the four-parameter equation (eq 2) is also linear with a slope of 1.030 ± 0.056. It should be noted that the 4-CF_3DNBS is *less* reactive than the 4-$CONH_2$ in both the enzymatic and nonenzymatic reactions; this order is the reverse of that predicted by the σ or σ^- substituent constants,[8] Exner (1972).

The inset in Figure 2 is a plot of the log of the product of the Michaelis constants for the two substrates vs. σ substituent constants. A linear regression yields a slope of −0.40 ± 0.36 with a correlation coefficient of only 0.44, a value too small for the correlation to be significant.

Deuterium Isotope Effects: Nonenzymatic Reaction. Table III lists the observed ratio of second-order rate constants (R) for oxidation of [4,4-^{1}H]NADH to that for [4,4-^{2}H]NADD (the dideuterated coenzyme). While most of these ratios are corrected for the degradation of the coenzyme, the degradation is responsible for most of the absorbancy change observed during the reduction of 4-Cl- and 4-H-DNBS by [4,4-^{2}H]-NADD and we were unable to accurately determine the H/D rate ratio for these sulfonates.

[8] In a highly analogous reaction (eq 10; Crampton et al., 1974, and references therein)



(10)

the substituent effect of p-CF_3 and p-CO_2CH_3 (very similar to p-$CONH_2$) on the Meisenheimer complex formation constant is also reversed from that expected. A possible explanation is suggested by the data reported by Holmes and Thomas (1975) who show that there is significant electron *donation* to the ring in trifluoremethylbenzenes, opposing the field effect of the large CF dipole moment. Reaction series with greater sensitivity to resonance than to field effects, such as ours, will respond differently to p-CF_3 than will series such as benzoic acid dissociation, where resonance stabilization is less important.

TABLE III: Primary Deuterium Isotope Effects.

X	σ^b	Rate ratio[c] (R)	DIE[e]
		Nonenzymatic Reaction[a]	
$-NO_2$	0.78	5.57 ± 0.18	4.81 ± 0.17
$-CN$	0.69	5.54 ± 0.17	4.78 ± 0.17
$-CONH_2$	0.46	5.43 ± 0.08	4.68 ± 0.10
$-CF_3$	0.53	3.74 ± 0.02	3.17 ± 0.06
$-COO^-$	(0.28)[d]	5.7 ± 0.6	4.9 ± 0.5
		Enzymatic Reaction[f]	
$-NO_2$	0.78	4.56 − 6.5[g]	4.3 − 6.3
$-CN$	0.69	5.57 ± 0.15	5.36 ± 0.34
$-CONH_2$	0.46	5.50 ± 0.34	5.67 ± 0.48
$-CF_3$	0.53	4.89 ± 0.38	4.92 ± 0.48
$-COO^-$	(0.28)[d]	4.79 ± 0.06	4.80 ± 0.29

[a] With 0.05 M K[PO_4], 10^{-4} M EDTA, pH 8.10, 25 °C. For 4-CN-DNBS the value found at pH 7.00 was the same within experimental error. The [4,4-^{2}H]NADD contained 1.91 ± 0.15 mol % protium at C-4. [b] Values given by Exner (1972). [c] Rate ratio = initial velocity ratio of NADH to [4,4-^{2}H]NADD before corrections for isotopic impurity, secondary isotope effect, and difference in chemical reactivity of A and B sides of the coenzyme (in the nonenzymatic reaction) but after correction for enzyme modification. [d] The σ for COO^- is ionic strength dependent. The value given was calculated from the Hammett equation defined by the other substituents (Kurz and Frieden, 1975). [e] For the nonenzymatic reaction; corrected for protium content and secondary isotope effect according to DIE = $(R/S)\,[XfS + (1-f)(1+X)]/[1+X-fR]$, where f is the atom fraction of H at C-4 in the [4,4-^{2}H]NADD preparation, and S and X are the secondary isotope effect (1.21) and A/B reactivity ratio (1.18) found for 4-CN-DNBS and assumed to be the same for the other sulfonates. For the enzymatic reaction; corrected for protium content and secondary isotope effect according to DIE = $(R/1.14)(1-f)/(1-Rf)$ where f is the protium content of [4,4-^{2}H]NADD (assumed to be [4,B-^{2}H]NADH) and 1.14 is the secondary isotope effect. This equation assumes the enzyme is specific for B transfer (Bates et al., 1970). [f] With 0.05 M K[PO_4], 10^{-4} M EDTA, pH 7.00, 25 °C, [coenzyme] ≈ 200 μM, [sulfonate] ≈ 1 mM. For 4-CN-DNBS the value found at pH 8.10 was the same within experimental error. [4,4-^{2}H]NADD contained 1.91 ± 0.15 mol % protium at C-4 for X = NO_2, CN and 3.21 ± 0.77 mol % for X = $CONH_2$, CF_3, COO^-. [g] Higher value assumes no contribution from modification reaction. Lower value assumes contribution from the modification is the same in the presence as in the absence of substrates.

