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Anomalous Equilibrium and Kinetic α-Deuterium Secondary Isotope Effects Accompanying Hydride Transfer from Reduced Nicotinamide Adenine Dinucleotide

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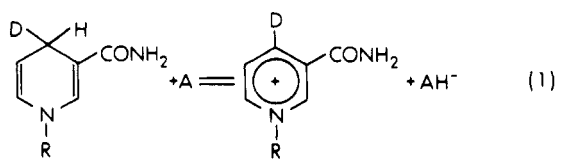
Abstract: The kinetic α-deuterium secondary isotope effect on the second-order rate constant has been measured for the nonenzymatic direct hydride transfer reduction of 4-cyano-2,6-dinitrobenzenesulfonate by NADH (deuterium substitution of the hydrogen bonded to the 4 carbon of NADH which is not transferred to the acceptor). Values of 1.156 ± 0.018 and 1.1454 ± 0.0093 were obtained using direct and intramolecular competition methods, respectively. The corresponding (enzyme catalyzed) equilibrium isotope effects were found to be 1.013 ± 0.020 and 1.0347 ± 0.0087 as determined by direct and intermolecular competition methods, respectively. Thus, the value of the kinetic effect is significantly greater than that on the equilibrium. It is suggested that this may arise either from participation of the α hydrogen in a hyperconjugative stabilization of an early transition state or from its participation in the reaction coordinate motion of a nonlinear activated complex. The values of the equilibrium effect allow calculation of a fractionation factor (relative to acetylene) for hydrogen bonded to the 4 carbon of NAD^+ of 1.448 ± 0.028 or 1.418 ± 0.020. This is larger than expected based on comparison with hydrogen bound to sp^2 carbon in propene (1.336) or benzene (1.368) but is consistent with the decreased aromatic character of pyridinium vibrational spectra. The lack of a significant inverse value for the equilibrium α-deuterium effect suggests complications in the interpretation of reported kinetic secondary effects of 0.85 and 1.2 for the forward ($sp^3 \rightarrow sp^2$) and reverse ($sp^2 \rightarrow sp^3$) rate constants for the nonenzymatic transhydrogenation of *N*-benzyl-1,4-dihydronicotinamide and its nicotinamide salt.

An understanding of the chemistry of enzyme cofactors should lead to a greater understanding of the possible roles these substances play in biological processes. Thus, nonenzymatic reductions of specific acceptors by dihydronicotinamides have been studied as models for the chemical mechanisms of the NAD^+-dependent dehydrogenases. Particular interest has been focused on the question whether the formal hydride transfer in these reactions (eq 1) takes place in a single kinetic



(1)

event (as a hydride ion transfer) or whether various one-electron intermediates are present. Multistep mechanisms have been proposed. These include electron transfer followed by atom transfer; electron transfer and proton transfer, followed by another electron transfer, and so on. The principal evidence for such decoupled mechanisms has been the observation[1] that, if a simple bimolecular mechanism were followed, then large inverse secondary α-deuterium isotope effects would be required to explain the kinetic data reported for several dihydronicotinamide reactions. Since the α hydrogen is bonded to a carbon which undergoes a sp^3 to sp^2 hybridization change in the course of the reaction, the secondary isotope effect was expected to have a value greater than one. It was initially proposed that the inverse effect was only apparent and was the

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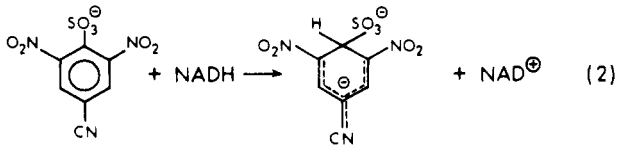
result of the kinetic complexity of a multistep mechanism. Recently, however, an inverse secondary effect has been directly observed in a model reaction.[2]

In contrast, other dihydronicotinamide reductions, such as the reductive desulfonation of 4-X-2,6-dinitrobenzenesulfonates (eq 2), are known to occur by direct hydride ion trans-



(2)

fer[3,4] with the transfer of negative charge and of the hydrogen nucleus taking place in a single kinetic event. Consistent with such a mechanism, we have reported secondary deuterium isotope effects greater than one for these reactions.[4] We now present a more thorough study of the α-deuterium secondary isotope effect in the reduction of 4-cyano-2,6-dinitrobenzenesulfonate by NADH. We also report a value for the corresponding equilibrium isotope effect and comment upon the mechanistic implications of the comparison of the kinetic and equilibrium values for this as well as for other dihydronicotinamide model reactions.

