Remote Nitrogen-15 Isotope Effects on Addition of Cyanide to NAD†

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ABSTRACT: The reversible reaction NAD + CN^- ⇌ NAD–CN was examined for remote secondary ^{15}N isotope effects caused by isotopic substitution at the ring nitrogen of the nicotinamide group. These were compared with analogous effects for dehydrogenase-catalyzed reactions, since both cyanide and the hydride ion add at the N-4 position of the nicotinamide ring. The ^{15}N effects on the rate constants for the forward and reverse processes were examined directly by conducting both the normal and isotopic reactions simultaneously under carefully controlled conditions in the sample and reference cells of a dual-beam spectrophotometer. In both cases, the ^{15}N kinetic isotope effect differed from 1.00 by considerably less than 0.01. The ^{15}N equilibrium isotope effect, ^{15}K, was obtained as the ratio of equilibrium constants measured separately with natural-abundance and labeled NAD by using a concentration jump procedure [1.004 ± 0.002 (cyanide addition)]. A similar value for ^{15}K of 1.010 ± 0.008 was obtained in an analogous manner for the reaction catalyzed by lactate dehydrogenase: NAD + lactate ⇌ pyruvate + NADH + H^+. The latter value is significantly smaller than a previously reported value obtained from kinetic studies [1.044 ± 0.012; Cook, P. F., Oppenheimer, N. J., & Cleland, W. W. (1981) *Biochemistry 20*, 1817]. The present value also is smaller than might be expected for a change in bond order from 4 to 3 [Cleland, W. W. (1980) *Methods Enzymol. 64*, 104–125] on the basis of the canonical resonance structures for NAD and NADH. However, quantum mechanical calculations [Huskey, W. P. (1985) Ph.D. Thesis, University of Kansas, Lawrence, KS] based on the crystal structure of NAD and NADH analogues are consistent with our lower value. The present results do not provide support for a mechanism for the activation of NAD by dehydrogenases that involves a geometric distortion of the nicotinamide ring.

Lactate dehydrogenase activates the 4-position of the nicotinamide ring of bound NAD by some 100-fold toward attack by nucleophiles such as hydride ion, cyanide, and pyruvate enolate [cf. Burgner & Ray (1984b)]. The magnitude of this rate enhancement was estimated by comparing the rate constants for the uncatalyzed addition of cyanide with those for the bimolecular enzyme-catalyzed process (Burgner & Ray, 1984a):

$$\text{NAD} + \text{CN}^- \rightleftharpoons \text{NAD–CN} \quad (1)$$

$$\text{E·NAD} + \text{CN}^- \rightleftharpoons \text{E·NAD–CN} \quad (2)$$

These authors suggest that the 100-fold rate effect is induced largely by *distortionless strain* (Fersht, 1974, 1977), which could be produced by holding one face of the nicotinamide ring in contact with hydrophobic side chains of the enzyme [cf. Holbrook et al. (1975) and Parker et al. (1978)]. By contrast, secondary kinetic isotope effects on the bond-making and -breaking processes have been reported when the nicotinamide ring nitrogen of NAD is replaced by ^{15}N and the [1-^{15}N]NAD is used in either the alcohol dehydrogenase or the formate dehydrogenase reactions. These isotope effects were interpreted in terms of a model where both the nicotinamide and dihydronicotinamide rings are *geometrically distorted* prior to transfer of hydride ion (Cook et al., 1981; Hermes et al., 1984). In attempting to reconcile these different suggestions for the activation of bound NAD, we investigated the remote secondary ^{15}N isotope effect for the *uncatalyzed* addition of cyanide to NAD. We expected that the ^{15}N kinetic isotope effect, i.e., ^{15}N KIE, or k_{14}/k_{15},[1] would be similar for the uncatalyzed addition of CN^- and H^- at the 4-position of NAD. If so, the CN^- adduct reaction would be a more accessible reference reaction than the uncatalyzed redox reaction for comparing with the enzyme-catalyzed reaction.

Using the cyanide adduct reaction as a reference for hydride addition seems reasonable, since the isotope effects that were studied are caused by a change in bond order at a nitrogen that is remote from the site of nucleophilic attack. However, none of the standard approaches for measuring kinetic and equilibrium isotope effects can be used with the cyanide adduct reaction. Thus, the equilibrium perturbation technique (Schimerlik et al., 1975) cannot be used because neither HCN nor NAD is sufficiently stable under the appropriate conditions to provide a stable equilibrium mixture. Moreover, the KIE's cannot be determined readily by isotope ratio mass spectrometry because the mobility of the above equilibrium precludes a simple isolation of either NAD or NAD–CN from the reaction mixture. Instead, we assessed kinetic isotope effects directly by conducting simultaneous reactions of cyanide with natural-abundance and [1-^{15}N]NAD in the sample and reference cells of a double-beam spectrophotometer.