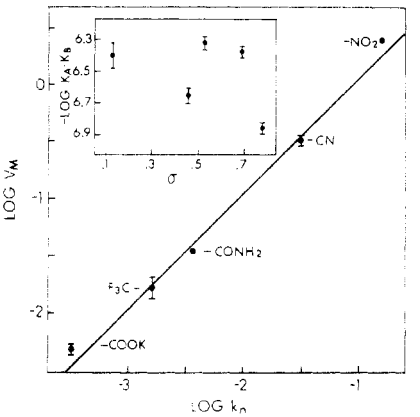


FIGURE 2: Correlation of the maximal velocity of the enzymatic reaction with the second-order rate constant of the nonenzymatic reaction. The slope of the least-squares line shown is 1.012 ± 0.055. The inset shows the lack of any correlation of the product of substrate binding constants with σ substituent constants.

TABLE IV: Substrate Concentration Dependence of the Deuterium Isotope Effect on the Enzymatic Reaction at pH 7.00 (0.05 M K[PO_4], 10^{-4} M EDTA, 25 °C).

[Coenzyme][a] ×10^{-4} (M)	[ρ-CN-Sulfonate][b] ×10^{-3} (M)	DIE[c,d]
1.68 (5.6 K)	5.40 (2.7 K)	5.39 ± 0.10
1.68 (5.6 K)	0.80 (0.4 K)	5.15 ± 0.11
0.42 (1.4 K)	5.40 (2.7 K)	4.95 ± 0.13
0.42 (1.4 K)	0.80 (0.4 K)	5.06 ± 0.06

[a] K for [4,4-^{2}H]NADD and [4,4-^{1}H]NADH = 30 μM. [b] K for 4-CN-DNBS = 2 mM. [c] Rates corrected for: nonenzymatic reaction minus ≤10%; coenzyme degradation minus ≤10%; enzyme modification plus ≤1%. [d] Rate ratios corrected for: isotopic impurity plus ~10%; secondary isotope effect minus 14%.

Table III also lists the primary isotope effects corrected for the difference in reactivity of the A and B hydrogens (1.18), the secondary isotope effect (1.20) and the protium content of the deuterated coenzyme (1.9%). The total magnitude of these corrections is about 16%.

With the exception of the 4-CF_3-DNBS, there is little substituent effect on the deuterium isotope effect. The difference between the value for 4-CF_3-DNBS and that for the other compounds lies outside experimental error.

Deuterium Isotope Effects: Enzymatic Reaction. Table III also lists the observed rate ratios for the oxidation of [4,4-^{1}H]NADH to that of [4,4-^{2}H]NADD catalyzed by glutamate dehydrogenase. These ratios were measured at pH 7.0 (rather than 8.1) in order to minimize complications arising from lysine modification (see Experimental Section). For 4-CN-DNBS, the rate ratio obtained at pH 8.10 was the same within experimental error as that obtained at pH 7.00.

The deuterium isotope effects were corrected for the secondary isotope effect (1.14) and the protium content of the deuterated coenzyme (2–3%). The magnitude of these corrections is less than 4%.

The secondary isotope effect, 1.14 ± 0.07, was determined from the ratio of rates (corrected for isotopic impurity) of oxidation of [4,B-^{2}H]NADH to [4,4-^{2}H]NADD using 4-CN-DNBS. Within the experimental error, this is the same value as obtained for the nonenzymatic reaction.

Table III shows that the primary deuterium isotope rate effects are similar in both enzymatic and nonenzymatic reactions. For the enzymatic reaction the isotope effect is substituent independent. The value for 4-CF_3-DNBS is not distinguishable from the values for the other sulfonates.

Table IV gives measurements of the deuterium isotope effect over a range of coenzyme and sulfonate concentration. The data show that the isotope effect is essentially independent of substrate concentration.

The Equilibrium ρ, a Lower Limit. For a single elementary step, a quantitative measure of the extent to which the transition state structure resembles that of products is the ratio of Hammett ρ values for the forward rate constant to that for the equilibrium constant, eq 14, (Leffler and Grunwald, 1963). The appropriate elementary step in the present case is the formation of the symmetrical sulfite complex, the immediate product of hydride transfer (**4** in Scheme I). Although we are not able to measure the equilibrium substituent effect directly, it can be shown that its magnitude (ρ_1) should be close to that on the σ complex *association* from the ultimate products (ρ_2), the hydrocarbons and sulfite. As shown below, there is likely to be relatively little substituent effect on the overall reaction (ρ_T). The relationship between these three ρ values is given by eq 11

$$\rho_1 = \rho_2 + \rho_T \qquad (11)$$

where ρ_1 describes the hydride transfer, ρ_2 the σ complex association and ρ_T the overall reaction (Scheme I and Figure 3).