Experimental Section

Materials. A list of abbreviations used in this article is found in ref 5. 4-Cyano-2,6-dinitrobenzenesulfonate (4-CN-DNBS), [4-^{2}H]-nicotinamide adenine dinucleotide ([4-^{2}H]NAD$^+$), and [4,A-^{2}H],-[4,B-^{2}H] and [4,4-^{2}H] reduced nicotinamide adenine dinucleotides ([4,A-^{2}H]NADH, [4,B-^{2}H]NADH, [4,4-^{2}H]NADH) were prepared as described previously.[4] Normal or recycling grade yeast alcohol dehydrogenase (YADH), type XI lactate dehydrogenase (LDH), glycerol 3-phosphate dehydrogenase (3PGDH), NAD$^+$ nucleosidase (NADase), grade V NAD$^+$, and DL-glycerol 3-phosphate were obtained from Sigma Chemical Co. Grade I NAD$^+$ and glutamate dehydrogenase (GDH) were purchased from Boehringer-Mannheim Corp. Other chemicals were reagent grade or better and were used without further purification. YADH was assayed in 5.0×10^{-2} M $Na[P_2O_7]$, pH 9.0, 2.2×10^{-2} M semicarbazide, 1.8×10^{-3} M NAD$^+$, and 0.33 M ethanol.

Purity and Concentration of Dinucleotides. Concentrations of NADH solutions were routinely measured by their absorption at 340 nm. However, they were frequently checked for residual absorption at 340 nm (probably resulting from the presence of the α isomer) after reaction with 3.3×10^{-3} M pyruvate in 5.0×10^{-2} M $K[PO_4]$, pH 8.00, 0.01 mg/mL LDH, or with 5.0×10^{-3} M α-ketoglutarate, 2.0×10^{-2} M NH_4Cl in 5.0×10^{-2} M $K[PO_4]$, pH 8.00, 0.01 mg/mL GDH. The concentration of NAD$^+$ solutions was obtained from the NADH produced after reaction with 0.33 M ethanol in 5.0×10^{-2} M $Na[P_2O_7]$, pH 9.0, 2.2×10^{-2} M semicarbazide, 0.1 mg/mL ADH. The presence of acetone and ethanol in commercial samples of NAD$^+$ was monitored by gas chromatography on Porapak Q according to directions from the manufacturer (Sigma Chemical Co.). Tris and glutamate in [4-^{2}H]NAD$^+$ were determined by the Protein Chemistry Facility of Washington University School of Medicine.

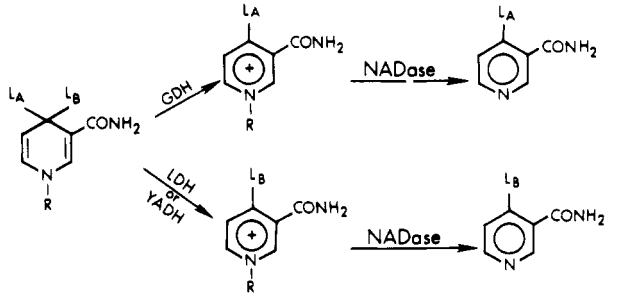
The isotopic purity of deuterated dinucleotides was determined by the H/D ratio present in nicotinamide after NADase digestion of NAD$^+$ or, for NADH, enzymatic oxidation followed by NADase digestion. No assumptions were made regarding the purity of final product based on the purity of reactants. For example, the H/D ratio in nicotinamide derived from [4,4-^{2}H]NADD was determined after both GDH (B-specific) and LDH (A-specific) catalyzed oxidation. See Scheme I.

Gas Chromatography–Mass Spectrometry. H/D ratios were measured using a computer-controlled Finnigan 3300 quadrupole mass spectrometer. The instrument was operated in the selected ion recording mode[6] and M − 1, M, and M + 1 mass peaks were monitored. The H/D ratio was calculated from the equation

$$H/D = (R - r_1)/(1 - r_2R) \quad (3)$$

where R is the peak height ratio, M/(M + 1), found for the unknown; r_1 and r_2 are the ratios (M − 1)/M and (M + 1)/M found for the protium standard (no excess deuterium). The greater part of the M

Scheme I



− 1 peak is an artifact which results from the asymmetrical mass peak shape characteristic of quadrupole instruments. Less than 0.2% is observed in a magnetic sector instrument (LKB 9000) and under optimum tuning and source conditions in the quadrupole instrument. As long as the artifact remained constant at or below a value of 1% of the parent, accurate results were obtained using eq 3. The complete mass spectrum was similar to that reported previously.[7]

The GC column for nicotinamide was similar to that used for the quantitative determination.[8] However, ghosting was found to compromise the accuracy of results if samples of widely varying isotopic content were injected into the same column. Therefore, separate columns were prepared for each sample. Identical results could be obtained on the same column if samples were separated by ~20 injections of solvent to reduce the size of the ghost peak below detection.

For 4-cyano-2,6-dinitrobenzenes we used a column (110 cm × 4 mm i.d.) packed with 1% (w/w) Dexsil 300 on 110/120 mesh Gas Chrom Q operated at 140 °C with a carrier gas flow of 30 mL/min. The sample elutes in 2 min. The remarks (above) about ghosting and mass peak shape also apply here.

Kinetic Isotope Effects. Rate-Constant Method. Rate constants for the reaction of 4-CN-DNBS with [4,4-^{1}H]NADH, k_c, [4,B-^{2}H]-NADH, k_d, [4,A-^{2}H]NADH, k_e, and [4,4-^{2}H]NADD, k_f, under initial velocity conditions were measured spectrophotometrically (25 ± 0.10 °C) as described earlier.[4] Corrections for isotopic content were applied.