Part of the present paper is devoted to demonstrating the feasibility of the above approach for *evaluating an intrinsic*

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[1] The following nomenclature, which is based on that suggested by Northrop (1977), is used for defining isotope effects: $^{15}k = k_{14}/k_{15}$, where k_{14} and k_{15} are the rate constants measured in the presence of the normal and heavy isotopes, respectively; $^{15}K = K_{14}/K_{15}$, where the equilibrium constants are defined in the same manner as the rate constants.

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KIE (i.e., ^{15}k-1) at the 1% level and for *detecting* considerably smaller KIE's; the sources of error in such a procedure also are considered as well as their minimization. In another part, both the concentration and pH-jump techniques are described that were used to obtain sufficiently accurate values of equilibrium constants to allow evaluation of the ^{15}N equilibrium isotope effects for the cyanide adduct reaction and for the lactate dehydrogenase reaction. Finally, the results are compared with published results for the alcohol and formate dehydrogenase reactions.

Experimental Procedures

Materials. The A_4 isozyme of lactate dehydrogenase was isolated by affinity chromatography from frozen muscle tissue of *Squalus acanthias*, the dogfish shark (Burgner & Ray, 1974, 1978). Lithium lactate and potassium cyanide were purchased from Sigma Chemical Co. and Alfa Inorganics, respectively, and were used without further purification. Stock solutions of KCN, 1 M, were stored frozen in liquid nitrogen, kept at 0 °C after thawing, and discarded a few hours later. The NAD was the best available grade from P-L laboratories. Samples of NAD–CN were prepared by adding 18.2 μmol of NAD to 1 mL of a solution prepared with 498 μmol of KCN and 240 μmol of HCl. The reaction mixture was incubated for 10 min at 30 °C and either used immediately or frozen in liquid nitrogen. Frozen samples were used within 2 days and immediately after thawing.

Methods. [1-^{15}N]NAD was prepared by NAD glycohydrolase catalyzed exchange of [1-^{15}N]nicotinamide with the thionicotinamide analogue of NAD (Oppenheimer et al., 1978; Oppenheimer & Davidson, 1980). Both the labeled NAD and commercial NAD were chromatographed twice on a 1 × 50 cm column of AG-1 × 8 resin, formate form (Bio-Rad). The isotopic purity of the [1-^{15}N]NAD was >99% by mass spectral analysis. A linear gradient from 0 to 1 M formic acid in the presence of 20% 1-propanol was used to elute the coenzyme [cf. Williams et al. (1976)]. The eluent containing the coenzyme was lyophilized, dissolved in water, and relyophilized; the dry powder was stored desiccated at −20 °C. Purity was checked by chromatography (0.1–0.5-μmol samples) on a SynChropak AX-100 anion-exchange column (250 × 4.1 mm). The sample was eluted isocratically with a buffer consisting of 15% (v/v) acetonitrile, 15 mM sodium acetate, pH 6, and 50 mM NaCl.

Kinetic isotope effects were assessed by conducting simultaneously isotopic and normal reactions in the sample and reference cells of a dual-beam spectrophotometer (Perkin-Elmer Model 575), which was equipped with an electronic temperature controller. Both cells (3 mL) contained 2.0 mL of the reaction mixture, and both were stirred continuously at 600 rpm. Slotted-disk magnets were used for the forward adduct reaction (the faster reaction) and small bar magnets for the reverse reaction (the slower reaction). (Abrasion of small particles from the surface of the disk magnets interfered with long-term measurements of small absorbance changes.) Reactions were initiated by simultaneous additions of the reactants. Two digitally adjustable pipets (SMI Co., Model Digital Adjusted Micro/pettor, 5–30 μL) capable of delivering a 20-μL sample with a precision of about ±0.3% (95% confidence level) were used. Since each glass barrel varies somewhat in size, the volume of one of the pipets was adjusted to that of the other by varying the plunger position with the digital adjustment. If the same dye is used in both pipets, a difference absorbance can be used as a monitor of a difference in volume. (Adjustment of the plunger position also was used to compensate for small differences in concentration of unlike reactants during the isotope studies.) The minisci were adjusted with the aid of a low-power dissecting microscope (6×) to the same position relative to the tip of the pipet by lightly blotting with tissue paper. To prevent premature mixing of the reactants, 2 μL of air was drawn into the pipets, and the outside of the glass barrel was wiped before placing the pipet tips in the reaction mixture. Depressing the plunger slowly over an interval of 1–3 s produced a more reproducible initial mixing than a rapid depression. Complete mixing (>99%) required about 3 and 15 s with the disk and bar magnets, respectively.