We estimate a lower limit of 11.5 on the ρ_2 value for σ complex association using the association constants for the sulfite complexes of 2,4,6-TNB (K_A = 250 M^{-1}; Crampton, 1967) and 4-CN-DNB (K_A = 23 M^{-1}; this work) and using σ substituent constants of 0.78 and 0.69, respectively. This ρ_2 value is a lower limit because only the asymmetric complex (7) can be detected in aqueous solutions of 4-CN-DNB and sulfite.[9]

The value of ρ_T can be estimated as follows. Using arguments developed by Hine (1962), ρ_T can be expressed in terms of σ values for the reactant and product groups ($-SO_3^-$, $-H$) and for the corresponding groups ($-CO_2H$, CO_2-) in the reference reaction, benzoic acid dissociation. Thus

$$\rho_T = \frac{\sigma_H - \sigma_{SO_3^-}}{\sigma_{CO_2^-} - \sigma_{CO_2H}} \sim -0.2 \text{ to } -0.4 \qquad (13)$$

The range of values results from uncertainty in the σ values for the charged substituents.

From these estimates for ρ_2 and ρ_T and eq 11, we obtain a lower limit of 11 for ρ_1 describing the hydride transfer step. This is larger (by about a factor of 2) than that ($\rho \sim 5$) describing dihydropyridine hydride transfer rate.

Discussion

The Nonenzymatic Transition State. Assuming the presence of a single rate-determining transition state, data reported in this work and elsewhere (see notes to Figure 3) allow construction of a reaction profile, Figure 3. The curve is drawn for values of the 4-NO_2-DNBS.

Charge Transfer. In comparisons with well understood equilibria and rate processes (Exner, 1972), the large ρ value found for the nonenzymatic reaction indicates that a great deal of negative charge has been transferred from the dihydropyridine to the sulfonate in the transition state (Kurz and Frieden, 1975). A more quantitative estimate is possible as follows.

Assuming (a) the absence of interactions in the transition state not found in reactants and/or products; (b) that a change in the standard free energy of the transition state is a linear combination of the changes in reactants and products (Leffler and Grunwald, 1963), then

$$\frac{\delta\Delta G^{\ddagger}}{\delta\Delta G^{\circ}} = \alpha = \frac{\rho^{\ddagger}}{\rho^{\circ}} \qquad (14)$$

where δ is a substituent stabilization operator, α measures the extent ($0 < \alpha < 1$) to which the transition state resembles product (with respect to the *transfer of negative charge* in this

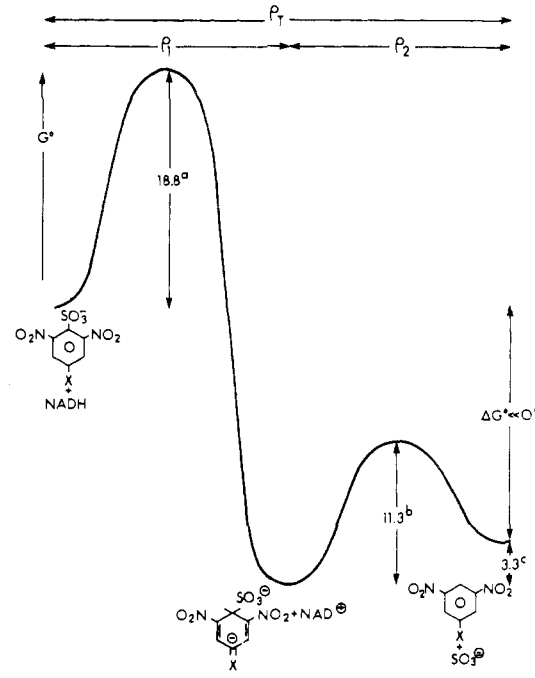
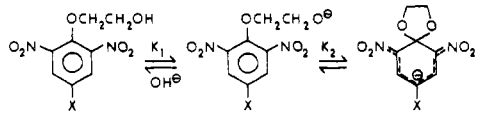


FIGURE 3: Relative standard Gibbs free energies of transition states and intermediates for the nonenzymatic reduction of 2,4,6-trinitrobenzenesulfonate (curve) by NADH. All numerical values are in kcal mol^{-1} and all species are in their standard states (1 M). ρ_1 is the reaction constant for the hydride transfer step. ρ_2 describes σ complex association. ρ_T describes the overall reaction. See text. (*a*) Calculated from rate constants given in Table II. (*b*) Calculated from data given by Bernasconi and Bergstrom (1973). (*c*) Calculated from data given by Crampton (1967). (*d*) No reverse reaction can be detected.

case), and $\rho^{\ddagger}$ and ρ° are Hammett ρ's for the rate and equilibrium, respectively. We find that $\alpha < 5/11 = 0.45$ for the nonenzymatic reduction of 4-X-dinitrobenzenesulfonates by NADH. Since the negative charge is not fully transferred in the rate-determining transition state, a mechanism with prior equilibrium electron transfer to form a radical pair intermediate followed by atom transfer is unlikely.

