Evidence That both Protium and Deuterium Undergo Significant Tunneling in the Reaction Catalyzed by Bovine Serum Amine Oxidase†

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ABSTRACT: The magnitudes of primary and secondary H/T and D/T kinetic isotope effects have been measured in the bovine serum amine oxidase catalyzed oxidation of benzylamine from 0 to 45 °C. Secondary H/T and D/T kinetic effects are small and in the range anticipated from equilibrium isotope effects; Arrhenius preexponential factors (A_H/A_T and A_D/A_T) determined from the temperature dependence of isotope effects also indicate semiclassical behavior. By contrast, primary H/T and D/T isotope effects, 35.2 ± 0.8 and 3.07 ± 0.07, respectively, at 25 °C, are larger than semiclassical values and give anomalously low preexponential factor ratios, A_H/A_T = 0.12 ± 0.04 and A_D/A_T = 0.51 ± 0.10. Stopped-flow studies indicate similar isotope effects on cofactor reduction as seen in the steady state, consistent with a single rate-limiting C–H bond cleavage step for V_{max}/K_m. The comparison of primary and secondary isotope effects allows us to rule out appreciable coupling between the primary and secondary hydrogens at C-1 of the substrate. From the properties of primary isotope effects, we conclude that both protium and deuterium undergo significant tunneling in the course of substrate oxidation. These findings represent the first example of quantum mechanical effects in an enzyme-catalyzed proton abstraction reaction.

Both deuterium and tritium isotope effects have been extensively characterized in organic and enzymatic reactions. Although unusually large isotope effects have occasionally been reported in enzymatic reactions [cf. Klinman (1978)], suggestive of hydrogen tunneling (Bell, 1980), the nature of this phenomenon has not been explored. Recently, Saunders has proposed that a comparative study of D/T and H/T isotope effects provides a sensitive probe for the detection of quantum mechanical tunneling in hydrogen-transfer reactions (Saunders, 1985). As discussed, the interdependence of mass, known as the Swain–Schaad relation (Swain et al., 1958), is expected to break down under conditions where moderate degrees of hydrogen tunneling occur. By use of this approach, unambiguous evidence has recently been advanced for room temperature tunneling in the yeast alcohol dehydrogenase reaction (Cha et al., 1989).

It becomes important to determine whether hydrogen tunneling is a general feature of enzyme-catalyzed hydrogen-transfer events as opposed to a selected feature of reactions catalyzed by dehydrogenases [cf. Huskey and Schowen (1983), Hermes and Cleland (1984), and Hermes et al. (1984) for further evidence of tunneling in dehydrogenase reactions]. Properties which determine tunneling probabilities include the mass of the transferred particle, the height and width of the reaction barrier, and the net thermodynamic driving force for hydrogen transfer (Bell, 1980). Proteins can, in principle, facilitate this phenomenon by a minimization in the mass of the transferred particle through solvent exclusion [cf. Cha et al. (1989)], by an equalization of energy states for reactants and products (Albery & Knowles, 1976; Nambiar et al., 1983), and by a reduction in barrier widths (Rodgers et al., 1982).

In the present report, we have examined the bovine serum amine oxidase (BSAO)[1] catalyzed conversion of amines to aldehydes. Recent studies of this enzyme system indicate the presence of a new redox cofactor, pyrroloquinoline quinone (Ameyama et al., 1984; Lobenstein-Verbeek, 1984), which oxidizes substrate via a proton abstraction mechanism (Hartmann & Klinman, 1986). An early observation of large primary isotope effects, which exceed the semiclassical limit (Palcic & Klinman, 1983), raised the possibility of hydrogen tunneling in the course of enzyme-catalyzed proton activation. In order to pursue this phenomenon, a systematic investigation of primary and secondary D/T and H/T isotope effects in the range of 0–45 °C was undertaken. Additionally, stopped-flow studies have permitted quantitation of deuterium isotope effects as a function of temperature. In contrast to the reactions catalyzed by dehydrogenases (Cook et al., 1981), we are unable to detect vibrational coupling between the primary and secondary hydrogens of substrate. The magnitudes of primary D/T and H/T isotope effects show little deviation from the Swain–Schaad relation; however, temperature dependencies appear highly anomalous. While such Arrhenius behavior can arise in reactions characterized by kinetic complexity (Koch & Dalhberg, 1980), the combination of very large primary isotope effects and highly inverse isotope effects on Arrhenius preexponential factors seen in the current study cannot be explained by semiclassical behavior. As we discuss, the data presented herein lead to the conclusion that both deuterium and protium undergo substantial tunneling in the BSAO reaction.

Experimental Procedures

Materials. Bovine serum amine oxidase was prepared as previously reported to a final specific activity of 0.3 unit/mg (Summers et al., 1979). [1,1-^{1}H]Benzylamines and [1,1-^{2}H]benzylamines were synthesized in parallel as previously described (Bardlsey et al., 1973). [*ring*-^{14}C(U)]Benzoic acid (Pathfinder, specific activity = 21 mCi/mmol) was reduced to [*ring*-^{14}C(U)]benzyl alcohol by $LiAlH_4$ (Aldrich) or $LiAlD_4$

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[1] Abbreviations: BSAO, bovine serum amine oxidase; HPLC, high-pressure liquid chromatography; PQQ, pyrroloquinoline quinone; NADH, nicotinamide adenine dinucleotide, reduced.

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(ICN, 99.3% D). The resulting [^{14}C]benzyl alcohols were mesylated (Crossland & Servis, 1970), followed by conversion to azides and reduction to amines under phase-transfer catalysis conditions (Rolla, 1982). Final specific activities of 6.8 and 10.9 mCi/mmol were obtained for protonated and deuterated alcohols, respectively. The isotopic content of the deuterated alcohol, measured by mass spectral analysis of the urethane derivative of benzyl alcohol, was 98.7% ± 0.3% deuterium at C-1. Randomly tritiated benzylamines were synthesized by first preparing [1-^{2}H]- and [1-^{1}H]benzaldehydes by a modification of the method of Burgstahler et al. (1972). The [1-^{2}H]benzaldehyde was deuterated at the level of 99.7% ± 0.3%, as determined by mass spectral analysis of the 2,4-dinitrophenylhydrazone derivative. [1-^{2}H]Benzaldehyde and [1-^{1}H]benzaldehyde were reduced to alcohol by NaB^3H_4 (New England Nuclear, specific activity = 68.3 Ci/mmol), followed by NaB^2H_4 (Cambridge Isotope Lab, 99.3%) and $NaBH_4$ (Sigma), respectively. Benzylamines were prepared from benzyl alcohols as indicated above.