Initially, temperature differences between the sample and reference cuvettes were about 0.1 °C as determined with a two-probe differential thermometer constructed from a pair of transducers (Analog Devices, No. AD590). This temperature difference remained constant to within 0.01 °C for several hours. The difference in temperature between the two cells subsequently was minimized by adjusting the offset voltage on the differential amplifiers in the temperature controller for the reference and sample cells. Thermal contact between the cuvette and two adjacent walls of its holder was improved by inserting small wooden wedges between the holder and the cells.

Finally, the temperature difference between the two cells was minimized by monitoring rate differences between reactions involving the decomposition of identical samples of NAD–CN in terms of the differential absorbance, ΔA (see protocol in the Figure 3 legend). If a maximum or minimum in the recorder trace of ΔA vs. time was observed at $t \simeq 1/k$, the temperature of one of the cells was adjusted as above. This procedure was repeated until the recorder output was "flat", i.e., until $\Delta A_{max} \leq 0.0005$ (during a time when the overall change in absorbance in both cuvettes was about 1.0, cf. curve C, Figure 1). For all kinetic studies, a 10-min time interval was allowed for temperature equilibration of the stirred solutions.

Results

Differential Rate Effects on the Cyanide Adduct Reaction. When similar reaction mixtures, which produce the same overall change in optical density, are present in the sample and reference cells of a spectrophotometer, time-dependent differences in optical density will be produced by differences in the rate of the two reactions. For small differences in rate between two first-order or pseudo-first-order processes, the maximum difference in absorbance, ΔA_{max} occurs at a time, t, such that $t \simeq 1/k_s^{obsd}$. Thus, the following relationship holds, where k_s^{obsd} and k_r^{obsd} are the rate constants for the reaction in the sample and reference cells, respectively, $\alpha = k_s/k_r$, and A_{tot} is the total change in optical density in either cell:[2]

$$\Delta A_{max} = A_{tot}(1 - \alpha)/e \qquad (3)$$

The above relationship is demonstrated in Figure 1 by using a concentration-induced rate difference. Thus, curve A shows the change in absorbance as a function of time when the cyanide adduct reaction was conducted only in the sample cell and provides values of A_{tot} and k_s. Curves D and E in this figure show a maximum and a minimum, which are charac-

[2] The difference in absorbance produced by two simultaneous first-order processes which produce the same overall absorbance change is given by $A_{tot}[\exp(-k_s t) - \exp(k_s t/\alpha)]$. The maximum deflection occurs at $t = \alpha \ln \alpha/[k_s^{obsd}(\alpha - 1)]$, and its magnitude is equal to $A_{tot}[\alpha^{-(\alpha/\alpha-1)} - \alpha^{1/\alpha-1}]$. These expressions are approximately equal to those in the text when $\alpha \simeq 1$.

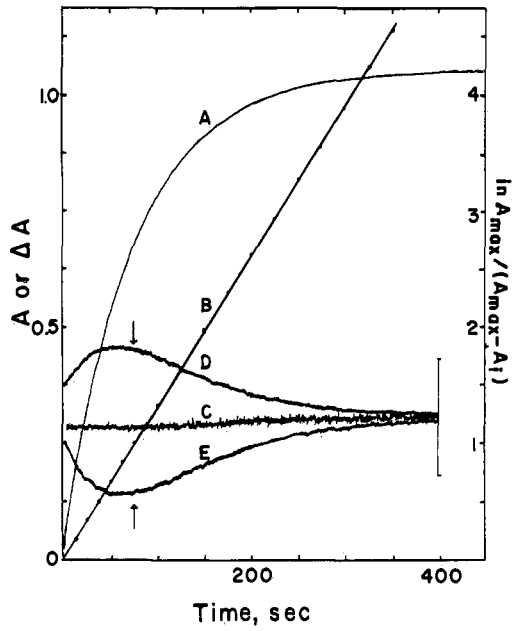


FIGURE 1: Direct, difference, and semilog plots for the cyanide adduct reaction. In all cases, reaction mixtures were made up with 240 μmol of KCN and 120 μmol of HCl in 2.00 mL (pH 9.3); absorbance changes at 327 nm were measured at 25 °C in stirred cells after the temperature difference between the two cells was minimized (see Experimental Procedures). Curve A, reaction initiated by adding 0.36 μmol of NAD in 20 μL to sample cell. Curve B, a plot of the linear first-order transform of the data from curve A; k_f^{obsd} calculated from the slope of this plot is 1.31×10^{-2} s^{-1}. Curve C, same as curve A except that the same amount of NAD was added simultaneously to both cells, and absorbance differences were recorded. Curve D, same as curve C except that the reference cell contained an additional 5 μmol of HCl (4% less CN^-). Curve E, same as curve C except that the sample cell contained an additional 5 μmol of HCl. The vertical arrows and bar, respectively, show the location of $t = 1/k$ and $\Delta A_{327} = 0.01$ for the difference plots.