Brown and Fisher (1976) have studied the effect of substituents in the 3 position of the dihydropyridine on the second-order rate constant for reduction of 4-NO_2-DNBS. For two of these dihydropyridines, NADH and reduced 3-acetylpyridine adenine dinucleotide, the oxidation potentials are known (Sund, 1968). Using these and data given by Brown and Fisher (1976) we calculate (eq 14) α = 0.37, a value remarkably close to that which we obtain from electronic substituent effects in the order reactant.[10] Such agreement would be ex-

[9] A ρ value for an analogous system is desirable for comparison. Crampton and Willison (1974) studied the formation of spiro complexes from para-substituted phenyl ethers in aqueous solutions (eq 12)



(12)

where K_1 is the acid dissociation constant of the ether and K_2 is the formation constant for the complex. From σ substituent constants and values of K_1K_2 for X = NO_2 and H, a ρ of 9.2 can be calculated. The ρ value for K_1 should be about the same as that for the ionization of substituted phenylacetic acids, where the acidic proton is located at about the same distance from the substituent. With this correction ($\rho \cong 0.3$), a ρ of 8.9 is calculated for K_2. While the steric interactions and nucleophile in this reaction series differ from ours, only the spiro complex analogous to the symmetrical complex in our reaction can be formed.

[10] While Brown and Fisher studied the nonenzymatic reaction using a number of substituted dihydropyridines, only two of these were studied in the enzymatic reaction. Unfortunately, one of these (NADH) deviated significantly from the linear free energy correlation between the log of the second-order rate constant and log of the cyanopyridine nucleotide complex dissociation constant. Because of this problem we restrict ourselves to pairwise comparisons between the enzymatic and nonenzymatic rate constants with the published oxidation potentials for the dihydropyridines. If we had used the slope of the free energy correlation established by the other substituents studied by Brown and Fisher (for which oxidation potentials are not known), the difference between the structures of the enzymatic and nonenzymatic transition states would be reduced ($\alpha_E/\alpha_N \sim$ 2.4 vs. 1.3). Until more than two coenzyme analogues are studied, we cannot know if NADH also deviates in the enzymatic reaction. Thus the quantitative difference between the sulfonate and dihydropyridine substituent effects in the enzymatic reaction as well as their similarities in the nonenzymatic reaction must be viewed with caution. It seems likely, however, that the qualitative comparisons will remain.

pected for a reaction profile for hydride transfer containing only one transition state. However, for a reaction profile for the hydride transfer step containing more than one transition state and therefore at least one intermediate, this agreement is not expected since $\Delta G^{\ddagger}$ in eq 14 would then refer to more than one elementary step and $\delta\Delta G^{\ddagger}$ would be a weighted average of contributions from different processes. In general, the values of the weighting factors would not be the same for different substituent stabilization operators, δ, and apparent α values, calculated from different substituent series, would not agree.

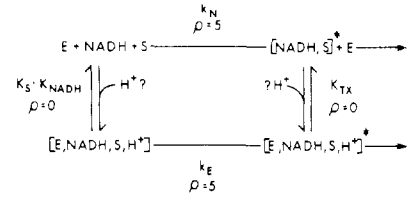
Hydrogen Nucleus Transfer. An indication of the extent of transfer of the hydrogen nucleus may be obtained from deuterium isotope effects. The large primary effects (Table III) and normal secondary effect (1.2) indicate that the hydrogen nucleus as well as the electrons (i.e., charge) are in transit in the activated complex. The magnitudes of these effects are similar (primary effects, 5.4–13; secondary effects, 1.06–1.16) to those obtained in the hydride transfer reductions of several quinones by *N*-methylacridan (Colter et al., 1976).

However, this pattern of isotope effects differs greatly from that found in the direct hydride transfer reductions of several other model substrates by dihydronicotinamides (Steffens and Chipman, 1971; Creighton et al., 1973; Hajdu and Sigman, 1975, 1976; Creighton et al., 1976). In these systems kinetically determined isotope effects (1.7) for the oxidation of monodeuterated dihydropyridine cannot be made compatible with product isotope partitioning ratios (~5) without inverse α-deuterium secondary isotope effects (0.4–0.7, values unprecedented for a sp^3 to sp^2 hybridization change) (Creighton et al., 1973). In these systems at least one and probably two kinetically important intermediates preceding hydrogen nucleus transfer (the step with an isotope effect) are required to explain these results and formation of a radical pair intermediate followed by hydrogen atom transfer has been suggested (Creighton et al., 1976).

