Deuterium Isotope Effects on Initial Rates of the Liver Alcohol Dehydrogenase Reaction†

Karen Bush,‡ Vernon J. Shiner, Jr., and H. R. Mahler§,*

ABSTRACT: Initial steady-state velocities using highly purified deuterated substrates and coenzymes as well as an isoenzymatically homogeneous enzyme preparation have been measured for liver alcohol dehydrogenase at 25° in 20 mM phosphate (pH 7.0) or 20 mM pyrophosphate (pH 8.8). Significant isotope effects of $v_{0,H}/v_{0,D}$ were observed. In the reduction of acetaldehyde by A-NADD the isotope effects were found to decrease with increasing acetaldehyde concentration, a result consistent with an ordered Theorell–Chance mechanism for the reduction of acetaldehyde *only* at saturating substrate concentrations. No kinetically significant interactions have been observed between the enzyme and the untransferred hydrogen of NADH.

The action of horse liver alcohol dehydrogenase, one of the most thoroughly studied NAD^+-requiring enzymes, can be represented by the following series of steps (eq 1–5). This

$$E + NAD^+ \rightleftharpoons E \cdot NAD^+ \quad (1)$$

$$E \cdot NAD^+ + \text{alcohol} \rightleftharpoons E \cdot NAD^+ \cdot \text{alcohol} \quad (2)$$

$$E \cdot NAD^+ \cdot \text{alcohol} \rightleftharpoons E \cdot NADH \cdot \text{aldehyde} \quad (3)$$

$$E \cdot NADH \cdot \text{aldehyde} \rightleftharpoons E \cdot NADH + \text{aldehyde} \quad (4)$$

$$E \cdot NADH \rightleftharpoons E + NADH \quad (5)$$

enzymatic reaction is generally considered to obey a modified Theorell–Chance mechanism with compulsory binding of the coenzyme as the initial step (Theorell and Chance, 1951). Although it has been proposed that the reaction sequence includes active ternary complexes (Baker, 1962; Mahler *et al.*, 1962; Silverstein and Boyer, 1964; Wratten and Cleland, 1965; Ainslie and Cleland, 1972), the kinetic importance of these is a matter of question (Silverstein and Boyer, 1964; Wratten and Cleland, 1965; Ainslie and Cleland, 1972; Dalziel, 1963b; Massey and Veeger, 1963; Strittmatter, 1966) and their concentration does not account for a stoichiometrically significant proportion of total enzyme. A strict interpretation of the Theorell–Chance mechanism requires the final enzyme–coenzyme dissociation reaction to be rate determining for primary alcohols and aldehydes.

However, all of the above interpretations have been based on experiments using alcohol dehydrogenase preparations containing as many as 12 isoenzymes (Pietruszko *et al.*, 1969; Pietruszko and Theorell, 1969; Lutstorf *et al.*, 1970) and thus are open to some questions. Since it is now possible to use a homogeneous alcohol dehydrogenase, we decided to determine deuterium isotope effects on the kinetic properties of such an enzyme (isoenzyme III or EE) (Pietruszko *et al.*, 1969; Pietruszko and Theorell, 1969; Lutstorf *et al.*, 1970). This isoenzyme accounted for 60–65% of the total amount of alcohol dehydrogenase present in commercial preparations. Using isoenzyme EE with stereospecifically deuterated reduced coenzymes (A-NADD[1] indicating deuterium replacement of the transferable hydrogen of NADH, B-NADD indicating deuterium replacement of the nontransferable hydrogen) we have already reported on the isotope effects on the equilibrium constant of step 5 of the above sequence (Bush *et al.*, 1971). We have now studied the initial reaction velocities using deuterated ethanol and the deuterated coenzymes at saturating substrate concentrations under steady-state conditions and have compared them with results of previous work from this laboratory (Baker, 1962; Mahler *et al.*, 1962).

Materials and Methods

Liver alcohol dehydrogenase was purified as follows. Immediately after dialysis of 9–10 mg of Boehringer alcohol dehydrogenase at 4° (60 hr *vs.* 300 vol of 20 mM phosphate (pH 7.0) with five buffer changes and 11 hr *vs.* 60 vol of 20 mM phosphate (pH 6.4)) the enzyme was eluted from a 0.7 × 18 cm column of Whatman CM52 (carboxymethylcellulose) (Dalziel, 1958) at 8° with 20 mM phosphate (pH 6.5). The major peak was collected in several fractions with either the leading edge or center of the peak used for kinetic studies. The selected dehydrogenase fraction was homogeneous upon rechromatography on carboxymethylcellulose as well as on Sephadex G-100 and starch gel electrophoresis. Dehydrogenase concentrations were determined from the absorbance at 280 nm using an extinction coefficient of 0.455 ml/mg of protein per cm (Taniguchi, 1967).