teristic of those produced by small rate differences when the same reaction is conducted simultaneously in both cells. (The rate difference was produced by a difference in cyanide concentration of ±4% in the sample cell; see figure legend.) The vertical arrow shows where $t = 1/k$. In both cases, ΔA_{max} occurs close to $t = 1/k$, as expected.

The size of ΔA_{max} produced by a small temperature difference between the two cells, ΔT, can be obtained by replacing α in eq 3 by $\exp[(-\Delta H^{\ddagger}/R)(\Delta T/T^2)]$. Since $\Delta H^{\ddagger}$ for the cyanide adduct reaction is about 12 and 20 kcal/mol for the forward and reverse processes, respectively (Burgner & Ray, 1984a), if $\Delta T = 0.08$ °C, a 0.5% rate difference in the forward reaction and a 1% rate difference in the reverse process will be observed. A 1% rate difference in either process produces a ΔA_{max} of 0.0036 under conditions where $A_{tot} \simeq 1.0$, which is a readily detectable change. Hence, temperature differences between the two cuvettes were reduced by minimizing ΔA_{max} by using the procedure described under Experimental Procedures. Curve C in Figure 1 shows a trace of the recorder output after the temperature differential had been minimized. We estimate that the temperature differential between the two cells was no more than 0.02 °C.

Very small differences in pH also could produce a differential rate effect on the cyanide adduct reaction, and such differences must be considered, since reactions were initiated with different samples of NAD in determining the ^{15}N KIE (see below) and since the added NAD was at a lower pH than the reaction mixture. In fact, a pH difference of only 0.008 pH unit would produce a rate difference of 1%. However, all samples of NAD were isolated in the same way (see Experimental Procedures) and used at a concentration only 0.003 of that for the CN^- present in the reaction mixture. Hence, the pH differences that are produced upon initiation of the reaction are likely to be too small to alter significantly the reaction rates.[3]

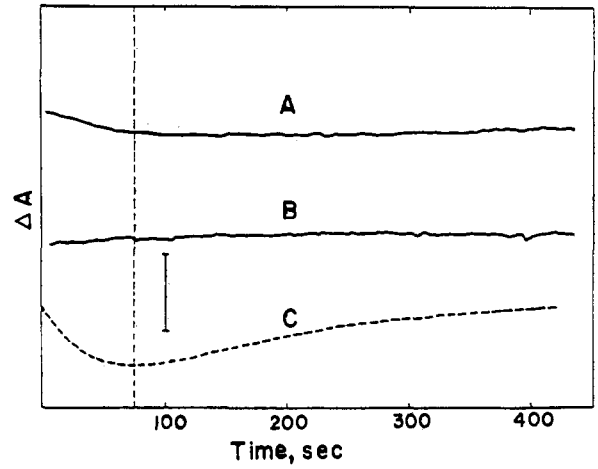


FIGURE 2: ^{15}N kinetic isotope effect in the formation of NAD–CN by direct assessment of rate differences. Plots of absorbance differences at 327 nm are shown. Reaction conditions were the same as for curve C of Figure 1 except that natural-abundance NAD was added to one cell and [1-^{15}N]NAD to the other. Curve A; [1-^{15}N]NAD in the reference cell; curve B, [1-^{15}N]NAD in the sample cell; curve C, the expected change in absorbance for an ^{15}N KIE of 1.010 with [1-^{15}N]NAD in the sample cell. The vertical dashed line shows where a maximum or minimum value of ΔA is expected when the reaction exhibits a KIE. The vertical bar defines an ΔA of 0.005.

The presence of an NAD-like contaminant in one of the added NAD samples could generate apparent rate differences if that contaminant produced a significant optical density change at 327 nm in the reaction mixture. Since the same purification procedure was used for all NAD samples and since no heterogeneity was detected when these were subjected to high-pressure liquid chromatography (HPLC) on an anion-exchange column (see Experimental Procedures), such a possibility is unlikely.

