Deuterium Isotope Effects on Lactate Dehydrogenase Using L-2-Hydroxysuccinamate and Effect of an Inhibitor in the Variable Substrate on Observed Isotope Effects†

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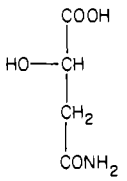
ABSTRACT: L-2-Hydroxysuccinamate, the 4-amide analogue of L-malate, is a substrate for beef heart lactate dehydrogenase with values for V and V/K at pH 8 which are 0.01 and 0.0002% of the corresponding constants for L-lactate and an equilibrium constant of 2.19×10^{-13} M. Similar values are observed for rabbit muscle lactate dehydrogenase. With beef heart lactate dehydrogenase, the pH-independent isotope effects on V and V/K of 3.7 ± 0.5 and 3.3 ± 0.4 indicate that hydride transfer is largely rate limiting for this reaction. L-2-Hydroxysuccinamate undergoes hydrolysis in mildly acidic or basic solution, and the pH vs. rate profile suggests intramolecular catalysis by the undissociated 1-carboxyl group in the pH range 1.5–3.5. The substrate activity of L-2-hydroxysuccinamate with pigeon liver malic enzyme reported by M. I. Schimerlik & W. W. Cleland [(1977) *Biochemistry 16*, 565] was caused by contaminating L-malate; the purified compound shows no activity (<0.015%). Theory has been developed for the effect on V and V/K deuterium isotope effects of having an inhibitor present in the variable substrate and tested by adding trifluoroethanol to deuterated or unlabeled cyclohexanol as substrates for alcohol dehydrogenase.

L-2-Hydroxysuccinamate (I) was reported by Schimerlik



I

& Cleland (1977) to be an alternate substrate for the forward reaction of pigeon liver malic enzyme (eq 1), presumably

$$\text{L-malate} + \text{NADP}^+ \overset{M^{2+}}{\rightleftharpoons} \text{pyruvate} + \text{NADPH} + CO_2 \quad (1)$$

$$\alpha\text{-hydroxy acid} + \text{NAD}^+ \rightarrow \alpha\text{-keto acid} + \text{NADH} \quad (2)$$

undergoing oxidation to 2-ketosuccinamate without subsequent decarboxylation. Lactate dehydrogenase, which catalyzes the oxidation of various α-hydroxy acids to the corresponding keto acids by eq 2 (Meister, 1950), will also utilize L-2-hydroxysuccinamate as a substrate. The present study was undertaken to determine the deuterium isotope effects on reactions 1 and 2 using this alternate substrate, but we have discovered that the reported activity with malic enzyme results from traces of malate arising from hydrolysis under acid conditions during crystallization. We thus report the isotope effects on the lactate dehydrogenase reaction and the pH–rate profile for hydrolysis of hydroxysuccinamate. During the course of this study, theory was also developed for the effect on V and V/K isotope effects of having an inhibitor present in the variable substrate (this situation occurs with a racemic mixture when one isomer is active and the other is inhibitory). This theory is extremely important for anyone contemplating measurement of isotope effects on enzyme-catalyzed reactions.

Materials and Methods

Lactate dehydrogenase (rabbit muscle type XI lyophilized powder and beef heart type III crystalline ammonium sulfate suspension), horse liver alcohol dehydrogenase, and chicken liver malic enzyme were from Sigma Chemical Co. Pigeon liver malic enzyme was isolated by the procedure of Hsu & Lardy (1967). L-Asparagine transaminase (0.3 unit/mg of protein) was isolated from rat liver by the procedure of Cooper (1977). 2,2,2-Trifluoroethanol and cyclohexanol were from Aldrich Chemical Co.

Synthesis of Substrates. Cyclohexanol-*1-d* was synthesized by reduction of cyclohexanone with $NaBD_4$. L-Malate-*2-d* was prepared by coupling the alcohol and aldehyde dehydrogenase catalyzed oxidation of perdeuterioethanol to oxalacetate reduction by malate dehydrogenase (Viola et al., 1979).