In summary, the data presented in this paper support a mechanism in which transfer of charge and hydrogen nucleus are synchronous, i.e., decoupled electron hydrogen atom transfers appear unlikely. The large primary isotope effect, which is approximately constant with the substituent, taken together with the free energy correlation which is linear over a 10^4–10^5 range are indicative of a *single* hydrogen nucleus transfer step as rate determining. In addition, less than half of the total charge has been transferred from the dihydropyridine to the aromatic nucleus in this rate determining transition state. A prior equilibrium electron transfer followed by rate-determining atom transfer is unlikely since this mechanism would require the kinetic $\rho(5)$ to approach the equilibrium value (>11) contrary to observation. A rate-determining atom transfer followed by a kinetically insignificant electron transfer is improbable since this mechanism would require a ρ of 5 for formation of a neutral radical transition state. The only examples of radical reactions with large positive ρ's also involve the transfer of negative charge (addition of one electron to the ring of 4-X-benzyl chlorides has a ρ of +14; Mohammed and Kosower, 1971). Other examples of neutral radical reactions with linear Hammett plots have been negative ρ values (Exner, 1972). However, it is possible that none of these are good models for hydrogen atom transfer to the ring of benzenesulfonates in which case the atom transfer mechanism could not be completely excluded.

The Enzymatic Transition State. Since the deuterium isotope effect on the rate of the enzymatic reaction is large and substrate concentration independent (Tables III and IV), the hydride transfer appears to be the rate-limiting step under all conditions and the mechanism of the enzymatic reactions proceeds via a prior equilibrium binding of substrates followed by the rate-limiting hydride transfer. Since the chemical transformation is rate limiting and the enzymatic data obey eq 1 or 2, we conclude that either substrate may bind to the enzyme in the absence of the other. This conclusion arises from the fact that a rapid equilibrium ordered binding of substrates yields an initial velocity equation different from eq 1 or 2.

Scheme II



The formalism (Kurz, 1972, Wolfenden, 1972) we will use to compare the enzymatic and nonenzymatic reactions is shown in Scheme II. The horizontal process at the top indicates the pseudo-equilibrium which exists between the nonenzymatic ground state and the nonenzymatic transition state. The vertical process on the left indicates the prior equilibrium association of the substrates with the enzyme to form the enzymatic ground state which is in pseudo-equilibrium with the enzymatic transition state. The vertical process on the right indicates the virtual equilibrium between the enzymatic and nonenzymatic transition states. Since the effects of changes in reaction variables on the thermodynamic constants of three members of the cycle (k_N, $K_{NADH}K_S$, k_E) can be observed, we can calculate the effects on the difference between the standard chemical potentials of the enzymatic and nonenzymatic transition states. This rigorous application of Scheme II requires that the enzymatic reaction must proceed by the prior equilibrium mechanism confirmed above.

Charge Transfer. The effects of para substituents in the sulfonate on the enzymatic maximal velocity, k_E, are exactly the same as those on the nonenzymatic second-order rate constant, k_N (Figure 2). Since there is no significant substituent effect on substrate binding ($K_{NADH}K_S$), there is no electronic substituent effect on the difference between the standard chemical potentials of the enzymatic and nonenzymatic transition states (as shown in Scheme II). Therefore, in the absence of a fortuitous cancellation of opposing effects, charge has been transferred to the same extent in both activated complexes.

It seems likely that the local dielectric constant in the vicinity of the enzymatic transition state is the same as that surrounding the nonenzymatic one, probably that of bulk water. As expected from the transfer of negative charge which occurs on formation of the transition state, these reaction rates are sensitive to electrostatic interactions. Brown and Fisher (1976) report that the nonenzymatic rate constant for reduction of 4-NO_2-DNBS by NADH increases with increase in solvent polarity and ionic strength. If there were significant differences in the dielectric properties of the medium surrounding the enzymatic transition state, we would expect ρ to change.

Hydrogen Nucleus Transfer. Deuterium isotope effects are sensitive to the extent of hydrogen nucleus transfer, and if the displacement of the hydrogen nucleus along the reaction coordinate is the same for both transition states, we expect the same isotope effects. Within experimental error, this was found for both primary and secondary effects.

Thus, by these criteria, the structures of enzymatic and nonenzymatic transition states are very similar.