^{15}N Kinetic Isotope Effect on the Cyanide Adduct Reaction. An estimate of the ^{15}N KIE in the forward cyanide adduct reaction was obtained by conducting simultaneous reactions with natural-abundance and [1-^{15}N]NAD in both spectrophotometer cells after conducting the control experiments described above. The results are shown in Figure 2 along with that expected for a KIE of 1.01 (dashed line). The upper tracing was obtained with labeled NAD in the sample cell; the middle tracing was produced with labeled NAD in the reference cell. The deviation from linearity in the upper plot can be rationalized primarily in terms of a concentration difference in the NAD added to the cells (less than 0.2% difference).[4] The middle plot shows no evidence of a concentration difference. Both plots show that the ^{15}N KIE for

[3] Potential problems caused by pH differences were investigated by using samples of commercial NAD either adjusted to pH 5 or left at pH 3. These samples, when used in the KIE assay, do not produce a detectable rate difference. Moreover, a 10% difference in contamination by formic acid in one sample (on a dry weight basis), which is the maximum possible and is highly unlikely, would decrease the cyanide concentration by less than 0.5%.

[4] A logarithmic change in ΔA with time is obtained when simultaneous first-order reactions are conducted as described here but at different initial concentrations of the varying reactant. Pipeting errors of 0.3% or less (see Experimental Procedures) thus produce a "base-line drift" without an inflection.

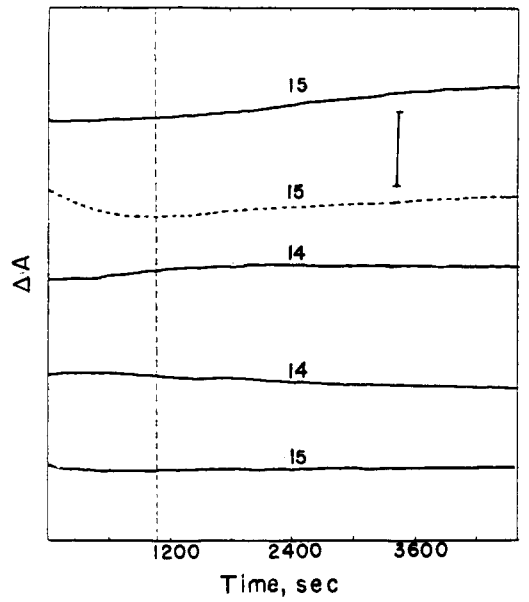


FIGURE 3: ^{15}N kinetic isotope effect in the decomposition of NAD–CN by direct assessment of rate differences. The reaction was initiated by adding 0.36 μmol of freshly prepared NAD–CN in 20 μL simultaneously to both sample and reference cells, which contained 2.0 mL of 0.1 M sodium phosphate, pH 7.0. The numbers above the curves indicate which isotope was present in the sample cell. The dashed curve and dashed horizontal line are analogous to the comparable features in Figure 2. The vertical bar equals an ΔA of 0.01.

the forward cyanide adduct reaction must be much closer to 1.00 than to either 1.01 or 0.99: cf. the dashed reference plot. Numerous other experiments produced comparable results.

^{15}N Kinetic Isotope Effect on the Reverse Cyanide Adduct Reaction. The decomposition of the NAD–CN adduct, which is prepared in the presence of excess cyanide at high pH (see Experimental Procedures), is induced by lowering the pH and reducing the concentration of cyanide (see Figure 2 legend). This reaction also was conducted simultaneously in the sample and reference cells of a spectrophotometer with natural-abundance and [1-^{15}N]NAD–CN. The results are shown in Figure 3. The dashed curve in this figure shows the absorbance differences expected for a KIE of 0.99 and the [1-^{15}N]NAD–CN in the sample cell. The experimental curves in the figure show that the KIE for the decomposition of NAD–CN is much closer to 1.00 than to 0.99 (dashed curve). However, an inspection, perhaps subjective, of the actual recorder tracings for these curves in Figure 3 plus numerous other plots for the reverse cyanide adduct reaction *suggests* that a small, inverse KIE of greater than 0.997 might occur during this process.

^{15}N Equilibrium Isotope Effect (EQIE) for the Cyanide Adduct Reaction. The ^{15}N EQIE for the cyanide adduct reaction was estimated directly from the ratio of equilibrium constants, K_{14}^{app} and K_{15}^{app}, that were measured under the same conditions with natural-abundance and [1-^{15}N]NAD, respectively. Both equilibrium constants were obtained by comparing the absorbance of equilibrium mixtures, where approximately 50% of the NAD was converted to NAD–CN, with the absorbance when the *same* reaction mixture was driven to completion. Complete conversion to NAD–CN was accomplished by increasing the CN^- concentration 100-fold by adding a small volume of concentrated KCN solution (see Table I). Replicate control experiments (seven) conducted in a similar manner with the normal lactate dehydrogenase (LDH) reaction (see below) demonstrate that the standard error of the estimate for equilibrium constants determined in this way can be maintained below 0.5% of the measured value for the LDH reaction and below 0.3% for the cyanide adduct reaction (data not shown).