L-Asparagine-*2,3,3-d*$_3$ was prepared by exchange in D_2O using L-asparagine transaminase. L-Asparagine (anhydrous; 6.60 g, 50 mmol) and phenylpyruvate (18.6 mg, 0.1 mmol) were dissolved in 300 mL of D_2O containing 33 mM potassium phosphate buffer, pD 7.8. Lyophilized asparagine transaminase (53 units, 0.3 unit/mg) was added, and the solution was stirred at 25 °C for 6 days, at which time exchange (monitored by proton NMR) appeared complete. The reaction was stopped by adding DCl to pH 2 and 0.5 mL of CCl_4 and vortexing. The solution was centrifuged, diluted to 500 mL, and applied to a 3 × 16 cm column of Dowex 50-H^+. After washing with 250 mL of 10 mM HCl and 100 mL of H_2O (acid and water effluent both gave a negative ninhydrin test), asparagine was eluted with 200 mM aniline. The eluate was extracted with 2 × 100 mL ether to remove aniline and flashed to dryness, and the product was recrystallized from H_2O: yield, 71%. During the course of the exchange reaction, all three protons were exchanged at roughly equal rates. Thus, rat liver L-asparagine transaminase appears similar to glutamate–pyruvate transaminase and differs from glutamate–oxalacetate transaminase in the ability to labilize the β-hydrogen atoms of the substrates under exchange conditions (Golichowski et al., 1978; Cooper, 1976).

L-2-Hydroxysuccinamate-*2,3,3-d*$_3$ and the corresponding unlabeled compound were synthesized from L-asparagine-*2,3,3-d*$_3$ and unlabeled L-asparagine, respectively, by a modification of the deamination procedure of Horton & Philips (1973). L-Asparagine (5.5 g, 40.7 mmol based on the monohydrate crystal) was dissolved in 500 mL of cold H_2O

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Table I: Kinetic Parameters for Lactate Dehydrogenase at pH 8[a]

substrate	V (mM min^{-1} mg^{-1})		V/K (min^{-1} mg^{-1})		K (mM)	
	BH	RM	BH	RM	BH	RM
L-lactate	0.069	0.125	0.13	0.017	0.54	7.5
L-2-hydroxysuccinamate	0.97×10^{-5}	1.0×10^{-4}	0.27×10^{-6}	0.83×10^{-6}	35	120
L-phenyllactate	0.66×10^{-5}	0.95×10^{-4}	0.60×10^{-6}	1.1×10^{-6}	11	82

[a] Beef heart lactate dehydrogenase (BH), 100 mM Tes, pH 8, 0.20 M ionic strength, 2 mM NAD^+, and 25 °C; rabbit muscle lactate dehydrogenase (RM), same conditions as in BH, except 0.25 M ionic strength.

containing 5.7 mL of concentrated HCl. A 25-mL volume of cold $NaNO_2$ solution (11.26 g, 163 mmol) was added dropwise over 15 min to the asparagine solution, stirring in an ice–salt bath at 0 °C, followed by dropwise addition over 15 min of glacial acetic acid (7.02 mL, 122 mmol). The temperature was maintained at 0–2 °C for 5 h; the reaction is signaled by vigorous bubbling. At this point the reaction had reached 70–80% completion, as monitored by the ninhydrin test. The solution was purged with N_2 gas, allowed to warm to room temperature over 2 h, and then concentrated to an orange syrup by rotary evaporation at 30 °C. NaCl was precipitated by adding 200 mL of glacial acetic acid containing 11.4 mL of concentrated HCl and removed by filtration. The filtrate was diluted to 500 mL, passed through a 3 × 16 cm column of Dowex 50-H^+ to remove Na^+, combined with an additional 500-mL H_2O wash, and concentrated to an orange syrup. Starlike crystals form at 4 °C within 2 h. Total yield including a second crop averaged 40–60%. L-2-Hydroxysuccinamic acid was decolorized with charcoal (1% w/w) and recrystallized from dilute HCl. Contaminating L-malate, assayed with malate dehydrogenase for which L-2-hydroxysuccinamate is not a substrate, was removed by chromatography at 4 °C on a 0.8 × 25 cm column of Dowex 1 Cl^-, eluting with a linear 60-mL, 0–200 mM KCl gradient, followed by continuing elution with 200 mM KCl. L-2-Hydroxysuccinamate, which was eluted at 160–200 mM KCl, was purified in 200-mg batches and neutralized to pH 6.5 with KOH. All contaminating malate was removed by this procedure.