NAD^+ and NADH from Sigma (grade III, ~98%) were used without further purification. NADH was also prepared from NAD^+ simultaneously with a preparation of A-NADD. NAD(D)$^+$ was prepared by cyanide exchange in D_2O (Colowick and Kaplan, 1957). The deuterated reduced

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‡ Present address: Department of Chemistry, University of Delaware, Newark, Del. 19711.

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[1] Abbreviations used are: A-NADD and B-NADD, reduced nicotinamide adenine dinucleotide with deuterium in the 4A and 4B nicotinamide positions, respectively; NAD(D)$^+$, NAD^+ with deuterium in the 4 nicotinamide position; glpc gas–liquid phase chromatography; pmr, proton magnetic resonance.

coenzymes were prepared as described by Colowick and Kaplan (1957) with the enzymatic method used for reduction and were purified by a modification of Winer's (1964) chromatographic method. They were stored desiccated *in vacuo* at −20° in the dark. Deuterium content (by pmr peak height integration) of the various deuterated coenzymes was >0.95 g-atom/mol for A-NADD and >0.90 g-atom/mol for NAD(D)$^+$ and B-NADD. For kinetic runs ethanol-d_6 from Isotopes, Inc. (Teledyne Co.) was further purified by glpc to eliminate two kinetically important impurities. Acetaldehyde was doubly distilled immediately before use and the purity of nondeuterated ethanol verified by glpc.

Assay mixtures were prepared with the final concentrations of reactants shown in Table I. Ethanol oxidations were performed at the highest alcohol concentration possible before substrate inhibition occurred. All coenzyme concentrations were also saturating. Kinetic measurements were performed by following the appearance or disappearance of NADH at 340 nm using a Gilford 240 spectrophotometer with the cell compartment maintained at 25°. A small amount of freshly purified alcohol dehydrogenase (15–30 μl depending upon the experiment) was placed in a plastic mixing spoon with reaction initiated by a few rapid up and down motions of the spoon in the cuvet containing 2 ml of the assay mixture. At least two sets of assays were run for each protio–deuterio pair with each set consisting of at least triplicate determinations for each compound. The order of individual assays was randomly alternated (protio–deuterio) in each set. Rate measurements were made by extrapolating the initial change in 340-nm absorbance to zero time. Initial velocities were linear for at least 30 sec. Blank reactions of enzyme–coenzyme and enzyme–substrate were negligible. Isotope effects were calculated from the ratios of the average linear rate per minute for each set of compounds.

TABLE I: Concentrations of Substrates for Alcohol Dehydrogenase Kinetic Studies at 26°.[a]

[Acetaldehyde] (mM)	[NADH] or [NADD] (mM)	[Enzyme] (μM)	[NAD$^+$] or [NAD(D)$^+$] (mM)	[Ethanol-d_5] (mM)
0.05, 0.50, 5.0	0.17	0.016		
		0.13[b]	8.0[b]	8.0[b]
		0.080[c]	0.13[c]	7.3[c]

[a] Acetaldehyde reduction run in 20 mM phosphate (pH 7.0). Alcohol oxidations run in 20 mM pyrophosphate (pH 8.8). NAD$^+$–NAD(D)$^+$ reduction checked in both buffers. [b] Ethanol-d_5–ethanol rate comparison. [c] NAD$^+$–NAD(D)$^+$ rate comparison.

Results

The isotope effects on initial velocities for various reaction sequences are shown in Table II together with the earlier results (Baker, 1962; Mahler *et al.*, 1962). The oxidation of ethanol by NAD$^+$ showed a small, but significant, isotope effect with ethanol-d_5 as the substrate. This agrees with the values determined previously by us. It also corresponds well with the tritium isotope effect of 1.41 observed by Palm *et al.* (1968) for the oxidation of ethanol-*1-t*, a result that, according to the relationship described by Swain *et al.* (1958), predicts a deuterium isotope effect of 1.27 for the corresponding deuterated ethanol. If the Theorell–Chance mechanism is valid for the oxidation of primary alcohols, then dissociation of enzyme–NADH should be rate limiting. In order for this step to exhibit an isotope effect, there must be some kinetically significant interaction between the transferred hydrogen (deuterium in the A-NADD form) and the enzyme. Such an interaction has been proposed for yeast alcohol dehydrogenase (Shiner *et al.*, 1960), lactate dehydrogenase (Thomson *et al.*, 1964; Thomson and Nance, 1965), malate dehydrogenase (Thomson *et al.*, 1962), and both the heterogeneous (Baker, 1962; Mahler *et al.*, 1962) and homogeneous (Bush *et al.*, 1971) liver alcohol dehydrogenase enzymes.