Table I: ^{15}N Equilibrium Isotope Effect for the Cyanide Adduct Reaction[a]

$^{14}K_{app}$	^{15}N EQIE[b]	
1.382	1.006	1.003
1.383	1.007	1.004
1.379	1.004	1.001
$^{15}K_{app}$	1.374	1.378

[a] The equilibrium process was initiated by adding 0.18 μmol of NAD in 20 μL to 1.0 mL of 0.1 M Na-CHES buffer [2-(*N*-cyclohexylamino)ethanesulfonate], pH 9.22, that contained 10 μmol of KCN and 5 μmol of HCl. The mixture was incubated at 25 °C for 1 h (ca. 10 half-times). After the absorbance increase at 327 nm was recorded at equilibrium (A_{eq}), 500 μmol of aqueous KCN in a volume of 0.100 mL subsequently was added to the above solution and the final absorbance was measured (A_∞). The K_{app} (at 4.92 mM CN^-) was calculated from $A_{eq}/(A_\infty - A_{eq})$ after correction of A_∞ for the volume change produced on addition of the KCN. [b] ^{15}N EQIE equals $^{14}K_{app}/^{15}K_{app}$.

A primary concern in this study was the stability of both the NAD and the NAD–CN present during the 60 min at pH 9.22 that was required to reach a 50:50 equilibrium (10 half-times). The stability of NAD–CN was verified by monitoring the absorbance for a sample of NAD–CN at the same temperature and pH during a comparable time interval. In addition, the absorbance of the above 50:50 equilibrium mixture did not change during an additional 60 min under the same conditions.

To evaluate the ^{15}N EQIE for the cyanide adduct reaction, three determinations were made of K_{14}^{app} and two of K_{15}^{app}. The same stock reaction mixture, precise temperature control, and nearly identical concentrations of both samples of NAD were used to eliminate as many causes of systematic error as possible. Measured values for these constants are shown in Table I, which also describes the method of calculation. A value for the ^{15}N EQIE of 1.004 ± 0.002 is calculated by averaging the ratios for all combinations of K_{14}^{app} and K_{15}^{app} in the table.

^{15}N Equilibrium Isotope Effect for the Lactate Dehydrogenase Reaction. The ^{15}N EQIE for lactate + NAD ⇌ pyruvate + NADH + H^+ was obtained in a manner analogous to that used above for the cyanide adduct reaction except that here the reaction was driven from about 50% completion at pH 8.20 to more than 99% completion by increasing the pH with added KOH. (Subsequent addition of a small amount of lactate dehydrogenase did not alter the final absorbance.) The calculated value of 4.18×10^{-12} M for the pH-dependent equilibrium constant agrees with values of (3–5) $\times 10^{-12}$ M measured under similar conditions by others (Nielands, 1952; Donovan et al., 1975; Cook et al., 1980). The ^{15}N EQIE for the lactate dehydrogenase reaction obtained as the average of all possible *ratios* of the five determinations of K_{14}^{app} and the four determinations of K_{15}^{app} in Table II is 1.010 ± 0.008.

Discussion

Although ^2H KIE's frequently can be measured with reasonable accuracy in separate experiments involving labeled and unlabeled reactants, the small size of the intrinsic KIE for the heavier isotopes of C, N, and O usually requires a competitive-type assay where both labeled and unlabeled reactants are present in the same reaction mixture, as in the equilibrium perturbation procedure (Schimerlik et al., 1975) or in the use of mass spectrometry to determine the relative amounts of the

Table II: ^{15}N Equilibrium Isotope Effect for the Lactate Dehydrogenase Reaction[a]

$^{14}K_{app}$	^{15}N EQIE[b]			
4.39	1.009	1.021	1.019	1.016
4.35	1.000	1.012	1.009	1.007
4.35	1.000	1.012	1.009	1.007
4.40	1.011	1.023	1.021	1.019
4.33	0.995	1.007	1.005	1.002
$^{15}K_{app}$	4.35	4.30	4.31	4.32