Comparison to Other Work. Brown and Fisher (1976) have studied the reduction of 4-NO_2-DNBS by two dihydropyridines which differ in the substituent at the three position of the dihydropyridine ring (3-$CONH_2$ vs. 3-$COCH_3$). They find that the turnover rate in the central complex of the enzymatic reaction is more sensitive to dihydropyridine substituent than is the rate of the nonenzymatic reaction. The V_{max} ratio is 66 while the corresponding nonenzymatic rate constant ratio is 6. In contrast, we find no differences in sensitivity to changes in the para substituents of the sulfonate substrates (Figure 2). On the basis of their results with the two substituted dihydropyridines, Brown and Fisher conclude that the enzymatic transition state is reached later with respect to carbon–hydrogen bond breakage than is that in the uncatalyzed reaction. Assuming that $\delta\Delta G°$ in eq 14 is the same for both enzymatic and nonenzymatic reactions, we calculate from their substituent effect data that $\alpha = 0.37 \pm 0.01$ and 0.87 ± 0.13 for the nonenzymatic and enzymatic transition states, respectively.[10,11] On the other hand, our results indicate that α values for charge transfer are the same for both transition states. Equation 14, which shows how α values can be calculated from substituent effects, is essentially a quantitative statement of the Hammond postulate (Jencks, 1969) which predicts that the transition state occurs earlier in catalyzed than in uncatalyzed reactions. Taken together with our results, those of Brown and Fisher are very interesting and raise the possibility of nonconservation of bond order in the enzymatic transition state.

Before this merits serious consideration more members of the dihydropyridine series should be studied. More importantly, measurement of $\delta\Delta G°$ for the enzymatic dihydropyridine reaction series is certainly required. Part of the difficulty may lie in the assumption that $\delta\Delta G°$ is the same for both catalyzed and uncatalyzed reactions. In order to bring the α values into agreement, $\delta\Delta G°$ for the enzymatic reaction would have to be greater than the free solution value by about 4 kcal. While there are no other reports of perturbed equilibrium substituent effects, $\delta\Delta G°$, several examples of large perturbations in standard free energy changes ($\Delta G°$) for reactions taking place in enzyme active sites are well documented: 8 kcal (10^6-fold) in the case of myosin catalyzed ATP hydrolysis (Bagshaw and Trentham, 1973; Alberty, 1969); 5.5 kcal (10^4-fold) in the case of lactate dehydrogenase catalyzed reduction of pyruvate by NADH (Shore et al., 1975).

Perhaps it is possible to explain how enzymatic vs. nonenzymatic substituent effects could differ for the coenzyme and not for the sulfonate substrates without invoking nonconservation of bond order. These differences could arise simply from a kind of nonproductive binding of the 3-acetyl analogues. For example, the enzyme may use the binding energy of the coenzyme to satisfy an entropic requirement for the reaction, perhaps holding the dihydropyridine ring in the proper orientation by hydrogen bonding to the amide hydrogens. If the 3-acetyl analogue were unable to pay this price (having no amide hydrogens to hydrogen bind), its reactivity on the enzyme might be diminished over that predicted from its free solution oxidation potential. It is clear that interactions between the dihydropyridine ring of NAD(P)H and the enzyme, detectable by difference spectroscopy (Fisher, 1971), are absent in the 3-acetyl analogue. The electronic substituent effect in the other reactant might not be very sensitive to these interactions. It seems reasonable that perturbations of highly specific binding interactions between enzyme and substrate are more likely to arise and cause difficulties in interpretation when structural changes are made in functional groups on a normal substrate.

The Source of Catalysis? With so few indications of structural differences between the enzymatic and nonenzymatic activated complexes, how can we account for the 11 kcal difference between the standard chemical potentials of the enzymatic and nonenzymatic transition states?[12] Interactions, such as those just discussed, which increase the probability of reaction by restricting the relative motion of the reactants (Jencks, 1975) may be important. It is not surprising that the importance of these would be manifest with small structural changes in the coenzyme (the more tightly bound substrate) and not with large changes in the sulfonate, a loosely bound, artificial substrate. In addition, it is necessary to keep in mind that the enzymatic transition state contains the enzyme. A difference in standard chemical potential between the species, $E^{\ddagger}$ and E, could contribute to catalysis. However, conventional chemical catalysts may be present. As indicated in Scheme II, the enzymatic activated complex includes a proton (at least one) not present in the nonenzymatic one. No pH dependence of the rate of the nonenzymatic reaction has thus far been detected (Brown and Fisher, 1976). For the enzymatic reaction, about a tenfold increase in transition state affinity is observed when the pH is lowered from 8 to 7. This effect is about equally divided between the binding constant of the sulfonate and the maximal velocity. The former effect is in agreement with the principle (outlined by Shore et al., 1975) that dehydrogenases maintain charge neutrality in their active sites and that binding of an anionic substrate is always accompanied by binding of a proton. The latter effect suggests acid catalysis. We have not yet detected any changes in electronic substituent effect with decreasing pH. If technical difficulties can be overcome, we will extend the pH range of our observations.