Assuming that the secondary isotope effect on deuterium transfer is the same as that on protium and that isotope effects on A-side hydrogen transfer are the same as those on B-side transfer, then the secondary isotope effect, k_H/k'_H, is given by (ref 4)

$$k_H/k'_H = [(k_e + k_d)/2k_f - [[(k_e + k_d)/2k_f]^2 - (k_c/k_f)]^{1/2} \quad (4)$$

and the primary isotope effect, k_H/k_D, by

$$k_H/k_D = (k_c/k_f)(k'_H/k_H) \quad (5)$$

where the rate constants are defined in the preceding paragraph.

Kinetic Isotope Effects. Product Ratio Method. The difference between the reactivities of the A and B sides of NADH is expected to be of the same order of magnitude as the secondary isotope effect.[4,9] To avoid assuming that isotope effects on A- and B-side transfer are equal, we performed an intramolecular competition isotope effect experiment (Scheme II). A tenfold excess (2×10^{-3} M) of 4-CN-DNBS was reduced with either [4,B-^{2}H]NADH or [4,A-^{2}H]NADH. After the desired extent of reaction (monitored spectrophotometrically), the 3-mL reaction mixture was extracted twice with 5 mL of ether. The extract was dried with a stream of N_2 gas and redissolved in methanol to give a concentration of ~1 μg/μL of 4-cyanodinitrobenzene. H/D product ratios were obtained in the mass spectrometer as described above. For [4,B-^{2}H]NADH, the H/D product ratio, PR_d, gave the ratio of rate constants:

$$PR_d = \frac{k'_{H,A}}{k_{D,B}} = \frac{\text{amount [1-}^1\text{H]4-CN-DNB produced}}{\text{amount [1-}^2\text{H]4-CN-DNB produced}} \quad (6)$$

where $k'_{H,A}$ is the rate constant for protium transfer from the A side of NADH in the presence of deuterium on the B side and $k_{D,B}$ is the rate constant for transfer of deuterium from the B side. A similar experiment with [4,A-^{2}H]NADH gave the ratio

$$PR_e = k'_{H,B}/k_{D,A} \quad (7)$$

When these ratios are combined with the apparent second-order rate constants determined spectrophotometrically:

$$k_c = k_{H,A} + k_{H,B} \quad (8)$$

$$k_d = k'_{H,A} + k_{D,B} \quad (9)$$

$$k_e = k_{D,A} + k'_{H,B} \quad (10)$$

isotope effects of the form of eq 11 and 12 can be calculated.

$$k_H/k_D = (k_{H,A} + k_{H,B})/(k_{D,A} + k_{D,B}) \quad (11)$$

$$k_H/k'_H = (k_{H,A} + k_{H,B})/(k'_{H,A} + k'_{H,B}) \quad (12)$$

Equilibrium Secondary Isotope Effect. Direct Determination. Taking advantage of the absolute stereospecificity of NAD-dependent dehydrogenases, the equilibrium constant can be determined for an enzymatically catalyzed reaction of the type shown in eq 13. With the proper stereospecifically labeled monodeuterio-NADH (or [4-^{2}H]-NAD^+) as cofactor, the deuterium will not be transferred to the hydride acceptor, A:

$$[4,A\text{-}^2H]NADH + A \overset{\text{B-specific enzyme}}{\rightleftharpoons} AH^- + [4\text{-}^2H]NAD^+ \quad (13)$$

The equilibrium constant using diprotio-NADH can be measured in the same experiment. Since A and AH^- are present in considerable excess, the desired equilibrium secondary isotope effect, eq 14, can be calculated from the equilibrium-constant ratio:

$$\frac{K_H}{K'_H} = \frac{[[4,(A \text{ or } B)\text{-}^2H]NADH][NAD^+]}{[NADH][[4\text{-}^2H]NAD^+]} = \frac{\phi NADH}{\phi NAD^+} \quad (14)$$

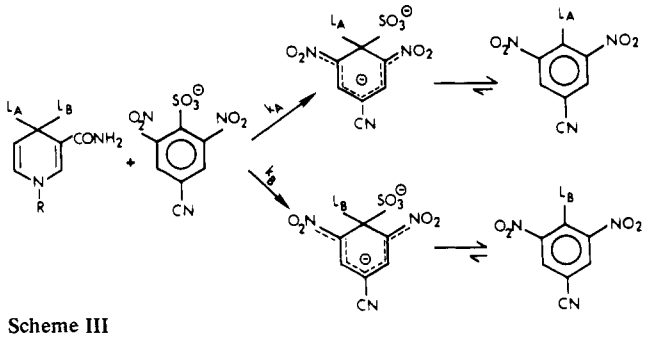
where ϕ is a fractionation factor for hydrogen on carbon 4 of NAD^+ or NADH. The result should not depend on the enzyme, the A,AH^- pair, or the direction of approach to equilibrium. YADH (A-specific) with ethanol/acetaldehyde or 2-propanol/acetone and 3PGDH (B-specific) with glycerol 3-phosphate/dihydroxyacetone phosphate were used with several different preparations of dinucleotides (containing differing levels of impurities) and varying buffer systems.