Methods. Kinetic measurements were carried out in buffered solutions containing 100 mM sodium pyrophosphate, pH 8.5, 1 mM (tritiated and ^{14}C-labeled) benzylamine (tritium specific activity = 6.2×10^6 cpm/μmol for protonated and 9.8×10^6 cpm/μmol for deuterated substrates, $^3H/^{14}C$ = 10/1), 50 mg/mL poly(ethylene glycol) 8000 (Sigma), 5 mM NADH (Boehringer), 13000 units/mL catalase (Boehringer), and sufficient liver alcohol dehydrogenase (Sigma) to maintain alcohol dehydrogenase at a 10-fold excess over bovine serum amine oxidase. Temperature control was maintained in a Neslab water bath to within 0.1 °C of the specified temperature. The final pH of reaction mixtures underwent small variations across the experimental temperature range, from pH 8.4 at 0 °C to pH 8.6 at 45 °C; since V/K has previously been shown to be insensitive to pH from 8 to 9 (Farnum et al., 1986), these small variations were assumed to be negligible. Aliquots were removed prior to injection of enzyme for the calibration of zero time points. Reaction was initiated by the addition of bovine serum amine oxidase in a volume of 0.11–0.015 mL, resulting in a dilution of the stock solution from 23% (0 °C) to 1.1% (45 °C). At appropriate time points (an average of eight per kinetic run), 0.05-mL aliquots were removed, quenched with dioxane and $NaBH_4$, and stored overnight on dry ice until analysis by HPLC. Neither benzaldehyde nor its air oxidation product, benzoic acid, could be detected by HPLC in product mixtures, consistent with the complete conversion of aldehyde to alcohol either by coupling of the enzymatic reaction to alcohol dehydrogenase or by subsequent reduction with $NaBH_4$. Tritiated solvent, benzyl alcohol, and benzylamine were separated on a reverse-phase C-18 column by HPLC, using a solvent comprised of 35 mM heptanesulfonic acid, 15% (v/v) acetonitrile, 5% (v/v) methanol, and 1% (v/v) acetic acid, pH 4.0.

Secondary isotope effects were determined from the comparison of ^3H to ^{14}C ratios in product alcohol at time t to substrate at zero time. Since complete enzymatic oxidation of starting amines to water and aldehyde routinely indicated a $^3H/^{14}C$ ratio in products which was equivalent to half of the $^3H/^{14}C$ ratio in reactants, the $^3H/^{14}C$ ratio of reactants was used as a frame of reference. Primary isotope effects were determined in a similar manner to secondary effects, except that tritium in water was compared to ^{14}C in product alcohol. Isotope effects were calculated according to eq 1 where f is

$$^T(V/K) = \ln (1 - f)/\ln \{1 - f[(^3H/^{14}C)_t/(^3H/^{14}C)_\infty]\} \quad (1)$$

the fractional conversion and was determined from the ^{14}C content of alcohol vs amine as eluted from HPLC. Experimental values for f varied from 0.03 to 0.6 for protonated amine and from 0.01 to 0.53 for deuterated amine. While the small contamination of protium in deuterated, tritiated benzylamine samples, 0.3%, was too low to be kinetically relevant, it was necessary to correct for a 1.3% protium contamination in deuterated, ^{14}C-labeled amines. It should be noted that protium contamination also occurs in nonradiolabeled amine as a result of reduction of deuterated aldehyde by NaB^3H_4 which was only ~50% tritiated. However, since all isotope effect measurements involve a comparison of ^3H to ^{14}C, this feature will not alter the results.

Correction for protium contamination in deuterated, ^{14}C samples involved a consideration of three possible reaction pathways with product arising from dideuterated material (S_D = 98.7% S_0) from amine with protium in the primary position (S_{H1} = 0.65% S_0) and from amine with protium in the secondary position (S_{H2} = 0.65% S_0), where S_0 is the sum of all ^{14}C-containing substrate at $t = 0$. The amount of material containing two hydrogens was insignificant. Since a large number of the experiments described in this study were carried out at or above K_m values for deuterated substrates, and, in some cases, contaminating protonated substrates, rate expressions must take into account competitive inhibition of one isotope against the other, eq 2–4, where K_D, K_{H1} and K_{H2} are

$$-dS_D/dt = \frac{V_D S_D}{K_D(1 + S_{H2}/K_{H2} + S_{H1}/K_{H1}) + S_D} \quad (2)$$

$$-dS_{H1}/dt = \frac{V_{H1} S_{H1}}{K_{H1}(1 + S_{H2}/K_{H2} + S_D/K_D) + S_{H1}} \quad (3)$$

$$-dS_{H2}/dt = \frac{V_{H2} S_{H2}}{K_{H2}(1 + S_D/K_D + S_{H1}/K_{H1}) + S_{H2}} \quad (4)$$

Michaelis constants for S_D, S_{H1}, and S_{H2}, respectively. A key feature of eq 2–4 is that rearrangement leads to a common term in the denominator, $C(t) = 1 + S_{H2}/K_{H2} + S_{H1}/K_{H1} + S_D/K_D$. As a result:

$$-dS_D/dt = \frac{(V/K)_D S_D}{C(t)} \quad (5)$$

$$-dS_{H1}/dt = \frac{(V/K)_{H1} S_{H1}}{C(t)} \quad (6)$$

$$-dS_{H2}/dt = \frac{(V/K)_{H2} S_{H2}}{C(t)} \quad (7)$$

If we assign an arbitrary value of A to $(V/K)_D$, eq 6 and 7 can be expressed in terms of A, such that $(V/K)_{H1} = (k_H/k_D)_1 \cdot A$ and $(V/K)_{H2} = (k_H/k_D)_2 \cdot A$. The magnitude of $(k_H/k_D)_1$ and $(k_H/k_D)_2$ can be estimated from initial measurements of tritium isotope effects, on the assumption that (to a close approximation) $k_H/k_D = (k_H/k_T)^{1/1.44}$. Given these interrelationships among eq 5–7, the integrated expression for total product formation (P_{tot}) becomes

$$P_{tot}(t) = 0.987S_0(1 - e^{-[A/C(t)]t}) + 0.0065S_0(1 - e^{-[A(k_H/k_D)_1/C(t)]t}) + 0.0065S_0(1 - e^{-[A(k_H/k_D)_2/C(t)]t}) \quad (8)$$

where $P_{tot}(t) = P_D(t) + P_{H1}(t) + P_{H2}(t)$. Although the rate constants shown in the exponents of eq 8 are not fixed, since $C(t)$ changes with time, *the relationship of the rate constants in each of the three exponents is constant with time.* This feature allows us to solve for the relative magnitude of each of the three terms in eq 8 at each experimental value for $P_{tot}(t)$. Once $P_D(t)$ (the first term in eq 8) has been calculated, the

Table I: Primary Isotope Effects for Oxidation of Benzylamine to Benzaldehyde by BSAO at 25 °C, pH 8.5[a]