Deamination of either D- or L-asparagine by this procedure results in >95% retention of configuration as measured by optical rotation and enzymatic assay. L- and D-2-hydroxysuccinamate gave $[\alpha]^{25°C}_D$ values of −32.4° and +32.3°, respectively (c 3.7, sodium salt in H_2O) compared to the reported value $[\alpha]^{18°C}_D$ −27.3° (c 3.25) (Lutz, 1902). A first-order ABX analysis of the 100-MHz proton NMR spectra (sodium salt in D_2O, d_{HDO} at 5.70 ppm standard) gave d_{Ha} at 3.44, d_{Hb} at 3.19 ppm, and d_{Hx} at 5.07 ppm (J_{ab} = −15.4 Hz, J_{ax} = +3.1 Hz, and J_{bx} = +9.5 Hz). Analysis of the deuterated compound by 270-MHz proton NMR showed 97.5% deuteration at the 2 position and 92.3% at carbon 3. Titration of L-2-hydroxysuccinamate yielded a pK of 3.50.

Enzymatic Reactions. Lactic dehydrogenase solutions (5 mg/mL) were made in 50 mM Tes,[1] 0.1 mM dithiothreitol, 1.0 mM EDTA, and 1 mg/mL bovine serum albumin, pH 8.0. This stock was diluted 1:200 into the same buffer for L-lactate assays. Alcohol dehydrogenase (1 mg/mL) was dissolved in 10 mM Tris-HCl, pH 8.0. Chicken liver malic enzyme was activated by dissolving the ammonium sulfate pellet in the buffer described above for lactic dehydrogenase and incubating at 4 °C overnight.

Initial velocity and inhibition patterns were obtained under the following conditions: lactate dehydrogenase, 100 mM buffer, 2 mM NAD^+, and 0.20 M constant ionic strength maintained with potassium acetate;[2] malic enzyme, 100 mM Pipes, pH 7.2, 20 mM $MgSO_4$, 333 μM $NADP^+$, and 67 μg/mL bovine serum albumin; alcohol dehydrogenase, 100 mM Tris-HCl, pH 8.0, and 2 mM NAD^+. Buffers used for the lactate dehydrogenase pH profiles were Mes (pH 6–7), Tes (pH 7–8), and Ches (pH 9). (Glycylglycine is a competitive inhibitor vs. L-lactate or L-2-hydroxysuccinamate with K_{is} = 13 mM at pH 8.3 and 33 mM at pH 8.8 and should not be used as a buffer for this enzyme.) In order to standardize the concentrated lactate dehydrogenase solutions, we developed an assay using L-phenyllactate which has V and V/K values roughly equal to those of L-2-hydroxysuccinamate with beef heart lactate dehydrogenase at pH 8 (Table I). A similar assay with the rabbit muscle enzyme provides an equally good standard; however, substrate inhibition by L-phenyllactate must be taken into account in this case.[3]

The reactions were monitored by using a Beckman DU monochrometer with deuterium lamp, a Gilford optical density converter, and a 10-mV recorder with adjustable zero and multispeed drive. Full-scale sensitivity of 0.025–0.10 OD and a chart speed of 0.4–2 in./min were used. The cell compartment was maintained at 25 °C with thermospacers, and the reactions were started by addition of enzyme (50 μL) via an adder mixer to a 3.0-mL volume preequilibrated at 25 °C in a 1.0-cm cuvette. The lactate dehydrogenase reaction at pH 6 favors production of lactate so that a 10.0-cm path length was used with a final volume of 5.5 mL. When L-2-hydroxysuccinamate was used as a substrate for lactate dehydrogenase, enzyme was preincubated in the reaction mixture for 5 min and substrate was added to start the reaction. This procedure eliminated the burst observed above pH 8.5 when either NAD^+ or enzyme was added last to initiate the reaction.[4]