Equilibrium Secondary Isotope Effect. Competition Method. Competitive methods for the determination of isotope effects (Scheme III) have the advantage that they cannot be affected by trace contaminants found in only one of the isotopically different preparations. In such experiments, a mixture of deuterio- and protio-NAD^+ is allowed to equilibrate with NADH (in the presence of other substrates, A and AH^-) in an enzymatically catalyzed reaction. The reaction is stopped, the products are separated, and H/D ratios are determined in both NAD^+ and NADH. The isotope effect can then be calculated from the equation

$$K_H/K'_H = [\text{H/D ratio in } NAD^+][\text{D/H ratio in NADH}] \quad (15)$$

In the present case it was found to be impossible to recover NADH from the reaction mixtures without significant degradation, a process

Scheme II



Scheme III

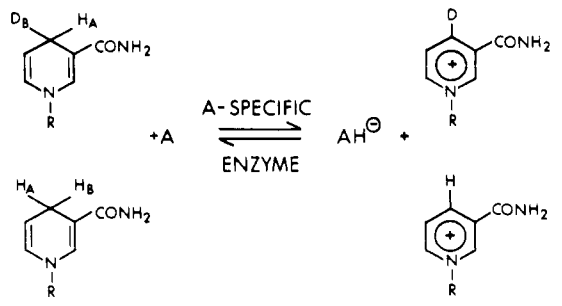


Table I. Rate Constants and Product Ratios for the Reduction of 4-Cyano-2,6-dinitrobenzenesulfonate

dihydronicotinamide	10^2k,[a] M^{-1} s^{-1}	product ratio H/D
[4,4-^{1}H]NADH	2.906 ± 0.011	
[4,B-^{2}H]NADH[b]	1.700 ± 0.016	6.36 ± 0.11[e]
[4,A-^{2}H]NADH[c]	1.3734 ± 0.0016	3.504 ± 0.049[e]
[4,4-^{2}H]NADD[d]	0.4837 ± 0.0077	

$$k_H/k'_H = \frac{1.1454 \pm 0.0093 \text{ (product-ratio method)}^f}{1.156 \pm 0.018 \text{ (rate-constant method)}^h}$$

$$k_H/k_D = \frac{5.423 \pm 0.038 \text{ (product-ratio method)}^g}{5.20 \pm 0.014 \text{ (rate-constant method)}^i}$$

[a] Rate constants obtained under initial velocity conditions in 0.025 M K[PO_4], 2×10^{-4} M EDTA, pH 8.00, 25.0 °C. Corrected for protium contamination of deuterated samples, degradation of dihydronicotinamide, and hydrolysis of sulfonate.[4] [b] Contained 5.4 mol % H on B side. [c] Contained 5.3 mol % H on A side. [d] Contained 1.0 mol % H on A side and 4.5 mol % H on B side. [e] Correction for isotopic impurity assumes that reaction goes to completion. [f] Using eq 12 and values given in Table I. [g] Using eq 11 and values given in Table I. [h] Using eq 4 and values given in Table I. [i] Using eq 5 and values given in Table I.

itself conceivably subject to an isotope effect. However, if the initial H/D ratio in NAD^+ and the final extent of reaction are known, the D/H ratio in NADH (the second term on the right of eq 15) can be calculated, yielding

$$K_H/K'_H = \frac{(H/D)[(H/D) + f(H/D)_0 + f + (H/D)_0]}{f(H/D)(H/D)_0 - f(H/D) - (H/D) + (H/D)_0} \quad (16)$$

where (H/D) is the protium to deuterium ratio found in NAD^+ at equilibrium, $(H/D)_0$ is the ratio present in NAD^+ initially, and f is the fractional conversion of NAD^+ to NADH; f = final [NADH]/initial[NAD^+]. It is necessary to completely inactivate the enzyme without perturbing the final concentrations so that the position of equilibrium does not change in the subsequent separation of reactants and products.

YADH was chosen for these experiments because of its extreme sensitivity to Ag^+ as an irreversible denaturant.[10] Reaction mixtures (3 mL) in 0.025 M Tris/SO_4^{2-}, 10^{-4} M EDTA, pH 8.00, were prepared containing 0.2 mg/mL YADH, 0.07 M acetone, and from 0.09 to 0.32 M 2-propanol. After a 15-min temperature equilibration time, 0.03 M NAD^+ ($(H/D)_0 \sim 1$) was injected to give a final nucleotide concentration of $\sim 4 \times 10^{-4}$ M. Equilibrium was reached in about 30 min and the optical density at 340 nm (a Cary 118 spectrophotometer, thermostated at 25 ± 0.1 °C, 4.8-nm spectral slit, was used) remained constant within ± 0.0001A over the next 20–30 min. A solution of $AgNO_3$ was then injected to give a final concentration of 2×10^{-4} M. The optical density at 340 nm immediately increased ~0.005A and continued to drift slowly upward over the next 60 min for an overall increase of ~0.01. This same change occurred in the absence of substrates (but in the presence of enzyme) and therefore does not represent any perturbation of the position of equilibrium. Studies of the degradation rate of the dinucleotides in the presence of Ag^+ did not indicate any significant effect.