$(k_D/k_T)_{obs}$	$(k_H/k_T)_{calc}$[b]	$(k_H/k_T)_{obs}$
3.01 ± 0.06		35.2 ± 0.3
3.02 ± 0.06		35.5 ± 4.7
3.06 ± 0.06		34.0 ± 5.0
3.03 ± 0.04	38.7 ● 2.9	36.2 ± 2.3
3.17 ± 0.03		35.0 ± 1.3
3.15 ± 0.09		
av: 3.07 ± 0.07[c]		av: 35.2 ± 0.8[c]

[a] Reactions were run at 1 mM benzylamine, as described under Methods. Each entry for $(k_D/k_T)_{obs}$ and $(k_H/k_T)_{obs}$ reflects a separate experiment. Within each experiment, approximately eight time points were analyzed, representing 3–60% conversion for protonated amine and 1–53% conversion for deuterated amine. [b] $[k_D/k_T)_{obs}]^{3.26} = (k_H/k_T)_{calc}$; error = $(3.26)(k_D/k_T)^{2.26}(\delta k_D/k_T)$. [c] Average values and standard deviations for measured isotope effects.

isotope effect for reaction of pure deuterated substrate is available:

$$(V/K)_D/(V/K)_T = \frac{\ln\{1 - [P_D(t)/P_D(\infty)]\}}{\ln\left\{1 - \frac{P_D(t)(^3H/^{14}C)_t[P_{tot}(t)/P_D(t)]}{P_D(\infty)(^3H/^{14}C)_\infty[P_{tot}(\infty)/P_D(\infty)]}\right\}} = \frac{\ln(1 - f_D)}{\ln\{1 - f_{tot}[(^3H/^{14}C)_t/(^3H/^{14}C)_\infty]\}} \quad (9)$$

where f_D is the fractional conversion of the dideuterated compound, $f_D = 0.987S_0(1 - e^{-[A/C(t)]t})/0.987S_0$, and f_{tot} is the observed fractional conversion including dideuterated and protonated compounds, $f_{tot} = f_{obs}$. $(^3H/^{14}C)_t$ is the observed ^3H to ^{14}C ratio at the time t, and $(^3H/^{14}C)_\infty$ is the observed ^3H to ^{14}C ratio at 100% conversion. It should be noted that in the absence of a correction, D/T isotope effects vary with fractional conversion as the protium-containing substrate is consumed first. Following correction, no systematic trends are observed in isotope effects.

Anaerobic stopped-flow measurements were carried out on a Union Giken RA-400 stopped-flow spectrophotometer interfaced to a Northstar Horizon microcomputer using the OLIS 3820 data system. All final solutions contained 100 mM sodium pyrophosphate, pH 8.5, 200 mM glucose (Calbiochem), and 50 mg/mL poly(ethylene glycol) 8000 (Sigma). The BSAO solutions (7–15 μM, 0.19 unit/mg) and benzylamine (0.02–20 mM) solutions were made anaerobic by freezing in liquid N_2, followed by evacuation and the readdition of 99.996% argon (Matheson). Solutions were evacuated 10 times in the first 5 min and then 5 times over the 45 min it takes for solutions to thaw. The solutions were then transferred to stopped-flow reservoirs using 5-mL gas-tight syringes containing 0.04 mL of glucose oxidase and catalase (Boehringer) to give 1000 units of each enzyme per 5 mL of solution. The reduction of BSAO by excess benzylamine was monitored at 480 nm, which results in a decrease in absorbance with time (Palcic & Klinman, 1983). The temperatures of the reservoirs and mixing chambers were monitored and found to be maintained within 0.2 °C. Three to five runs were carried out at each temperature and averaged before fitting to single first-order exponential decays.

Results

Measurement of D/T and H/T Isotope Effects at 25 °C. Primary D/T and H/T isotope effects are summarized in Table I. The magnitude of the average primary H/T ratio is very large and in the range expected from previous stopped-flow estimates of $(k_H/k_D)^{1.44} = 12.5^{1.44} = 38$ for the product of primary and secondary effects (Palcic & Klinman, 1983). As noted earlier (Palcic & Klinman, 1983), these primary effects are outside the range of values anticipated from semiclassical effects which predict upper limits of 27 for k_H/k_T and 2.7 for k_D/k_T (Bell, 1980). Simultaneous measurement of D/T effects shows highly reproducible values, with an average effect of 3.07. The relationship of D/T to H/T values is illustrated in the second column of Table I. As discussed by Saunders (1985), semiclassical D/T and H/T isotope effects are expected to be related by reduced mass differences; in the event of a measurable tunnel correction, one expects $(k_H/k_T)_{calc} < (k_H/k_T)_{obs}$. An unexpected and surprising feature of Table I is that, within experimental error, $(k_H/k_T)_{calc} = (k_H/k_T)_{obs}$. Thus, while the large magnitude of k_H/k_T implicates tunneling, the interrelationship of k_D/k_T to k_H/k_T is close to the semiclassical range. We note that if a trend can be discerned outside of experimental error, it is opposite to that anticipated from tunneling such that $(k_D/k_T)^{3.26} > (k_H/k_T)_{obs}$.

Table II: Secondary Isotope Effects for Oxidation of Benzylamine to Benzaldehyde by BSAO at 25 °C, pH 8.5[a]

$(k_D/k_T)_{obs}$	$(k_H/k_T)_{calc}$[b]	$(k_H/k_T)_{obs}$
1.030 ± 0.020		1.182 ± 0.016
1.050 ± 0.007		1.174 ± 0.031
1.060 ± 0.013		1.208 ± 0.008
1.060 ± 0.009	1.176 ± 0.040	1.203 ± 0.032
1.058 ± 0.012		1.206 ± 0.025
1.052 ± 0.010		
av: 1.051 ● 0.011[c]		av: 1.195 ± 0.016[c]

[a] See footnote *a*, Table I. [b] $[(k_D/k_T)_{obs}]^{3.26} = (k_H/k_T)_{calc}$; error = $(3.26)(k_D/k_T)^{2.26}(\delta k_D/k_T)$. [c] Average values of the isotope effects.

Secondary tritium isotope effects are summarized in Table II. The average H/T ratio of 1.195 appears normal, falling significantly below an anticipated upper limit of 1.4 (Klinman, 1978) for full conversion of amine (sp^3 at C-1) to the oxidized imine (sp^2 at C-1). This result, together with the unusually large primary effects seen in Table I, suggests little or no coupled motion between the primary and secondary hydrogens at C-1 of amine in the course of the proton abstraction reaction.[2] The D/T values summarized in Table II are seen to be quite reproducible with an average value of 1.051. Estimation of $(k_H/k_T)_{calc}$ using the reduced mass equation, $(k_D/k_T)^{3.26} = k_H/k_T$, gives a value which is within experimental error of $(k_H/k_T)_{obs}$.