L-2-Hydroxysuccinamate Hydrolysis. Samples 150 mM in L-2-hydroxysuccinamate were prepared in 100 mM buffer at intervals of 1 pH unit from 2 to 11. For pH 12 to 13, 10

[1] Abbreviations used: Tes, *N*-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid; Pipes, piperazine-*N*,*N*′-bis(2-ethanesulfonic acid); Mes, 2-(*N*-morpholino)ethanesulfonic acid; Ches, cyclohexylaminoethanesulfonic acid. ^DV and $^DV/K$ are the deuterium isotope effects on V and V/K, respectively. HSA (L-2-hydroxysuccinamate) is used for brevity as a subscript on V and V/K.

[2] Acetate was chosen because it showed only half the inhibition seen with KCl (at constant ionic strength) on $V/K_{lactate}$ at pH 7.5 with the rabbit muscle enzyme (V was unaffected). Although acetate forms a complex with enzyme and NADH below pH 8 (Winer et al., 1959), this will not cause difficulties when a slow substrate like hydroxysuccinamate is used and initial velocities are measured, since the level of E–NADH present in the steady state will be very low.

[3] Substrate inhibition by L-lactate was not observed with either rabbit muscle or beef heart enzymes (pH 7–10) with NAD, but it was seen with acetylpyridine–NAD with the rabbit muscle enzyme at pH 7. L-Phenyllactate gave substrate inhibition with either enzyme and NAD.

[4] This burst was not stoichiometric with enzyme, was independent of the source of NAD, buffer concentration, or enzyme level, and was not observed at pH 8 or below. The same behavior, only more pronounced, was seen with acetylpyridine–NAD and either L-2-hydroxysuccinamate or L-2-phenyllactate.

and 100 mM KOH were used, while for pH 0 to 1, 1 M and 100 mM HCl were used. Samples were incubated at 25 °C in tightly stoppered test tubes (a drop of toluene was added to inhibit bacterial growth), and aliquots were assayed for L-malate content at regular intervals. The assay consisted of 5 mM NAD^+, 40 mM L-glutamate, 100 mM Caps, pH 10, 25 units of glutamate–oxalacetate transaminase, 25 units of malic dehydrogenase. Data were plotted as ln $[(a_\infty - a_t)/(a_\infty - a_0)]$ vs. time to determine the pseudo-first-order rate constant (k_{obsd}) for hydrolysis, where a_0 and a_t are the concentrations of L-malate at time zero and time t and a_∞ = 150 mM. No pH change was observed at the extent of reaction used to determine the rate constants.

Data Processing. The nomenclature used in this paper is that of Cleland (1963). Reciprocal initial velocities were plotted vs. reciprocal substrate concentrations and linear plots were obtained in all cases. Single-reciprocal plots were fitted to eq 3, while data conforming to linear competitive inhibition

$$v = \frac{VA}{K + A} \tag{3}$$

$$v = \frac{VA}{K(1 + I/K_{is}) + A} \tag{4}$$

$$v = \frac{VA}{K(1 + F_iE_{V/K}) + A(1 + F_iE_V)} \tag{5}$$

$$\log Y = \log [C/(1 + H/K_1)] \tag{6}$$

were fitted to eq 4. Kinetic deuterium isotope effects were determined from fits to eq 5, where F_i is the fraction of deuterium in the substrate, $E_{V/K}$ and E_V are the isotope effects minus 1 on V/K and V, respectively, and A is the concentration of the particular form of the substrate that was used. Equation 6 describes a pH profile which decreases below pK_1, with H as the hydrogen ion concentration, and C as the value of Y attained in the plateau region above pK_1. In eq 3–5, K is the Michaelis constant for substrate, while V is the corresponding maximum velocity.

Experimental data were fitted to eq 3–6 by the least-squares method assuming equal variances for the velocities and log Y values (Wilkinson, 1961), using a digital computer and the Fortran programs of Cleland (1979). The points in the figures are the experimentally determined values, while the lines are calculated from fits to the corresponding equations.