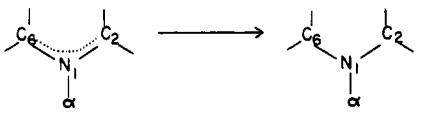
[a] The initial equilibration mixture contained 100 μmol of lithium lactate, 0.14 μmol of NAD, and 10 units of lactate dehydrogenase in 1.025 mL of solution that also contained 50 mM Bicine [*N,N*-bis(2-hydroxyethyl)glycine] and triethylamine sulfate; the pH of the mixture was 8.20 and the temperature 25 °C. The equilibrium process was initiated either by addition of 5 μL of the enzyme solution or by addition of 20 μL of the NAD solution. The increase in absorbance at 340 nm, A_{eq}, was recorded after 10 min (at least 20 half-times). Subsequently, the pH was increased to 10.3 by adding 14.7 μL of 5.4 N KOH, and A_∞ was recorded. After a correction was made for the volume of the added base, K_{app} was calculated for pH 8.20 as [Pyr]-[NADH]/[NAD][Lac], i.e., as $A_{eq}^2/(A_\infty - A_{eq})$[Lac], where [Lac] = 0.1 M. [b] ^{15}N EQIE is equal to $^{14}K_{app}/^{15}K_{app}$.

isotopic reactant remaining or the isotopic product produced [cf. Cleland (1980, 1982)]. For reasons noted in the introduction, neither of these approaches is readily applicable to measuring the remote secondary ^{15}N KIE for the cyanide adduct reaction with NAD. Hence, a spectrophotometric procedure was developed, which is similar in principle to that used by Albery and Robinson (1969) and by Rosenberg and Kirsch (1979) for measuring ratios of rate constants. However, it is necessary to exercise careful control of reaction conditions to successfully employ this procedure to evaluate the [1-^{15}N]effect on the cyanide adduct reaction.

If separate reactions involving labeled and unlabeled reactants are compared by conducting them sequentially in the same spectrophotometer cell, temperature *drift* is the primary problem. When both the sample and the reference cells are used simultaneously, the temperature *difference* between the two cells is critical. In either case, prevention of spurious temperature-induced rate effects, which mimic KIE's, is required for accurate measurement of isotope effects. In fact, the temperature difference between the two cells can be accurately measured, if the activation energy for the process is known precisely [see Methods as well as Albery & Robinson (1969)]. In the case of a first- or pseudo-first-order reaction such as the cyanide adduct reaction, temperature differences (between sample and reference cells) produce an absorbance difference, ΔA, that changes with time (Figure 1) in a manner precisely analogous to that obtained in an equilibrium perturbation experiment (Schimerlik et al., 1975). Reducing the *temperature-induced* maxima or minima in such plots to an undetectable level was the basis of our procedure for minimizing the temperature difference between the reference and sample cells (see Results). In addition, pH differences must be minimized, since pH affects the reaction rate in the present system. Finally, the effect of pipetting errors and impurities also must be minimized. The procedures and controls described under Results indicate that we have dealt adequately with these problems and that intrinsic KIE's of only a few tenths of a percent can be detected in the reation of NAD and cyanide. Unfortunately, this procedure is not useful for accurate measurement of intrinsic KIE's smaller than about 1%.

In the following discussion, we assume that the NAD–CN adduct is structurally similar to NADH. The basis for this assumption has been considered only obliquely by a number of authors (Lindquist & Cordes, 1968; Bruice & Benkovic, 1966; Sund et al., 1964; Johnson & Smith, 1976), but nothing we observe here gives us cause to question this assumption.[5]

Scheme I



The intrinsic ^{15}N KIE's for both the forward and reverse cyanide adduct reaction are much less than 1%. Thus, bonding that involves the ring nitrogen of nicotinamide is not much different in the transition state than in the ground state for either NAD or NADH. This conclusion is not particularly surprising, except by way of contrast with the KIE's reported for the hydride transfer to and from NAD and NADH catalyzed by alcohol and formate dehydrogenase (see below).

Values of KIE's close to 1.00 also are consistent with our estimate of the ^{15}N EQIE for the cyanide adduct reaction of 1.004 ± 0.002 (Table I). However, an EQIE so close to 1.00 was not expected on the basis of conventional comparisons of ^{15}N EQIE's in other systems and on the measured ^{15}N EQIE for the alcohol dehydrogenase reaction. Thus, the canonical resonance structures around the ring nitrogen of the nicotinamide moiety for NAD and NADH, which are shown in Scheme I, show a change in bond order at N-1 from 4 to 3. Such changes in simple systems can provide an EQIE of about 1.04 (Cook et al., 1981; Hermes et al., 1984).[6] However, canonical structures do not provide a good estimate of the bond order at N-1 for NAD and NADH. Thus, crystallographic studies show that the lengths of the covalent bonds to N-1 do not change substantially on conversion of NAD to NADH: N_1–C_2, 1.34–1.38 Å; N_1–C_6, 1.35–1.43 Å; N_1–C_α, 1.49–1.47 Å (Karle, 1961; Voet, 1973; Reddy et al., 1981). In fact, a comprehensive analysis of vibrational modes for the nicotinamide and dehydronicotinamide groups of NAD and NADH provides an ^{15}N EQIE of only 1.008 (Huskey, 1985), which is in accord with our value of the EQIE for the cyanide adduct reaction (1.004; see above) as well as our value of the ^{15}N EQIE for the lactate dehydrogenase reaction (1.01 ± 0.008) (Table II).[7] Why the ^{15}N EQIE of 1.044 ± 0.012 obtained from equilibrium perturbation studies on alcohol dehydrogenase reaction is so different from the above three values is not known.