Temperature Dependence of D/T and H/T Isotope Effects. The data in Tables I and II at 25 °C were extended to include the temperature range of 0–45 °C. The limits of this range were determined by the need for cryosolvents below 0 °C and the complication of protein denaturation above 45 °C. Although cryosolvents could, in principle, have been used with BSAO to extend the temperature range below 0 °C, all low-viscosity solvents examined lead to extensive inhibition of enzyme activity. This is very likely due to the presence of pyrroloquinoline quinone as the active-site cofactor, since PQQ is known to undergo covalent adduct formation, as well as redox chemistry, with organic reagents. As noted under Experimental Procedures, poly(ethylene glycol) was added to reaction mixtures to minimize temperature effects on protein stability.

Plots of ln (k_L/k_T) vs $1/T$ are given in Figure 1 for primary isotope effects where L is either H (upper line) or D (lower

[2] As previously demonstrated in several dehydrogenase reactions, the phenomenon of tunneling plus coupled motion leads to inflated values for secondary kinetic isotope effects which exceed equilibrium limits (Cook et al., 1981; Hermes & Cleland, 1984; Hermes et al., 1984).

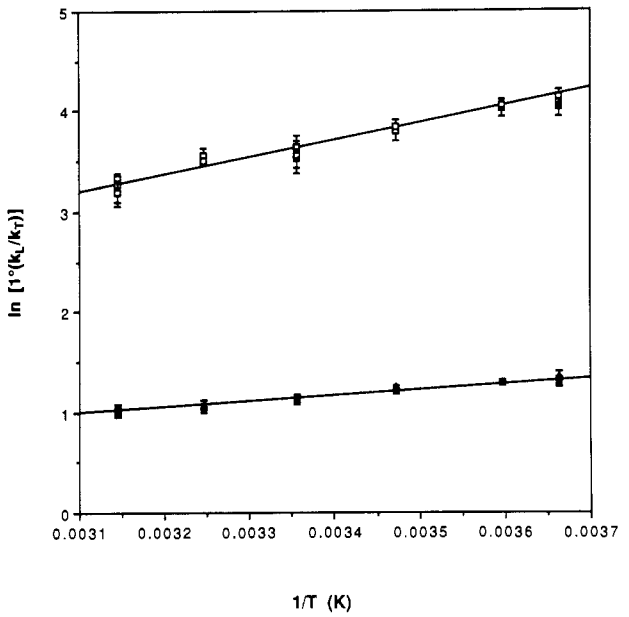


FIGURE 1: Arrhenius plots for primary L/T measurements. H/T isotope effects (□) and D/T isotope effects (▲).

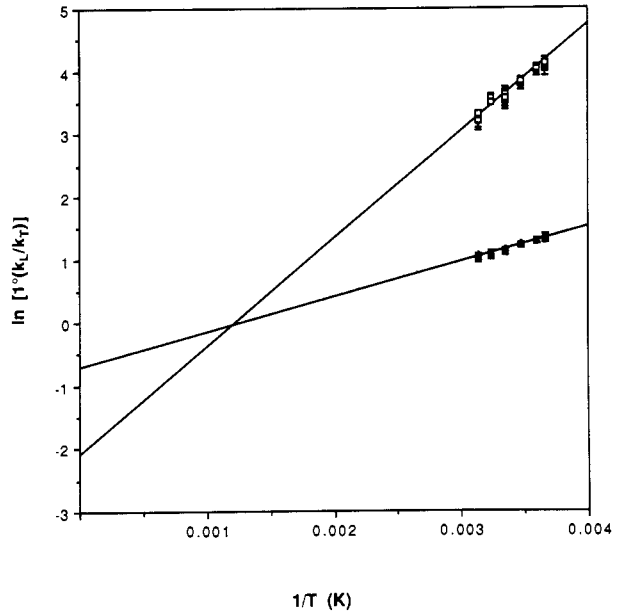


FIGURE 2: Arrhenius plots for primary L/T measurements expanded to show the y intercepts, H/T isotope effects (□), and D/T isotope effects (▲).

line). The experimental points show little experimental scatter at all temperatures, and Arrhenius plots are clearly linear in the experimental temperature range. An expanded version of Figure 1 is shown in Figure 2, allowing extrapolation to infinite temperature and, hence, estimation of $A_D/A_T = 0.51 \pm 0.10$ and $A_H/A_T = 0.12 \pm 0.04$ (Table III). Both values fall below semiclassical limits of $A_D/A_T = 0.9$ and $A_H/A_T = 0.6$ (Schneider & Stern, 1972), indicative of tunneling.

The temperature dependence of secondary D/T and H/T isotope effects is shown in Figures 3 and 4. Comparison of Figures 1 and 3 reveals greater experimental error with secondary than primary measurements, making the determination of slope and intercept values more difficult. The full temperature range for secondary isotope effects is shown in Figure 4, allowing visualization of A_L/A_T in the limit of infinite temperature. The magnitudes of A_H/A_T and A_D/A_T have been summarized in Table III, indicating values which fall within the semiclassical range. Consistent with the size of secondary

Table III: Arrhenius Parameters for the Primary and Secondary H/T and D/T Kinetic Isotope Effects

	A_L/A_T	$E_a(T) - E_a(L)$ (kJ/mol)
1° H/T	0.12 ± 0.04[a]	14.2 ± 0.7[b]
1° D/T	0.51 ± 0.10[a]	4.51 ± 0.48[b]
2° H/T	0.81 ± 0.05	0.96 ± 0.15
2° D/T	1.02 ± 0.06	0.068 ± 0.136

[a] Semiclassical lower limits are 0.7 for A_H/A_D, 0.6 for A_H/A_T, and 0.9 for A_D/A_T (Schneider & Stern, 1972). [b] Semiclassical upper limits are 5.8 kJ/mol for $E_a(D) - E_a(H)$, 8.3 kJ/mol for $E_a(T) - E_a(H)$, and 2.5 kJ/mol for $E_a(T) - E_a(D)$ (Bell, 1980).

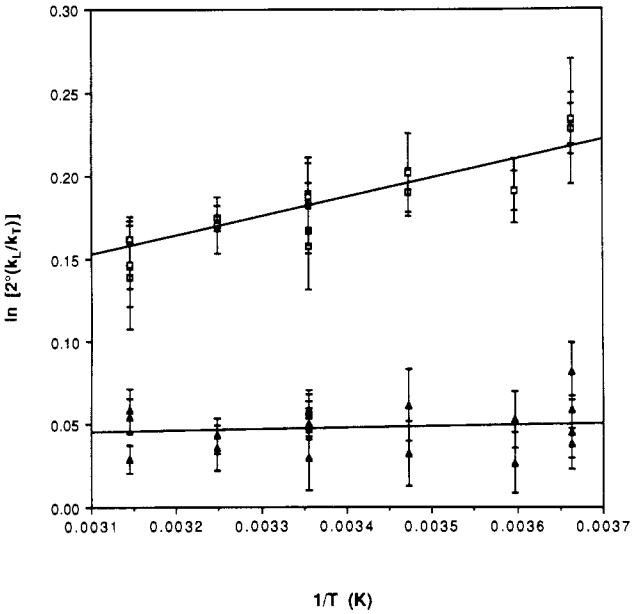


FIGURE 3: Arrhenius plots for secondary L/T measurements, H/T isotope effects (□), and D/T isotope effects (▲).