After 60 min, the reaction mixture was diluted 50-fold with ice-cold H_2O and applied to a 1 × 25 cm column of low capacity (0.51 mequiv/g) DEAE-HCO_3^- (Bio-Rad Cellex D). Aliquots were withdrawn after the addition of Ag^+ and until all the sample was on the column in order to check for YADH activity. No activity (<0.1% of the original) could be found at any time after addition of $AgNO_3$.[11] NAD^+ was eluted with 2×10^{-3} M NH_4HCO_3 and NADH with 0.05 M NH_4HCO_3. Yields of >90% of both nucleotides were obtained. The fractions containing NAD^+ were combined and freeze-dried until all the solvent and excess salt had sublimed. The residue was dissolved in 3 mL of H_2O and digested at 37 °C overnight with ~0.25 unit NADase (previously exhaustively dialyzed against H_2O). The pH was adjusted to 7 and the sample carefully evaporated (nicotinamide sublimes) to dryness. The residue was triturated with 2 × 0.5 mL of methanol, centrifuged to clarify, and dried with a stream of N_2 gas. The sample was taken up in 1 mL of H_2O, pH adjusted to 7, and ap-

Table II. Equilibrium Secondary Isotope Effect—Direct Determinations

enzyme and A/AH⁻ [a]	conditions	K_H/K'_H (N)[b]
YADH (0.2 mg/mL) $H_3COCH_3/CH_3CHOHCH_3$[c]	0.1 M Bicine/K^+ 2×10^{-4} M EDTA pH 8.02, 25.0 °C	1.013 ± 0.011 (2)[d,f] 1.0093 ± 0.0021 (2)[e,f] 1.020 ± 0.010 (2)[d,g] 1.014 ± 0.012 (2)[e,g]
	0.1 M Tris/Cl^- 2×10^{-4} M EDTA pH 8.10, 25.0 °C	1.023 ± 0.014 (2)[e,h] 0.980 ± 0.027 (8)[e,i] 0.978 ± 0.010 (6)[j,k,l]
YADH (0.02 mg/mL) CH_3CHO/CH_3CH_2OH[m]	0.1 M Bicine/K^+ 2×10^{-4} M EDTA pH 8.02, 25.0 °C	1.018 ± 0.003 (2)[d,f] 1.0307 ± 0.0087 (2)[e,f] 1.0334 ± 0.0039 (2)[d,g] 1.032 ± 0.0070 (2)[e,g]
	0.05 M $Na[P_2O_7]/Cl^-$ pH 9.10, 25.0 °C	1.029 ± 0.010 (8)[e,h]
3PGDH[o] (0.04 mg/mL) $CH_2OHCOCH_2OPO_3^{2-}$/ $CH_2OHCH_2OHCH_2OPO_3^{2-}$ [n]	0.1 M glycine/K^+ pH 9.00, 25.0 °C	0.985 ± 0.061 (6)[e,i,p]

[a] Substrate pair. [b] K_H is the equilibrium constant for the reaction [4,4-^{1}H]NADH + A ⇌ [4-^{1}H]NAD^+ + AH^-; K'_H is the equilibrium constant for the reaction [4,A or B-^{2}H]NADH + A ⇌ [4-^{2}H]NAD^+ + AH^-; N is the number of determinations in the set. For $N = 2$, the uncertainty given is the average deviation. For $N > 2$, the uncertainty given is the standard deviation. [c] 0.089 M $CH_3CHOHCH_3$ and 0.067 M CH_3COCH_3 present initially. Reaction started with 2×10^{-4} M NAD^+. Final extent ~0.5. Addition of 2×10^{-4} M glutamate had no effect. [d] [4-^{2}H]NAD^+ contained 3.9 mol % H. [e] [4-^{2}H]NAD^+ contained 2.2 mol % H. [f] [4,-^{1}H]NAD^+ (Boehringer) contained 0.005 wt % CH_3CH_2OH and 0.007 wt % CH_3COCH_3. [g] [4-^{1}H]NAD^+ (Sigma grade V) contained 2.0 wt % CH_3COCH_3 and <0.1 wt % CH_3CH_2OH. [h] [4-^{1}H]NAD^+ (Sigma grade V) contained 1.0 wt % CH_3COCH_3 and <0.1 wt % CH_3CH_2OH. [i] [4-^{1}H]NAD^+ (Sigma grade III). [j] [4,B-^{2}H]NADH contained 2.2 mol % H on B side. [k] NADH (Sigma grade III). [l] 0.052 M $CH_3CHOHCH_3$, 0.049 M CH_3COCH_3, and 1×10^{-4} M NADH present initially. Final extent ~0.5. Reaction started with addition of enzyme. [m] 0.14 M CH_3CH_2OH and 2×10^{-4} M NAD^+ present initially. Reaction started with addition of NAD^+. Final extent ~0.5. Addition of 2×10^{-4} M glutamate had no effect. Purging all solutions with N_2 had no effect. [n] 0.027 M $CH_2OHCOCH_3OPO_3^{2-}$ and 3×10^{-4} M NAD^+ present initially. Final extent 0.5. Glutamate (2×10^{-4} M) or Tris had no effect. Reaction started with addition of NAD^+. [o] Preparation contains triose phosphate isomerase (EC 5.3.1.1) but no detectable aldolase (EC 4.1.2.13). [p] Drift in final position of equilibrium (triosephosphate isomerase present) resulted in large uncertainty.