Results and Discussion

Substrate Activity of L-2-Hydroxysuccinamate with Lactate Dehydrogenase. The kinetic parameters V_{HSA} and V/K_{HSA} for L-2-hydroxysuccinamate were determined from fits to eq 3 under conditions of saturating NAD^+ (2 mM = 10 K_{NAD^+}). The data in Figure 1 show that V and V/K decrease below apparent pK values of 7.35 ± 0.03 and 7.14 ± 0.06. The same behavior is seen for both the beef heart and rabbit muscle enzymes. This pK is similar to those of 6.8–7.0 observed in the pK_{is} vs. pH profiles for various competitive inhibitors of pyruvate (Holbrook & Stinson, 1973), the V and V/K profiles for L-lactate oxidation (Schwert et al., 1967), and chemical modification studies (Holbrook & Ingram, 1973) and can be ascribed to the active-site histidine residue. The similar pK for V and V/K indicates that L-2-hydroxysuccinamate can bind with nearly equal affinity to either protonation state of the enzyme, while only the unprotonated ternary complex is catalytically active. When deuterated and unlabeled hydroxysuccinamate were compared as substrates, however, $^DV_{HSA}$ and $^D(V/K_{HSA})$ were pH independent, with weighted averages of the values derived from fits to eq 5 of 3.72 ± 0.46

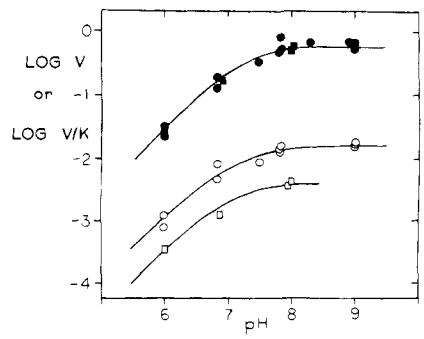
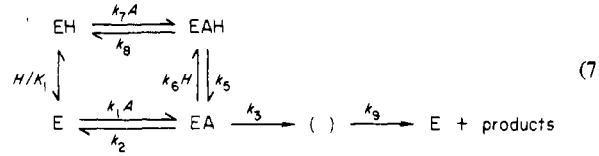


FIGURE 1: pH dependence of V (closed symbols) and V/K_{HSA} (open symbols) for beef heart (circles) and rabbit muscle (squares) lactic dehydrogenase with L-2-hydroxysuccinamate as the substrate. The lines are fits to eq 6 with pK values of 7.35 ± 0.03 for V and 7.14 ± 0.06 for V/K.

and 3.31 ± 0.36, respectively, for beef heart lactate dehydrogenase. For the rabbit muscle enzyme, a single measurement at pH 8 gave $^DV_{HSA}$ = 2.86 ± 0.42 and $^D(V/K_{HSA})$ = 2.62 ± 0.42. The equilibrium constant for oxidation of hydroxysuccinamate by DPN was found to be 2.19×10^{-13} M, compared to the value for lactate of $\sim 4 \times 10^{-12}$ M and the value for L-malate of about 6×10^{-13} M.

The observed pH behavior of the kinetic parameters and deuterium isotope effects can be accounted for in terms of the simple model described by Cleland (1978) and shown in eq 7:



(7)

where E is E–NAD^+, A is L-2-hydroxysuccinamate, k_3 is the isotope dependent step (which may include non-isotope-sensitive steps prior to and following hydride transfer, including release of the first product, as long as none are pH dependent), and k_9 includes all steps after release of the first product which result in regenerating free E (or E–NAD^+ under conditions of saturating NAD^+). This model predicts that ^DV and $^DV/K$ should both become equal to $^Dk_3$, the actual isotope effect on k_3, at pH values below pK_1. The values on the plateau region above pK_1, however, will only be equal when $k_2 = k_9$ or when $k_2, k_9 \gg k_3$. In the former case, the isotope effects will be pH dependent, while in the latter case, $^DV = {}^D(V/K) = {}^Dk_3$ and the isotope effects do not vary with pH. Since $^DV_{HSA}$ and $^D(V/K_{HSA})$ are constant and roughly equal over the entire pH range, it appears that $^DV = {}^D(V/K) = {}^Dk_3$, and thus the rate constant k_2 for L-2-hydroxysuccinamate dissociation and k_9 [which probably represents release of NADH (Schwert et al., 1967)] are both much greater than k_3, which contains the hydride transfer step.