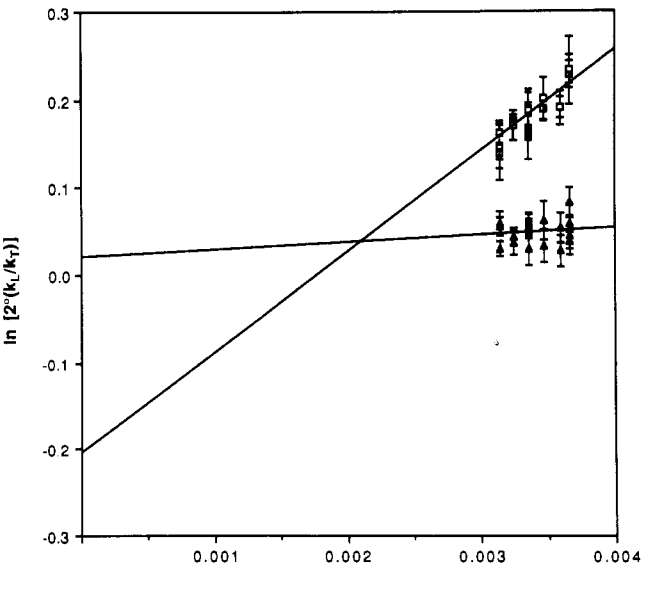


FIGURE 4: Arrhenius plots for secondary L/T measurements expanded to show the y intercepts. H/T isotope effects (□) and D/T isotope effects (▲).

isotope effects observed at 25 °C (Table II), the temperature dependence of these effects fails to reveal any abnormalities indicative of coupled motion and tunneling.

Stopped-Flow Measurement of Deuterium Isotope Effects. Stopped-flow studies were undertaken as a measure of the

Table IV: Comparison of Isotope Effects Determined under Stopped-Flow and Steady-State Conditions

temp (°C)	$(k_H/k_D)_{stopped\ flow}$[a]	$(k_H/k_D)_{steady\ state}$[b]
0	14.8 ± 0.7	19.0 ± 0.8
5	15.7 ± 1.2	18.7 ± 0.2
15	16.6 ± 1.4	16.5 ± 0.4
25	16.1 ± 1.8	13.5 ± 0.4
35	13.8 ± 1.1	13.0 ± 0.4
45	10.4 ± 0.9	10.4 ± 0.2

[a] Determined at 20 mM benzylamine under conditions described under Experimental Procedures. The concentration of 20 mM benzylamine is saturating relative to K_m values estimated as 2.2, 1.7, 1.4, 1.4, 1.2, 1.1, and 1.2 mM at 0, 5, 10, 15, 25, 35, and 45 °C. As indicated in the text, these measurements were carried out with dideuterated benzylamines and therefore reflect both primary and secondary isotope effects. Error bars refer to the deviation of multiple measurements from average values. [b] These values are determined from the tritium isotope effects in Tables I and II, according to the relationship $[(k_H/k_T)_{1°}(k_H/k_T)_{2°}]^{1/1.44} = (k_H/k_D)_{steady\ state}$ (see text).

nature of rate-limiting steps in the temperature range of 0–45 °C. Although the very large magnitude of H/T isotope effects on V/K implies rate limitation by the C–H bond cleavage step (Table I and Figures 1–2), the parameter V/K contains rate constants for substrate binding and release as well as for the chemical step. We therefore examined the pre-steady-state oxidation of [1,1-^{1}H]- and [1,1-^{2}H]benzylamines under conditions of substrate saturation (20 mM substrate) in the temperature range of 0–45 °C, obtaining the isotope effects summarized in Table IV as $(k_H/k_D)_{stopped\ flow}$. Since these measurements have employed dideuterated substrate, they reflect both primary and secondary isotope effects. They have, therefore, been compared to the product of primary and secondary tritium isotope effects (Tables I and II), using the expression $[(k_H/k_T)_{1°}(k_H/k_T)_{2°}]^{1/1.44} = (k_H/k_D)_{steady\ state}$. The use of the Swain–Schaad relationship in calculating $(k_H/k_D)_{steady\ state}$ is justified by the very close agreement of the experimental relationship between D/T and H/T measurements to the value expected from reduced mass considerations. Additionally, deviation from the 1.44 exponent relating H/D and H/T isotope effects as a result of tunneling is expected to be smaller than any deviations appearing in the exponent relating D/T and H/T isotope effects (Saunders, 1985). In conjunction with the measurement of deuterium isotope effects by stopped flow, the temperature dependence of the C–H bond cleavage step was explored. A plot of k_{obs} vs $1/T$ for dideuterated benzylamine is shown in Figure 5, characterized by an enthalpy of activation of 66 kJ/mol. The enthalpy of activation for protonated substrate can be estimated from $E_a(D) - E_a(H) = [E_a(D) - E_a(T)] - [E_a(H) - E_a(T)]) = 10.6$ kJ/mol (Table III), yielding 55 kJ/mol. This result is not very different from $E_a(H) = 58$ kJ/mol obtained directly from an Arrhenius plot of k_{obs} vs $1/T$ for protonated benzylamine (data not shown).

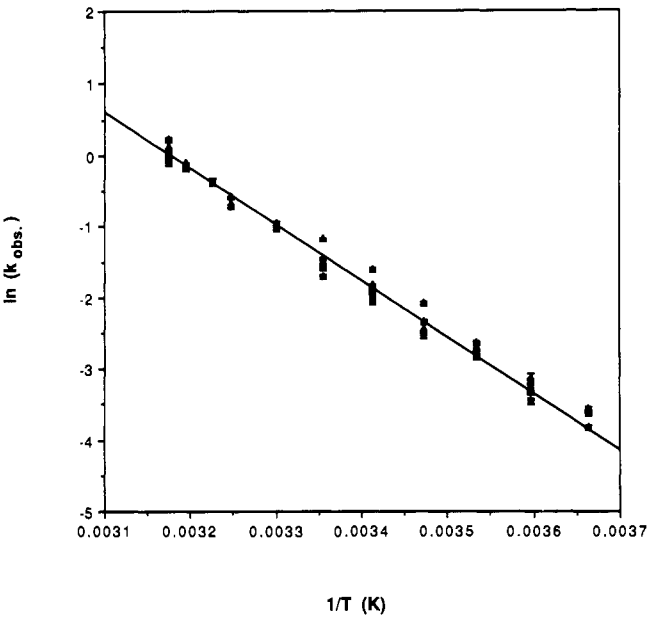


FIGURE 5: Arrhenius plot of the rate of enzyme reduction by dideuterated benzylamine. Data were collected under conditions of substrate saturation and refer to k_3, eq 15 in the text.