plied to a 3 × 0.7 cm column of AGl-X-8 (Bio-Rad). Nicotinamide was eluted with H_2O. Fractions, monitored at 261.5 nm, were combined, reduced to 3 mL, and millipored. The solutions were then carefully dried. The residue was taken up in 2 × 1 mL of acetone, centrifuged, and dried with N_2 gas. The samples were dissolved in 100 μL of MeOH just prior to analysis in the mass spectrometer. Yields of 70–80% (theoretical yield of 75 μg) were obtained.

Results

Kinetic Isotope Effects. The second-order rate constants and product ratios found for the reduction of 4-CN-DNBS are given in Table I. The rate-constant method (using eq 4 and 5) gave a secondary isotope effect $k_H/k'_H = 1.156 \pm 0.018$ and a primary isotope effect $k_H/k_D = 5.20 \pm 0.014$. These values are the average of isotope effects calculated from five sets of four kinetic runs. However, exactly the same values are obtained from the average rate constants given in Table I.

The product ratios were found to be independent of the extent of reaction from 50 to >90% of completion. Using eq 11 and 12 then, we obtain values for the secondary effect, $k_H/k'_H = 1.1454 \pm 0.0093$, and primary effect, $k_H/k_D = 5.423 \pm 0.038$. The two methods give essentially the same result.

Equilibrium Secondary Isotope Effects. The results of direct determination of the equilibrium isotope effect using various systems are given in Table II. The average value is 1.013 ± 0.020 (all determinations weighted equally). The competitive method (eq 16) yielded the results shown in Table III. The average value (weighted according to the standard deviation) found for the isotope effect was 1.0347 ± 0.0087. The results of the two types of experiments are in substantial agreement.

There are, however, some difficulties with the intermolecular competition method. As shown by the first two entries of Table III, a small (but probably real) difference is found between H/D ratios initially present and those isolated from blank reaction mixtures to which Ag^+ was added before NAD^+. The enzyme may not be instantaneously and irreversibly inactivated and a small amount of change in the position of equilibrium may have occurred during product isolation.[11] However, the value of the equilibrium effect is very insensitive to determinant error in the value of f. Even a 10% error in f will change the final value by only about 0.003. Furthermore, determinant errors in $(H/D)_0$ and (H/D) cancel almost exactly.

Table III. Secondary Equilibrium Isotope Effect—Competition Method

$(H/D)_0$[a]	f[b]	(H/D)[c]	K_H/K'_H[d]
1.0943 ± 0.0092	*e*	1.0788 ± 0.0093	
1.0943 ± 0.0092	*e*	1.0851 ± 0.0082	
0.0129 ± 0.0090	*f*	0.0121 ± 0.0013	
1.0449 ± 0.0038	0.5448	1.0586 ± 0.0048	1.024 ± 0.011
1.0449 ± 0.0038	0.7270	1.0699 ± 0.0060	1.0331 ± 0.0095
1.0449 ± 0.0038	0.8079	1.0805 ± 0.0040	1.0423 ± 0.0067

[a] H/D ratio initially present in NAD^+. [b] Fractional conversion of NAD^+ to NADH = final [NADH]/initial [NAD^+]. [c] H/D ratio found in NAD^+ at equilibrium. [d] Secondary isotope effect calculated from eq 16. Uncertainties are standard deviations propagated from those found in the mass peak ratios (representing five injections into the GC–MS) and used in eq 3 and 16. [e] A reaction mixture of initial composition the same as that represented by $f = 0.8079$ but in which Ag^+ was added before NAD^+. [f] A synthetic reaction mixture containing 1.98×10^{-4} M [4-^{2}H]NAD^+, 1.90×10^{-4} M [4,B-^{2}H]-NADH, 5.5×10^{-2} M acetone, and 9.3×10^{-2} M 2-propanol. No enzyme was present.

That no deuterium is lost from NAD^+ through any nonenzymatic process (such as transhydrogenation or exchange with solvent) is shown by the results (third entry in Table III) of an experiment in which a synthetic reaction mixture containing all components but enzyme was carried through the incubation and isolation procedure.