L-2-Hydroxysuccinamate Substrate Activity with Malic Enzyme. The substrate activity reported earlier by Schimerlik & Cleland (1977) for L-2-hydroxysuccinamate with pigeon liver malic enzyme has been shown to be an artifact due to contaminating L-malate. The low levels of L-malate which arise from hydrolysis in weakly acidic or basic solution were sufficient to give apparent substrate activity with both pigeon and chicken liver malic enzymes. Close analysis showed, however, that the ratio $(V/K_{HSA})/(V/K_{L\text{-malate}})$ was exactly equal to the ratio [L-malate]/[L-2-hydroxysuccinamate] for the samples used (L-malate assayed with malate dehydrogenase) and further that removal of the contaminating L-malate by ion-exchange chromatography resulted in a com-

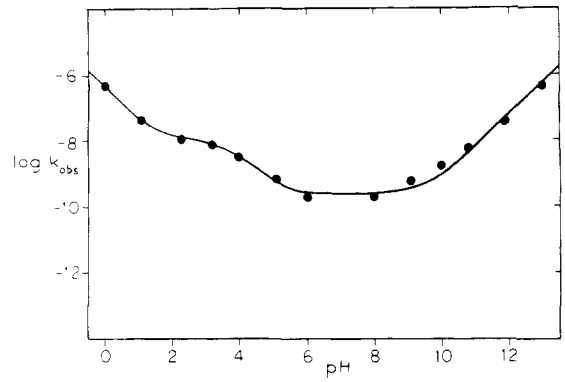


FIGURE 2: Plot of log k_{obsd} (s^{-1}) vs. pH for hydrolysis of L-2-hydroxysuccinamate at 25 °C. The curve is a least-squares fit to eq 8 with $k_H = (4 \pm 1) \times 10^{-7}$ M^{-1} s^{-1}, $k_{OH} = (6 \pm 1) \times 10^{-6}$ M^{-1} s^{-1}, $k_W = (2.3 \pm 0.6) \times 10^{-10}$ s^{-1}, $k_0 = (1.0 \pm 0.3) \times 10^{-8}$ s^{-1}, and $K = 10^{-3.5}$.

plete loss of substrate activty (<0.015%) with chicken liver malic enzyme. Both D- and L-2-hydroxysuccinamate are competitive inhibitors vs. L-malate at pH 7.2 with K_{is} values (65 ± 15 mM and 30 ± 5 mM, respectively) which are roughly 15-fold higher than, and in the same ratio as, the dissociation constants for D- and L-malate.

pH Profile for Hydrolysis of L-2-Hydroxysuccinamate. The data in Figure 2 were obtained in order to find the optimal conditions for the preparation and storage of solutions of this compound. The data can be described by

$$k_{obsd} = k_H H^+ + k_{OH}(OH^-) + k_W + k_0/(1 + K/H^+) \quad (8)$$

where K is the acid dissociation constant and k_H, k_{OH}, k_W, and k_0 are the rate constants for catalysis by H^+, OH^-, and water (pH-independent rate), and intramolecular catalysis by the undissociated form of the carboxyl group, respectively. Intramolecular catalysis of this type was also observed for the hydrolysis of succinamic acid by Higuchi et al. (1966), although the pH profile was not extended into the base-catalyzed region. It is apparent from Figure 2 that even a short exposure to acid or base results in L-malate contamination of the sample. Since L-2-hydroxysuccinamate is crystallized from dilute acid, the procedure adopted was to purify the crystalline material by ion-exchange chromatography at neutral pH and then to store the solution at 4 °C. The rate of hydrolysis under these conditions is quite slow, but the sample was checked for contamination by using the malate dehydrogenase assay before use.