Relation to the Physiological Reaction. Because of the lack of obvious analogy between the structures of 4-X-2,6-dinitrobenzenesulfonates and α-ketoglutarate plus ammonia (the normal substrates of glutamate dehydrogenase), it seems reasonable to question the validity of any mechanistic conclusions about the latter reaction derived from study of the former. However, considerable evidence supports the validity of such comparisons. Both enzymatic reactions proceed with stereospecific transfer of hydrogen from the B side of the coenzyme (Bates et al., 1970). The normal substrate, α-ketoglutarate, is a competitive inhibitor of the sulfonate reduction (Bates et al., 1970). The nucleotide effectors, GTP and ADP, have very similar effects on the two reactions not only with respect to the values of the inhibitor and activator apparent dissociation constants, but also with respect to the final extents

[11] Brown and Fisher (1976) report a nonenzymatic isotope effect for reduction of 4-NO_2-DNBS by NADH of 3.4—substantially smaller than the 4.9 they report for the enzymatic reaction. We fail to find any differences (Table III).

[12] The 11 kcal is calculated, using values in Table II, from the relation $\Delta G° = -RT \ln K_{TX}$ where K_{TX}, the transition state dissociation constant of Scheme II, is given by $(V_{max}/K_S K_{NADH})/k_N$. The question may be raised as to how this (and the other data) should be interpreted in terms of the "catalytic rate enhancement" provided by the enzyme in these reactions. Since this requires comparison of reaction rates of different order, it does not appear (to the authors) possible to answer this question properly. However, two comparisons are commonly used. From the first, we calculate that the "effective concentration" of substrate in the active site (V_{max}/k_N) is about 100 M at pH 7. The second method compares the two reactions under conditions of the same reaction order (Jencks, personal communication). This method gives a value of about 13 000 at pH 7 ($V_{max}/K_S k_N$). Clearly the two methods of calculation give different results and indicate some difficulty with the concept of "catalytic rate enhancement."

of inhibition and activation (Bates et al., 1970; Bates, 1972; Kurz and Frieden, unpublished observations). It is always possible that these similarities merely reflect a common binding site for benzenesulfonates and the natural substrates and not any similarity in chemical mechanisms.

Those qualitative differences which do exist between the two reactions are crucial to our investigation of the mechanism by which the enzyme catalyzes the hydride transfer step. Firstly, the nonenzymatic reduction of the sulfonates by the coenzyme is readily studied and proceeds with a mechanism which we propose is very similar to that on the enzyme. The reduction of the normal substrates does not occur at all in the absence of the enzyme. The large substituent-independent V_{max} isotope effects on the sulfonate reduction together with no significant isotope effects on the other kinetic constants confirm a mechanism with prior equilibrium association of the substrates with the enzyme followed by rate-determining hydride transfer. In contrast, with α-ketoglutarate and NH_3 as substrates the apparent hydrogen isotope effects are very small or absent (Bates, 1970): thus while we do not know with certainty what steps are rate determining in the natural reaction, they are not hydride transfer. Of course hydride transfer does occur in the natural reaction but it is difficult to study because the natural reaction is both chemically (C–N bond making is involved) and kinetically (substrate–product handling steps are not fast compared to bond making–breaking steps) more complex. Therefore we see no reason why deductions about the mechanism of catalysis by glutamate dehydrogenase of the reduction of dinitrobenzenesulfonates should not also be applicable to the hydride transfer step of the normal reaction. Certainly any part of the enzyme's catalytic apparatus concerned directly with the coenzyme should remain intact in our reaction.

In summary, we believe the following conclusions can be drawn from the present work. For those sulfonates thus far examined: (a) The structures of nonenzymatic and enzymatic transition states are very similar. (b) In these activated complexes the transfers of negative charge and the hydrogen nucleus are nearly synchronous.

Acknowledgments

We are grateful for the many helpful suggestions and discussions provided by Professors George Drysdale and Joseph L. Kurz. We are indebted to Dr. Craig Jackson whose Cary 118 made many of these experiments practical.