Effect of Determinant Error. If errors were random, the

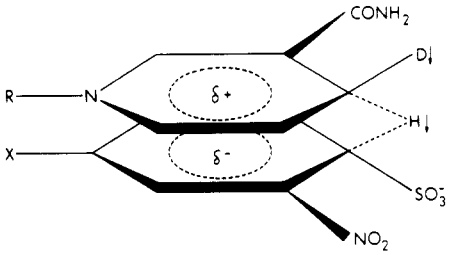


Figure 1. Activated complex for nonlinear hydride transfer.

secondary kinetic isotope effect would be significantly larger than the corresponding equilibrium effect. We have been concerned about the possibility of determinant error. The value of the secondary kinetic isotope effect is most sensitive to an error in the diprotio rate constant, k_c, which is used in both the rate-constant and product-ratio methods. To make the kinetic effect even equal the largest of the two equilibrium values (1.0347) requires the measured value of k_c to be about 10% too high (the precision of the measurement is ±0.3%). However, this 10% change would have large and opposing effects on the value of the primary effect calculated by the two methods, raising the primary effect from 5.20 to 5.32 for the rate-constant method and lowering it from 5.42 to 4.89 for the product-ratio method. Since the opposite ordering of the experimental values of the primary effect is in fact observed, it seems unlikely that a determinant error in k_c alone is responsible for raising the value of the secondary kinetic effect. Of course, we can never rule out the possibility of multiple determinant errors.

Discussion

Fractionation Factors for NADH and NAD^+. These data require an unusual fractionation factor for deuterium on the 4 carbon (sp^2) of NAD^+. The secondary equilibrium isotope effect is the ratio of the fractionation factor for deuterium on the 4 carbon of NADH to that for NAD^+ (eq 14). We can obtain a value for NADH as follows. A primary equilibrium isotope effect, $K_H/K_D = 0.89 \pm 0.03$, has been reported[12] for the reduction of acetaldehyde by [4,A-^{2}H]NADH catalyzed by YADH to give [2-^{2}H]ethanol. Since

$$K_H/K_D = \phi\text{NADH}/\phi\text{CH}_3\text{CHDOH} = 0.89 \pm 0.03 \quad (17)$$

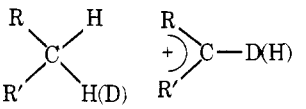
and we can estimate ϕ for ethanol, using fractionation factors relative to acetylene,[13,14] as

$$\phi\text{CH}_3\text{CHDOH} = \phi\text{CH}_3\text{CH}_2\text{D}(\phi\text{CH}_2\text{DOH}/\phi\text{CH}_3\text{D}) = 1.579 \quad (18)$$

we obtain ϕNADH = 1.406 ± 0.047. A primary equilibrium isotope effect of 0.851 ± 0.007 has been reported for the reduction of acetone by [4,A-^{2}H]NADH to give [2-^{2}H]-2-propanol.[15] Proceeding in a manner analogous to eq 18 to estimate ϕ for $CH_3CDOHCH_3$, we obtain a value of ϕNADH = 1.482 ± 0.012. If ϕNADH = 1.467 ± 0.028 (the weighted average of the two values), and the equilibrium secondary effect is 1.013 ± 0.020 or 1.0347 ± 0.0087, then ϕNAD^+ = 1.448 ± 0.28 or 1.418 ± 0.020.

The fractionation factor for NADH, 1.467 ± 0.028, is close to that calculated[13] for CH_3CHDCH_3, 1.501. However, that which we have obtained for NAD^+ is higher than that calculated for propene (CH_2=$CDCH_3$),[16] 1.336, or for benzene,[14] 1.368. The presence of the positive charge on the pyridinium nitrogen in NAD^+ may serve to increase the fractionation factor. Infrared data for several *N*-alkylnicotinamide salts have been interpreted[17,18] to indicate a considerable decrease in the aromatic character of these compounds in comparison to nicotinamide. Force-field calculations based on these data would be of considerable interest.

The Equilibrium Secondary Effect. Consequence for the Interpretation of Kinetic Secondary Effects. For any reasonable mechanism, the ratio of the values of the secondary kinetic isotope effects determined for both the forward and reverse direction should be equal to the equilibrium effect. Recently a report has been made of a study of the nonenzymatic transhydrogenation (exchange) between *N*-benzyldihydronicotinamide and its nicotinamide salt.[2] A value of 0.849 ± 0.015 was reported for the secondary kinetic isotope effect in the forward ($sp^3 \rightarrow sp^2$) direction and a value of 1.200 ± 0.077 for the reverse ($sp^2 \rightarrow sp^3$) direction. The value of the ratio,[19] 0.708 ± 0.047, cannot be reconciled with the value of the equilibrium effect, 1.01–1.03, reported in the present work. It would be valuable to have the results of the following experiment as a consistency test. Starting with deuterium in either dihydronicotinamide or salt, allow the transhydrogenation to come completely to equilibrium. Measure the separate equilibrium concentrations of all species and construct the appropriate ratio (as in eq 14) to be compared with the values calculated from rate data. Inverse secondary isotope effects have also been implicated in the reduction of several other oxidants by dihydronicotinamides.[1] For those cases which can be studied in the reverse direction, it would also be of interest to determine whether a similar discrepancy would be found between kinetically and directly determined equilibrium isotope effects.