Effects on ^DV and $^DV/K$ of an Inhibitor in the Variable Substrate. When we first attempted to determine deuterium isotope effects on V_{HSA} and V/K_{HSA} with pigeon liver malic enzyme by comparison of the initial velocities for DL-2-hydroxysuccinamate-*2,3,3-d*$_3$ and 95% optically pure L-2-hydroxysuccinamate, an apparent large isotope effect on V was observed. We attributed this effect to the presence of the inhibitory D isomer in the deuterated hydroxysuccinamate sample and, as a result, developed a theory to describe the effect on ^DV and $^D(V/K)$ or having an inhibitor present in the variable substrate. In retrospect, the observed V ratio was the result of a larger L-malate content in the unlabeled substrate, and ^DV would have been inverse had the relative L-malate contents been reversed, but since this theory is of interest in its own right, and important for anyone measuring isotope effects, we present it here.

The effects on the apparent kinetic parameters of having a linear competitive, linear uncompetitive, or linear noncompetitive inhibitor present in constant ratio to the variable substrate (Cleland et al., 1973) are shown in eq 9, 10, and 11,

$$1/v = (K/V)(1/A) + (1/V)(1 + rK/K_{is}) \quad (9)$$

$$1/v = (K/V)(1/A) + 1/V + rA/(VK_{ii}) \quad (10)$$

$$1/v = (K/V)(1/A) + (1/V)(1 + rK/K_{is}) + rA/(VK_{ii}) \quad (11)$$

respectively, where K_{is} and K_{ii} are the slope and intercept inhibition constants and $r = [I]/[A]$. In all cases with inhibitor present in the variable substrate, the reciprocal plot has an asymptote parallel to the uninhibited line where $r = 0$. For linear competitive inhibition, the intercept of the plot is increased above that of the uninhibited line and the increase is linear with r. Thus, while V/K remains unchanged, apparent $1/V$ and apparent $1/K$ both increase linearly with increasing r. For linear uncompetitive inhibition, the asymptote is identical with the uninhibited line while apparent substrate inhibition is observed at high levels of variable substrate. Linear noncompetitive inhibition gives reciprocal plots which show both substrate inhibition and an elevated asymptote intercept.

When reciprocal plots with deuterated and hydrogen-containing substrates are compared, $^D(V/K)$ is the ratio of the slopes and ^DV is the ratio of intercepts. It has been assumed (Cleland, 1979; Northrop, 1977) that the isotope effects measured in this manner were valid provided both compounds were *equally free* from inhibitory contaminants originating either from impurities or as a result of a racemic mixture in which one isomer is active and the other is inhibitory. Since the coefficient of the $1/A$ term in eq 9–11 is K/V in each case, the ratio of slopes or asymptote slopes is always $^D(V/K)$, and impurities have no effect on the V/K isotope effect, regardless of whether they are competitive, uncompetitive, or noncompetitive. Likewise, the constant term in eq 10 is just $1/V$, so the ratio of intercepts or asymptote intercepts is simply ^DV and an uncompetitive inhibitor will not give an incorrect value of the V isotope effect, even though it will induce differing levels of substrate inhibition if present to different degrees in the deuterated and nondeuterated substrates.

For a competitive or noncompetitive inhibitor, however, the constant term in eq 9 or 11 is $(1/V)(1 + rK/K_{is})$, and thus when deuterated and unlabeled substrates are compared, ^DV is given by

$$\text{app } ^DV = \frac{V_H(1 + r_D K_D/K_{is})}{V_D(1 + r_H K_H/K_{is})} \quad (12)$$

where K_{is} should be the same for deuterated or nondeuterated inhibitors. This equation can be rearranged into

$$\text{app } ^DV = \frac{(K_{is}/K_H)^DV + r_D{}^D(V/K)}{K_{is}/K_H + r_H} \quad (13)$$

Examination of eq 13 shows that apparent $^DV = ^DV$ only when $K_{is}/K_H \gg r_H, r_D$ or when $r_H/r_D = ^D(V/K)/^DV$ [the most likely case of this being when $r_H = r_D$ and $^D(V/K) = ^DV$]. When r_D is large and greater than r_H, one can get large apparent isotope effects which can exceed either ^DV or $^D(V/K)$, while if r_H is large and greater than r_D, one can see sizable apparent inverse isotope effects. If both deuterated and nondeuterated substrates are racemates and the inactive enantiomer is inhibitory, $r_D = r_H = 1$, and apparent ^DV varies hyperbolically from ^DV when $K_{is} \gg K_H$ to $^D(V/K)$ when $K_{is} \ll K_H$, with apparent ^DV being the average of ^DV and $^D(V/K)$ when $K_{is} = K_H$.