Transient kinetic techniques have been used to show a significant isotope effect (k_H/k_D = 5.2) on the rate of formation of the alcohol dehydrogenase–(A-NADD) complex from dehydrogenase and saturating concentrations of NAD$^+$ and ethanol-d_5 (Shore and Gutfreund, 1970). Therefore, hydrogen transfer must be involved in that process. However, the rate of that step is so large and the isotope effect in the steady-state reaction is so small that hydrogen transfer from ethanol to NAD$^+$ cannot contribute significantly to the observed isotope effect on the overall steady-state process. This conclusion is in agreement with previous observations regarding the rapid rate of hydrogen transfer within the ternary complex (Silverstein and Boyer, 1964; Wratten and Cleland, 1965; Dalziel and Dickenson, 1966).

From the absence of any isotope effect with either NAD(D)$^+$ or B-NADD we may conclude that any secondary interaction of the untransferred C-4 hydrogen with the enzyme is kinetically negligible or nonexistent. The discrepancy between our value and that found earlier (Baker, 1962; Mahler *et al.*, 1962) may be attributed to impure coenzymes. With older B-NADD preparations we observed small isotope effects and anomalous optical characteristics which increased with the age of the coenzyme. It is possible that some type of competitive inhibitor developed (Dalziel, 1963a) or epimerization of the C-4 hydrogens occurred (Ludowieg and Levy, 1964) as the preparations were stored.

Reduction of acetaldehyde by reduced coenzymes as reported in Table II was followed at saturating coenzyme concentrations and initially at 0.5 mM acetaldehyde, a concentration close to the reported K_m value (Dalziel and Dickinson, 1966). In agreement with earlier observations the reduction of acetaldehyde by A-NADD at this aldehyde concentration resulted in a significant decrease in initial velocity compared to commercial (Sigma) NADH, an effect which was constant for several different A-NADD preparations even after storage for many months.

Further studies with A-NADD were carried out over a range of acetaldehyde concentrations with all velocities compared to reactions with NADH which was prepared identically with the deuterated coenzyme. A double reciprocal plot of the data is shown in Figure 1. In these studies the isotope effect at 0.5 mM acetaldehyde increased to 1.78, indicating that the reduced coenzyme prepared in the laboratory did not contain the kinetically significant impurity reported to be present in commercial coenzyme (Dalziel, 1963a). Most importantly, the isotope effect was 2.21 at 0.05 mM, 1.20 at 5.0 mM, *and disappeared at extrapolated infinite aldehyde concentration.* These results are substantiated by the recent studies of Gershman and Abeles (1973) who reported a kinetic isotope effect of 1.08 on the maximum velocity of the reduction of acetaldehyde by NADH and A-NADD using heterogeneous alcohol dehydrogenase.

TABLE II: Deuterium Isotope Effects upon Initial Velocities of Various Alcohol Dehydrogenase Reaction Sequences.[a]

Coenzyme	Substrate	Products	Previous results (2) $V_{max,H}/V_{max,D}$	This work $v_{0,H}/v_{0,D}$
NAD^+	Ethanol-d_5	Acetaldehyde-d_4 + A-NADD	1.32	1.34 ± 0.02
$NAD(D)^+$	Ethanol	Acetaldehyde + B-NADD		0.99 ± 0.01
A-NADD	Acetaldehyde (0.5 mM)	1-Monodeuterioethanol + NAD^+	1.59	1.60 ± 0.03
A-NADD	Acetaldehyde (0.05 mM)[b]	1-Monodeuterioethanol + NAD^+		2.21 ± 0.11[b]
	Acetaldehyde (0.5 mM)[b]	1-Monodeuterioethanol + NAD^+		1.78 ± 0.06[b]
	Acetaldehyde (5.0 mM)	1-Monodeuterioethanol + NAD^+		1.20 ± 0.02[b]
B-NADD	Acetaldehyde (0.5 mM)	Ethanol + $NAD(D)^+$	1.47	1.01 ± 0.01

[a] All comparisons were made relative to Sigma coenzyme unless otherwise indicated. [b] Rate comparison relative to NADH prepared simultaneously with A-NADD.