Discussion

Experimental Probes of Tunneling. In this paper, we have explored the origin of the anomalously large primary isotope effects observed in the bovine serum amine oxidase reaction (Palcic & Klinman, 1983). While such isotope effects can be highly suggestive of hydrogen tunneling (Bell, 1980), they do not, in and of themselves, constitute sufficient evidence for quantum mechanical effects in hydrogen-transfer reactions. As a consequence, two additional approaches have been exploited as probes of room temperature hydrogen tunneling. The first, more frequently explored approach involves a comparison of temperature dependencies for H and D transfer. In the event of tunneling, isotope effects on Arrhenius preexponential factors are expected to deviate from semiclassical limits, leading to A_H/A_D values <0.7. Such effects arise from a greater contribution of tunneling to observed reaction rates for H than D transfer, resulting in a crossing of Arrhenius plots and values for A_H/A_D less than 1. It is important to note that these intercept isotope effects are not reflections of a "true" A_H/A_D but reflect the slopes of the respective H and D lines in the experimental temperature range. As has been discussed by Stern and Weston (1974), the magnitude of A_H/A_D is, in fact, expected to *change* with temperature as the contribution of tunneling to the reaction coordinate varies: at very high temperatures, semiclassical conditions are expected to prevail and $A_H/A_D = 1.0$; in the intermediate temperature range where protium begins to tunnel in excess of deuterium tunneling, $A_H/A_D \ll 1.0$; and finally, at very low temperatures where all nuclei (H, D, and T) can be expected to tunnel, $A_H/A_D \gg 1.0$.

A second technique which has seen more recent application to the detection of room temperature hydrogen tunneling involves a comparison of D/T to H/T isotope effects. As described by Saunders (1985), semiclassical behavior leads to a predictable mass interdependence for D/T and H/T effects:

$$(k_D/k_T)^{3.26} = (k_H/k_T)_{calc} \quad (10)$$

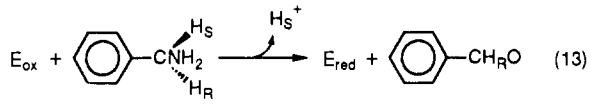
By contrast, the presence of a mass-dependent tunneling probability leads to a much greater contribution of tunneling to H/T than D/T effects, producing the inequality shown in eq 11. Evidence has recently been presented, using this approach, for room temperature tunneling in the hydride-transfer reaction catalyzed by yeast alcohol dehydrogenase (Cha et al., 1989). Importantly, it has been shown that the inequality in eq 11 cannot occur artifactually due to kinetic complexity, since the presence of a commitment leads to the opposing inequality:

$$(k_D/k_T)^{3.26} = (k_H/k_T)_{calc} < (k_H/k_T)_{obs} \quad (11)$$

$$(k_D/k_T)^{3.26} = (k_H/k_T)_{calc} > (k_H/k_T)_{obs} \quad (12)$$

Primary Isotope Effects in BSAO Indicate Tunneling. In the present investigation, a detailed analysis of both D/T and H/T isotope effects and temperature dependencies on these effects has been carried out in the BSAO-catalyzed conversion

of benzylamine to benzaldehyde:



(13)

where H_S and H_R represent the primary and secondary positions of substrate. Although the net reaction shown in eq 13 involves the oxidative deamination of amine to aldehyde, the reaction proceeds via two steps, involving an isotope-sensitive conversion of amine to imine, followed by a rapid hydrolysis of imine to benzaldehyde (Farnum et al., 1986).

In contrast to primary isotope effects, little has been known regarding the nature of secondary effects in the BSAO reaction. In the absence of vibrational coupling between the primary and secondary positions, the magnitude of the secondary effect is expected to lie between unity and an equilibrium value [ca. 1.4 (Klinman, 1978)] for the $sp^3 \rightleftharpoons sp^2$ conversion of an amine to an imine. Alternatively, highly anomalous secondary isotope effects may arise [cf. our recent results on the yeast alcohol dehydrogenase reaction (Cha et al., 1989)] in the event of vibrational coupling to a primary hydrogen which is exhibiting tunneling. As summarized in Table II, the data obtained for secondary isotope effects in the BSAO reaction are in the semiclassical range and give an exponential relationship between k_H/k_T and k_D/k_T consistent with reduced mass considerations. Further, the temperature dependence of secondary H/T and D/T values (Figures 3 and 4 and Table III) indicates preexponential Arrhenius factors which are close to unity, as predicted for isotopic discrimination arising solely from zero point energy vibrational effects. Thus, there is no evidence for vibrational coupling between H_R and H_S and, hence, propagation of tunneling from the primary position into H_R.

The data for primary isotope effects summarized in Table I indicate very different properties from those seen with secondary effects. First, as previously reported (Palcic & Klinman, 1983), the magnitude of these effects lies outside the semiclassical limit of 27 for k_H/k_T and 2.7 for k_D/k_T at 25 °C (Bell, 1980). Second, the temperature dependences of k_H/k_T and k_D/k_T (Figures 1 and 2) indicate preexponential Arrhenius factors which fall far below semiclassical values of 0.6 and 0.9, respectively (Schneider & Stern, 1972). These temperature dependences are in the direction expected for hydrogen tunneling, strengthening the argument based on the magnitude of the primary isotope effects that hydrogen tunneling contributes to the BSAO reaction coordinate.

An unexpected feature of the data in Table I is our failure to see $(k_H/k_T)_{obs} > (k_H/k_T)_{calc}$, as predicted by Saunders for reactions characterized by tunneling. This feature makes it especially important to question whether the observation of large primary isotope effects and inverse isotope effects on Arrhenius preexponential factors can arise from origins other than tunneling. First, it should be noted that large isotope effects and moderately inverse values for A_H/A_T can, in principle, derive from a loose transition state in which bond order has not been conserved (McLennan, 1979). However, very small bond orders, which appear particularly unrealistic for enzyme-catalyzed reactions, are necessary to generate these effects, and further, the values for A_H/A_T observed in the present study are too inverse to be explained by such an effect. Second, highly anomalous Arrhenius curves can arise in reactions characterized by more than one rate-limiting step. For example, values for A_H/A_D as small as 0.32 have been simulated for a reaction such as shown in eq 15 (see below), in the event that $k_2/k_3 = 0.56$ and $A_2/A_3 = 0.1$ (Koch & Dahlberg, 1980). However, in such instances, expressed isotope effects are suppressed due to the presence of more than one rate-limiting step, falling significantly below the semiclassical limit. These considerations indicate that the *combination of very large primary isotope effects and inverse isotope effects on Arrhenius preexponential factors seen in the BSAO reaction cannot be explained by semiclassical effects.*[3] By contrast, the combined presence of these two features is predicted for a reaction characterized by tunneling.