To test this theory describing the effect of an inhibitor in the variable substrate, we added 2,2,2-trifluoroethanol (a competitive inhibitor vs. alcohols) to solutions of deuterated and nondeuterated cyclohexanol and determined the apparent

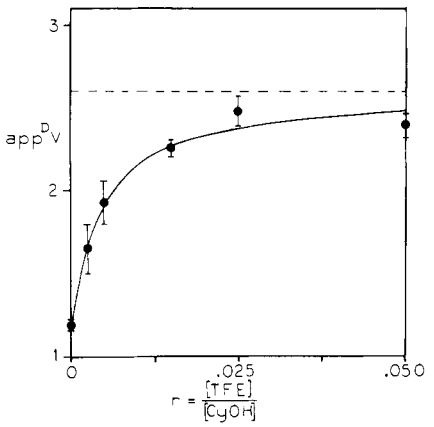


FIGURE 3: Plot of apparent ^{D}V vs. r = [2,2,2-trifluoroethanol]/[cyclohexanol] for cyclohexanol-1-(*h*,*d*) with horse liver alcohol dehydrogenase, 2 mM NAD^+, and 100 mM Tris-HCl, pH 8, at 25 °C. The points are shown with standard errors calculated from fits to eq 5, and the curve is a fit of the data to a hyperbola with vertical intercept = 1.2 ± 0.1 and asymptote = 2.6 ± 0.1.

isotope effects with horse liver alcohol dehydrogenase at pH 8. As predicted by eq 13, apparent ^{D}V increases with increasing r (Figure 3). The values calculated for apparent ^{D}V at $r = 0$ (1.2 ± 0.1) and $r = \infty$ (2.6 ± 0.1) are, within experimental error, equal to thee true ^{D}V of 1.19 ± 0.04 and the true $^{D}(V/K)$ of 2.2 ± 0.1 for cyclohexanol measured in the absence of trifluoroethanol and the values reported by P. F. Cook and W. W. Cleland (unpublished experiments) [^{D}V = 1.15 and $^{D}(V/K)$ = 2.5 at pH 8]. Clearly, it is not sufficient to have labeled and unlabeled substrates which are equally free of inhibitory impurities in order to be sure that the apparent ^{D}V measured is a real effect.

The above theory assumes only a single substrate but is equally applicable in a multireactant mechanism when one varies the labeled substrate at fixed levels of the unlabeled ones, as was done above with alcohol dehydrogenase. When one varies an unlabeled substrate at fixed levels of a deuterated or nondeuterated substrate, however, the situation may differ. In an ordered mechanism in which A is labeled but B is varied at high fixed A levels, the apparent $^{D}(V/K_B)$ value is sensitive to impurities in A, since a competitive inhibitor will decrease the level of EA available to react with B. The equation for apparent $^{D}(V/K_B)$ will be similar to eq 12 with $^{D}(V/K_B)$ replacing V_H/V_D and K_{ia} (the dissociation constant of A from EA) replacing K_D and K_H. The true values of $^{D}(V/K_B)$ will then be seen only when $r_D = r_H$. The apparent ^{D}V value is also affected by impurities in A, and eq 12 applies with K_H and K_D being the Michaelis constants of unlabeled and deuterated A. If B contains a competitive inhibitor but A is free from impurities, $^{D}(V/K_B)$ is unaffected, but apparent ^{D}V is given by eq 12 with $r_D = r_H$ and K_H and K_D being the Michaelis constants of B with unlabeled and deuterated A, even though the same solutions of B are varied with labeled and unlabeled A. These considerations are particularly important for isotope effects on dehydrogenase reactions where NADH and NADD are used at fixed levels and the other substrate is varied.

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