References

Abeles, R. H., Hutton, R. F., and Westheimer, F. H. (1957), *J. Am. Chem. Soc. 79,* 712.
Alberty, R. A. (1969), *J. Biol. Chem. 244,* 3290.
Bagshaw, C. R., and Trentham, D. R. (1973), *Biochem. J. 133,* 323.
Bates, D. J. (1972), Ph.D. Thesis, Washington University, St. Louis, Mo.
Bates, D. J., Goldin, B. R., and Frieden, C. (1970), *Biochem. Biophys. Res. Commun. 39,* 502.
Bernasconi, C. F., and Bergstrom, R. G. (1973), *J. Am. Chem. Soc. 95,* 3603.
Biemann, K. (1962), Mass Spectrometry, New York, N.Y., McGraw-Hill, p 223.
Blankenhorn, G. (1976), *Eur. J. Biochem. 67,* 67.
Brown, A., and Fisher, H. F. (1976), *J. Am. Chem. Soc. 98,* 5682.
Busing, L. A., and Levy, H. A. (1962), Oak Ridge National Laboratory Report No. ORNL-TM-271.
Coffee, C. J., Bradshaw, R. A., Goldin, B. R., and Frieden, C. (1971), *Biochemistry 10,* 3516.
Colter, A. K., Saito, S., Sharom, F. J., and Hong, A. P. (1976), *J. Am. Chem. Soc. 98,* 7833.
Crampton, M. R. (1967), *Proc. R. Soc. London, Ser. B,* 1341.
Crampton, M. R., Ghariani, M. A., and Willison, M. J. (1974), *J. Chem. Soc., Perkin Trans. 2,* 441.
Crampton, M. R., and Khan, H. A. (1972), *J. Chem. Soc., Perkin Trans. 2,* 733.
Crampton, M. R., and Willison, M. J. (1974), *J. Chem. Soc., Perkin Trans. 2,* 1681.
Creighton, D. J., Hajdu, J., Mooser, G., and Sigman, D. S. (1973), *J. Am. Chem. Soc. 95,* 6855.
Creighton, D. J., Hajdu, J., and Sigman, D. S. (1976), *J. Am. Chem. Soc. 98,* 4619.
Exner, O. (1972), in Advances in Linear Free Energy Relationships, Chapman, N. B., and Shorter, J., Ed., New York, N.Y., Plenum Press.
Fisher, H. (1971), *Adv. Enzymol. 39,* 369.
Foreman, M. I., and Foster, R. (1969), *Can. J. Chem. 47,* 729.
Goldin, B. R., and Frieden, C. (1971), *Biochemistry 10,* 3527.
Golumbic, C., Fruton, J. S., and Bergman, M. (1946), *J. Org. Chem. 11,* 518.
Gunstone, F. D., and Tucker, S. H. (1963), Organic Syntheses Collect. Vol. IV, New York, N.Y., Wiley, p 160.
Hajdu, J., and Sigman, D. S. (1975), *J. Am. Chem. Soc. 97,* 3524.
Hajdu, J., and Sigman, D. S. (1976), *J. Am. Chem. Soc. 98,* 6060.
Hine, J. (1962), Physical Organic Chemistry, 2nd ed, New York, N.Y., McGraw-Hill, Chapter 4.
Holmes, S. A., and Thomas, T. D. (1975), *J. Am. Chem. Soc. 97,* 2337.
Jencks, W. P. (1969), Catalysis in Chemistry and Enzymology, New York, N.Y., McGraw-Hill, pp 193–195.
Jencks, W. P. (1975), *Adv. Enzymol. 43,* 279.
Kurz, J. L. (1972), *Acc. Chem. Res. 5,* 1.
Kurz, L. C., and Frieden, C. (1975), *J. Am. Chem. Soc. 97,* 677.
Leffler, J. E., and Grunwald, E. (1963), Rates and Equilibria of Organic Reactions, New York, N.Y., Wiley, p 157.
Matsumoto, I. (1965), *Yakugaku Zasshi 85,* 544.
Mohammad, M., and Kosower, E. M. (1971), *J. Am. Chem. Soc. 93,* 2709.
Newman, M. S., and Blum, J. (1964), *J. Am. Chem. Soc. 86,* 5600.
Okuyama, T., and Satake, K. (1960), *J. Biochem. (Tokyo) 47,* 454.
Oppenheimer, N. J., Arnold, L. J., and Kaplan, N. O. (1971), *Proc. Natl. Acad. Sci. U.S.A. 68,* 1971.
San Pietro, A., Kaplan, N. O., and Colowick, S. P. (1955), *J. Biol. Chem. 212,* 941.
Shore, J. D., Weidig, C. F., Lodola, A., Parker, D. M., and Holbrook, J. J. (1975), *Proc. 10th FEBS Mtg., 40,* 63.
Steffens, J. J., and Chipman, D. M. (1971), *J. Am. Chem. Soc. 93,* 6694.
Strauss, M. J. (1970), *Chem. Rev. 70,* 667.
Suelter, C. H., and Metzler, D. E. (1960), *Biochim. Biophys Acta 44,* 23.
Sund, H. (1968), in Biological Oxidations, Singer, J. P., Ed., New York, N.Y., Wiley, p 606.
Velick, S. F. (1958), *J. Biol. Chem. 233,* 1455.
Welsh, L. H. (1941), *J. Am. Chem. Soc. 63,* 3276.
Wolfenden, R. (1972), *Acc. Chem. Res. 5,* 10.