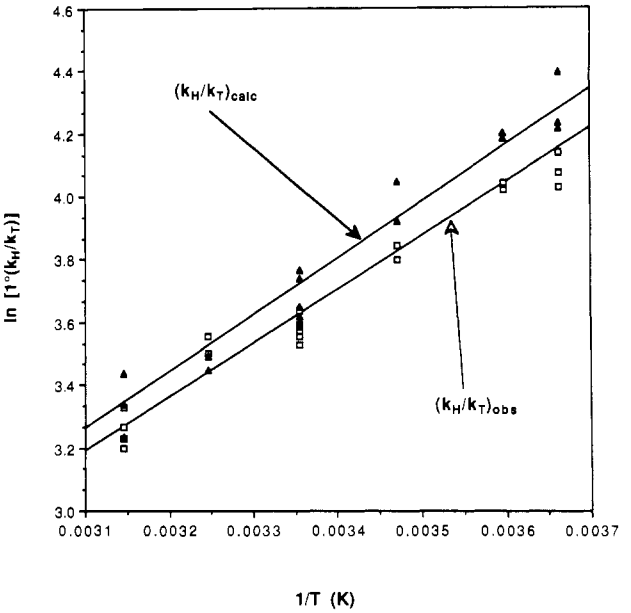


FIGURE 6: Comparison of the Arrhenius plot for H/T isotope effects (□) to that for $(k_D/k_T)^{3.26}$ (▲).

The simultaneous measurement of both D/T and H/T isotope effects introduces an additional probe for the demonstration of hydrogen tunneling in the BSAO reaction. Following the method of Northrop, observed tritium isotopes, $^T(V/K)$, are conveniently expressed in terms of an intrinsic isotope effect, ^Tk, and a commitment to catalysis, C_H:

$$^T(V/K) = \frac{^Tk + C_H}{1 + C_H} \qquad (14)$$

As previously noted by Cha et al. (1989), application of eq 14 to a D/T isotope effect leads to a change in both the ^Tk and C_H terms. Importantly, since a D/T isotope effect uses C–D cleavage (rather than C–H cleavage) as a frame of reference, the value of C_D is diminished relative to C_H by the magnitude of the primary deuterium isotope effect. For reactions in which the C–H bond cleavage step is already close to or fully rate limiting, substitution of H by D in the primary position of substrate ensures that a measured isotope effect will be free of potentially complicating, non-isotope-sensitive kinetic steps. Thus, while an H/T isotope effect could, fortuitously, reflect a combination of a commitment (leading to an inverse A_L/A_T value) and a highly expanded transition state (leading to a very large H/T value), *the magnitude of A_L/A_T for the D/T isotope effect is expected to be free of ambiguity regarding the nature of rate-limiting steps.* We have therefore compared the temperature dependence of $(k_D/k_T)^{3.26}$ =

[3] In a recent theoretical study, Thibblin (1988) has shown that unusually large isotope effects and anomalously small isotope effects on Arrhenius preexponential factors can arise for branched reactions which lead to two or more products via a common intermediate. All evidence in the BSAO reaction indicates a single product which is either reduced cofactor (stopped flow) or aldehyde product (steady state).

Table V: Exponential Relationship of Observed D/T and H/T Isotope Effects[a]

temp (°C)	primary effects	secondary effects
45	3.16 ± 0.10	3.13 ± 1.10
35	3.31 ± 0.05	4.40 ± 0.61
25	3.17 ± 0.07	3.56 ± 0.80
15	3.13 ± 0.08	4.17 ± 1.79
5	3.14 ● 0.02	4.80 ± 2.21
0	3.11 ± 0.08	4.15 ± 1.40

[a] $\ln[(k_H/k_T)_{obs}]/\ln[(k_D/k_T)_{obs}]$ = exponent. Derived from data in Tables I and II and Figures 1 and 3.

$(k_H/k_T)_{calc}$ to that of $(k_H/k_T)_{obs}$. As shown in Figure 6, the data for $(k_H/k_T)_{calc}$ and $(k_H/k_T)_{obs}$ are virtually superimposable, within experimental error, at each temperature. If there is a trend in the commitment on the proton abstraction step, it is very small, increasing with decreasing temperature. Importantly, the effect of such a trend would be to minimize anomalous behavior, such that the magnitudes of $(k_H/k_T)_{obs}$ and their temperature dependence would appear more semiclassical than those seen in the absence of a commitment. As will be discussed below, this behavior of the temperature dependence of $(k_D/k_T)^{3.26}$ (Figure 6) is a consequence of deuterium as well as protium tunneling, as reflected in the Arrhenius parameters for D/T isotope effects (Figures 1 and 2, and Table III).

Evidence for a Single Rate-Limiting Step. In Table V, the magnitudes of the exponents relating D/T and H/T isotope effects have been summarized for both primary and secondary effects. Although the magnitudes of the exponents for secondary isotope effects appear to fall primarily above 3.26, as predicted for hydrogen tunneling (eq 11), the errors on these exponents are very large. Thus, with the exception of the data at 35 °C, exponential values are seen to lie well within experimental error of the semiclassical range. Turning to the exponents relating primary isotope effects, these are seen to fall systematically below the semiclassical limit of 3.26 with trends outside of experimental error in the 0–25 °C range. Since this observation is in the direction expected for kinetic complexity, eq 12, we considered it important to pursue the nature of rate-limiting steps by an independent method.

Stopped-flow kinetic measurements have been used previously to determine rate constants for cofactor reduction under conditions of either low or high substrate concentrations, reflecting the terms $k_1k_3/(k_2 + k_3)$ (equivalent to V/K) and k_3, respectively:

$$E + S \underset{k_2}{\overset{k_1}{\rightleftharpoons}} E{\cdot}S \xrightarrow{k_3} E{\cdot}P \qquad (15)$$

In the event that k_2 is not much greater than k_3, measured isotope effects on k_3 are expected to be larger than isotope effects determined under V/K conditions. Previously, Palcic and Klinman showed that at 25 °C, deuterium isotope effects appear identical under conditions of either V/K or stopped flow (both at high or at low substrate concentrations), implying that $k_2 \gg k_3$.