Discussion

These last results for aldehyde reduction strongly suggest that E·NAD^+ dissociation is rate limiting (Theorell–Chance mechanism) *only* at high acetaldehyde concentrations and that at lower acetaldehyde concentrations some prior step with an isotope effect becomes at least partly rate determining. The most likely possibility is, of course, the interconversion of the ternary complexes. Shore and Gutfreund (1970) observed an isotope effect of $k_H/k_D \simeq 3$ in the ("burst") oxidation of E·(A-NADD) by acetaldehyde. Even though they set a very high lower limit for the first-order rate of conversion of E·NADH·aldehyde to E·NAD^+·alcohol they were not able to detect any stoichiometrically significant concentration of ternary complexes so that the rate of loss of aldehyde from the E·NADH·aldehyde ternary must also be very fast. Thus, it appears that under steady-state conditions with low aldehyde concentrations the conversion of ternary complexes might be at least partly rate controlling. The reaction E + A-NADD does not appear a likely candidate for the rate controlling step because the initial rates of reaction are linear and independent of [E·A-NADD]. Also, this step should not be dependent on acetaldehyde concentration. Since the concentration of NAD^+ increases continuously from zero to finite values as the reaction proceeds, competition by these species would be expected to lead to monotonic *decreases* in rate. The formation of the E·(A-NADD)·aldehyde ternary complex or the loss of ethanol-*1*-*d* from the E·NAD^+ alcohol complex would also appear unlikely to be rate controlling under these conditions because neither of these would be expected to exhibit (primary) isotope effects large enough to explain the observations. Thus, it seems to us, the following argument can be used to reconcile the various deuterium isotope effects observed in this system. (a) The large isotope effect on the ("burst") production of E·A-NADD from E·NAD^+ and ethanol-d_5 (Shore and Gutfreund, 1970) is determined by step 3, hydrogen transfer within the ternary complexes. (b) The small isotope effect on the initial rate in the steady-state oxidation of ethanol-d_5 by NAD^+ and catalytic amounts of alcohol dehydrogenase may result from the dissociation of the E·A·NADD complex being rate determining and indicates a special interaction between the transferrable hydrogen (or deuterium) in this complex. An isotope effect (k_H/k_D) of 0.58 on the dissociation constant of the E·A-NADD complex, as determined by equilibrium binding measurements (Bush *et al.*, 1971), also suggests an interaction between the enzyme and the transferrable hydrogen. A comparison of this effect with the above isotope effect of 1.34 (dissociation of A-NADD from the enzyme) predicts that an isotope effect of 2.3 should be observed for the binding of A-NADD to the enzyme. Studies to check this prediction are in progress. (c) The absence of an isotope effect on the initial rate of reduction of high concentrations of acetaldehyde by A-NADD and catalytic concentrations of enzyme results from the reverse of step 1, dissociation of the E·NAD^+ complex being rate determining. This conclusion is consistent with the Theorell–Chance mechanisms. (d) The moderate isotope effect on the initial rates in the reduction of low concentrations of acetaldehyde by A-NADD and catalytic concentrations of enzyme may result from the conversion of ternary complexes (reverse of step 3) being at least partly rate determining.

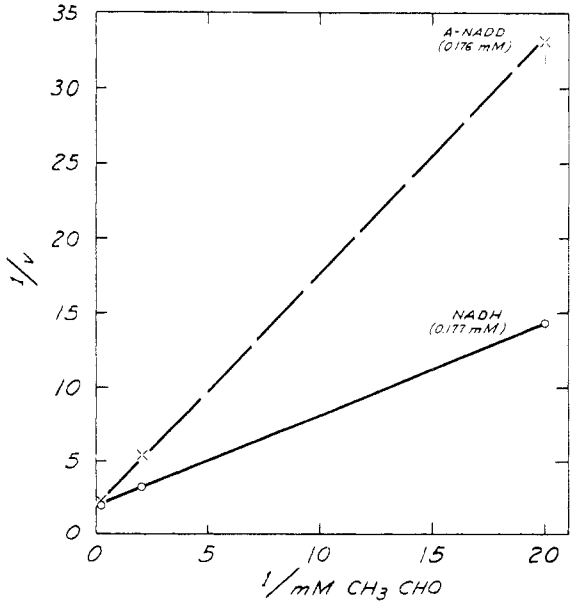

FIGURE 1: Double reciprocal plot of steady-state initial velocities for the reduction of acetaldehyde by (○) 0.177 mM NADH and (×) 0.176 mM A-NADD at 25° in 20 mM phosphate (pH 7.0). Both coenzymes were prepared and purified under identical laboratory conditions. The liver alcohol dehydrogenase concentration was 0.016 μM. The only error large enough to be significant is that for A-NADD at the lowest aldehyde concentration.

In conclusion, studies utilizing a homogeneous liver alcohol

dehydrogenase fraction reaffirm earlier mechanistic observations from this laboratory related to steady-state isotope effects on the reduction of deuterated ethanol. Discrepancies between these results and those reported previously (Baker, 1962; Mahler *et al.*, 1962) for the reduction of acetaldehyde by the stereospecifically deuterated reduced coenzymes may be related to two factors: (1) a homogeneous isoenzyme preparation was used and (2) coenzyme preparations of much higher purity were employed in the present studies.

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