Mechanism of Hydride Ion Transfer in the Reduction of 4-Cyano-2,6-dinitrobenzenesulfonate by NADH. To our knowledge, this is the first report of an α-deuterium secondary kinetic effect whose value (1.15) exceeds that of the corresponding equilibrium effect (1.01–1.03). Several indexes of activated complex structure are consistent with the idea that the transition states in these reductions are reached relatively early (i.e., they resemble the structures of reactants to a greater extent that those of products). Thus, the effect of substituents in both the sulfonate[3,4] and dihydronicotinamide[20] on the second-order rate constants have been compared[4] to those on the equilibrium constants and indicate a Leffler α value <0.5 for the fractional displacement of the transition state along the reaction coordinate. If α = 0.5 and the secondary kinetic effect is 1.15, simple application of the Leffler relation predicts the value of the corresponding equilibrium effect to be 1.31. Clearly, the substitution of deuterium for protium in the nontransferred 4 hydrogen of NADH cannot be regarded as a simple substituent change.

An explanation of these data has been suggested:[21] Since the transition state is early (occurring before much movement of the α-H into the plane of the ring), a hyperconjugative interaction in which the α-H stabilizes the developing positive charge on the dihydropyridine nitrogen could occur. Such an interaction, possible in the transition state but not in reactants or products, would lead to a smaller fractionation factor for the α-H in the transition state than we would expect otherwise. This explanation has two unusual features. Firstly, hyperconjugation is invoked to justify an α (rather than β, or more remote) deuterium effect. Secondly, the center of positive charge responsible for the effect is more than four bonds removed from the isotopic atom. Remote deuterium isotope effects on the rates of several solvolysis reactions have been explained by hyperconjugation with the reaction center transmitted through carbon–carbon multiple bonds.[22]

However, we would like to suggest a more intriguing explanation. While the idea of regarding the α-D as a substituent and the consequent *linear* relationship between the kinetic isotope effect and degree of reaction has been questioned, the

secondary equilibrium effect is still thought to closely approximate an upper limit on the value of the secondary kinetic effect even in the presence of special transition-state effects.[23] However, this conclusion is based primarily on cases (S_N2 reactions) in which the reaction coordinate motion is dominated by heavy atoms (carbon and halogen). It seems reasonable to question whether the situation might be different when the masses of the isotopic and leaving atoms are comparable and ask whether it is possible that the bending motion of the α-H of NADH is part of the reaction coordinate motion. Perhaps the observed α effect is in some sense a primary one.

Such a suggestion appears more plausible when we consider earlier proposals[24,25] that hydride ion transfer reactions are likely to have "nonlinear" or triangular transition states. Such an activated-complex geometry would be particularly likely in the present case (Figure 1) because it would allow a favorable electrostatic interaction between the developing positive charge in the dihydropyridine ring and the developing negative charge in the nitrobenzene ring. The reaction coordinate would then correspond to a wagging of the $CH_2(D)$ at the 4 position of the dihydropyridine ring so that the α-H(D) moves into the plane as the hydride ion is transferred to the nitrobenzene ring.

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(5) Abbreviations used: 4-CN-DNBS, 4-cyano-2,6-dinitrobenzenesulfonate; NAD^+, oxidized nicotinamide adenine dinucleotide; NADH, reduced nicotinamide adenine dinucleotide; [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; YADH, yeast alcohol dehydrogenase (E.C. 1.1.1.1); LDH, lactate dehydrogenase (E.C. 1.1.1.27); 3PGDH, glycerol 3-phosphate dehydrogenase (E.C. 1.1.18); NADase, NAD nucleosidase (E.C. 3.2.2.5) from *N. crassa;* GDH, glutamate dehydrogenase (E.C. 1.4.1.2); Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetic acid; Bicine, *N,N*-bis(2-hydroxyethyl)-glycine.
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Structure of Natural Antibiotic CP-47,444

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Abstract: The structure of the first member of a new class of natural antibiotics is deduced by the systematic application of high-resolution NMR techniques.

Antibiotic CP-47,444, isolated from *Nocardia argentinensis* Huang sp. nov., has been recently described in the patent literature.[1] This paper describes the systematic application of conventional NMR methods to deduce its chemical structure, which is of novel class.[2] The argument proceeds through distinct stages which are common to most problems of this nature.

1. Molecular Formula

The highest peak observed in the mass spectrum of CP-47,444 is at *m/e* 515.2493 corresponding within 2.6 ppm to the molecular formula $C_{28}H_{37}NO_8$. This agrees reasonably with chemical analysis, which yields 64.3% carbon (~65.2%), 7.6% hydrogen (~7.2%), and 2.6% nitrogen (~2.7%). The ^{13}C

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