Since the value of the ^{15}N EQIE reported for the alcohol dehydrogenase reaction differs from the values reported here, at least one of the KIE's for the alcohol dehydrogenase reaction must differ substantially from the corresponding KIE's for the cyanide adduct reaction. In fact, both of the KIE's do. Es-

[5] Of course, the bond between C-4 of the nicotinamide ring and CN is much more polar than the corresponding bond to H, but we view this as a secondary effect. On the other hand, the puckering behavior of the dihydronicotinamide ring in those NADH and NAD–CN conformers without a stacked-ring structure may not be the same (N. J. Oppenheimer, unpublished observations).

[6] The ^{15}N isotope effect for $NH_4^+ \rightarrow NH_3 + H^+$ originally was reported as 1.039 (O'Leary, 1977). Later this value was reduced to 1.019 (Hermes et al., 1985). However, an increased isotope effect is expected when bond scission occurs between carbon and nitrogen rather than hydrogen and nitrogen [Cleland, 1980 (Table I, footnotes r and x); Hermes et al., 1985 (Table IV)]; i.e., the effect for $CH_3NH_3^+ \rightarrow CH_4 + NH_3$ is expected to be 1.035.

[7] In general, equilibrium isotope effects are determined by bond "stiffness" as opposed to bond order (Hartshorn & Shiner, 1972). However, in the present *ring* systems, one can assume (Huskey, 1985) that torsional (out of the ring bending) force constants increase with increasing bond order. Although the increase is more rapid than for stretching force constants, bond order and stiffness are somewhat related concepts in such a system.

pecially important is the difference in KIE for the reverse process (NADH → NAD). In the alcohol dehydrogenase reaction, a normal KIE was reported for the reverse process. This suggests that the bond order around the ring nitrogen in the transition state is *reduced* relative to that in NADH, even though bonding at N-1 must *increase* in the overall conversion of NADH to NAD. On the basis of this observation, the tantalizing suggestion was made that alcohol dehydrogenase, in particular, and possibly dehydrogenases in general distort the bound NADH so that in the transition state it is further from NAD, structurally, than NADH. While our present results do not rule out this possibility, the much larger value of the ^{15}N EQIE that was reported for those studies relative to that determined here suggests that such a mechanism has not been adequately verified. On the other hand, if the transition state for dehydrogenase reactions turns out *not* to be intermediate between NAD and NADH, it would stand in sharp contrast with the transition state for the uncatalyzed cyanide adduct reaction studied here where bonding at N-1 likely is close to that of both NAD and NAD–CN if not intermediate between them (see above). Thus, in addition to the small ^{15}N KIE's that we observe for the cyanide adduct reaction, $\Delta H°$ for this process is relatively modest in size. Although the Hammond (1955) postulate deals with energetics rather than structure, a relatively small absolute value of $\Delta H°$ suggests that the energetics if not the structure of the transition state for the adduct reaction are intermediate between those of NADH and NAD and probably NADH-like. Similar conclusions have been drawn from the effect of solvents and substituents on the cyanide adduct reaction (Lindquist & Cordes, 1968). Although it is obvious that enzymes induce major changes in the energetics of the transition states for the reactions they catalyze, documenting the induction of a substantial change in geometrical structure of the transition state by an enzyme would be intriguing.

Added in Proof

The results of a subsequent study, where an isotope ratio mass spectrometer was used, indicate that the ^{15}N EQIE for the nicotinamide ring nitrogen is about 1.005 for oxidation of lactate by NAD. This isotope effect was determined by measuring the change in natural-abundance ratios of ^{15}N to ^{14}N in both the reactant and product coenzymes (J. W. Burgner, P. M. Weiss, and W. J. Ray, unpublished observations).

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Registry No. NAD^+, 53-84-9; H^-, 12184-88-2; CN^-, 57-12-5; ^{15}N, 14390-96-6; dehydrogenase, 9035-82-9; lactate dehydrogenase, 9001-60-9.

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