In the present study, a similar comparison has been made in the temperature range of 0–45 °C, leading to the data in Table IV. As shown, only one condition (25 °C) was observed where the isotope effect measured under stopped-flow conditions was larger than that seen in the steady state. Although this could imply that substrate binding is partially rate limiting in the steady state, the magnitude of the error on $(k_H/k_D)_{stopped\ flow}$ is large, and stopped-flow and steady-state isotope effects can be seen to lie within experimental error. In fact, as noted above, Palcic and Klinman (1983) already demonstrated that $(k_H/k_D)_{steady\ state}$ is within experimental error of $(k_H/k_D)_{stopped\ flow}$ for several substrates at 25 °C and hence that $k_2 \gg k_3$ under this temperature condition. From the present study, the excellent agreement between $(k_H/k_D)_{steady\ state}$ and $(k_H/k_D)_{stopped\ flow}$ at 45, 35, and 15 °C (Table IV) indicates that $k_2 \gg k_3$ also pertains in this temperature range.

Examination of Table IV indicates an unexpected deviation of $(k_H/k_D)_{steady\ state}$ from $(k_H/k_D)_{stopped\ flow}$ at 5 and 0 °C, such that the stopped-flow isotope effect is seen to be smaller than the value obtained in the steady state. Although this discrepancy cannot arise from the kinetic scheme shown in eq 15, several origins for this behavior have been considered. First, it is possible that new spectral intermediates begin to accumulate below 10 °C, such that a single exponential for cofactor reduction can no longer be monitored at 480 nm. Recent rapid-scanning stopped-flow studies have indicated this type of behavior for substrates other than benzylamine at 25 °C (Hartmann, 1988). A second more plausible explanation is an onset of substrate inhibition at low temperature, with inhibition occurring at a lower concentration for protonated than deuterated benzylamine.[4] Although double-reciprocal plots have been seen to approximate linearity in the concentration range of 2–20 mM, small degrees of substrate inhibition could easily produce the effects shown in Table IV.

The significant conclusion from the comparison of stopped-flow and steady-state parameters is that isotope effects are the same in the temperature range of 15–45 °C (Table IV). Below 15 °C, stopped-flow isotope effects are actually diminished relative to those seen in the steady state, attributed to a small degree of isotope-sensitive substrate inhibition. Importantly, independent of whether we have monitored cofactor reduction at 480 nm (stopped flow) or aldehyde and tritium release to solvent (steady state), observed isotope effects agree within experimental error. *This ability to observe identical isotope effects by differing methods is of considerable importance, ruling out experimental artifacts as the source of abnormal isotope effects and providing further evidence that these effects arise from a single rate-limiting proton abstraction step.*

Deuterium Tunneling. All lines of inquiry pursued thus far support both protium tunneling and a single rate-limiting C–H bond cleavage step in the BSAO reaction. In light of these features, it becomes important to question why we have been unable to detect the expected breakdown between D/T and H/T primary isotope effects (eq 11). One possible explanation may lie with the relationship between the magnitude of the measured isotope effect and the extent of breakdown. From the simulations of Saunders (1985), it is clear that exponential breakdowns become magnified for small isotope effects, becoming increasingly difficult to detect as the magnitude of the isotope effect increases. We have already seen this behavior in the yeast alcohol dehydrogenase reaction, where the extent of breakdown of eq 11 for small secondary isotope effects was far in excess of that seen with the larger primary isotope effects (Cha et al., 1989).

We have also considered it important to examine the origin of the prediction that the exponent relating D/T and H/T can only equal or exceed 3.26. In the original study of Saunders (1985), the Bell equation was invoked to model tunneling, where an observed rate is formulated as the product of a semiclassical rate, k_s, multiplied by a tunnel correction, Q_t:

$$k_{obs} = Q_t k_s \qquad (16)$$

[4] Steady-state studies at 5 and 25 °C clearly indicate a more rapid onset of substrate inhibition for protonated benzylamine (above 4 mM) vs deuterated benzylamine (above 20 mM).

The isotope effect on k_{obs} is expected to reflect the product of two terms, leading to eq 17. From eq 17, it can be seen

$$k_{obs}(L)/k_{obs}(T) = [Q_t(L)/Q_t(T)][k_s(L)/k_s(T)] \quad (17)$$

that the exponential relationship between D/T and H/T isotope effects will depend on exponential values relating both $Q_t(D)/Q_t(T)$ to $Q_t(H)/Q_t(T)$ and $k_s(D)/k_s(T)$ to $k_s(H)/k_s(T)$. Since the relationship between $k_s(D)/k_s(T)$ and $k_s(H)/k_s(T)$ is fixed at ca. 3.26, the key determinant lies with the Q_t term.

In Saunders' calculations, data were modeled using relatively low frequencies for the reaction coordinate and correspondingly small tunnel corrections. In this range, it can be shown that the exponent relating $Q_t(D)/Q_t(T)$ to $Q_t(H)/Q_t(T)$ rises rapidly from 3.26 to an inflated value whose absolute value depends on the magnitude of the isotope effect being measured. Thus, in the range of small tunnel corrections, the exponent relating D/T and H/T isotope effects can only exceed 3.26. Recently, we have extended this work to include greater tunnel corrections.[5] Our simulations show an important trend in the relationship of $[Q_t(D)/Q_t(T)]^y$ to $Q_t(H)/Q_t(T)$, such that the value of y first rises above 3.26 as protium begins to tunnel, declines again to below 3.26 as deuterium begins to tunnel substantially, and finally levels off at a minimum value of 2.30 when all three isotopes (H, D, and T) undergo quantum mechanical tunneling. These results indicate that while Saunders' prediction is a good indicator of moderate amounts of hydrogen tunneling, reaction coordinates which promote considerable deuterium (and possibly tritium) tunneling can be expected to yield a wide range of values for y.

The above considerations suggest that the observation of y values equal to or below 3.26 in the BSAO reaction may be a consequence of *significant* deuterium, as well as protium, tunneling. This somewhat startling conclusion is, in fact, supported by the temperature dependences of primary D/T effects (Figures 1 and 2 and Table III); in the event that deuterium transfer were occurring by an over the barrier process, these temperature dependences are expected to yield semiclassical values for Arrhenius parameters. *By contrast, a similar degree of deviation from semiclassical Arrhenius parameters is seen in the study of D/T as in H/T isotope effects.*

Catalytic Consequences. Given the increasing evidence for nuclear tunneling events in enzyme reactions, as evidenced by the present study and our earlier work on the yeast alcohol dehydrogenase reaction (Cha et al., 1989), the relationship of this phenomenon to our understanding of enzyme catalysis becomes of considerable importance. One particularly useful parameter in developing models is the magnitude of the enthalpy of activation, which as indicated in Figure 5 is equal to 66 kJ/mol for deuterated benzylamine in the BSAO. This sizable enthalpy term may be an approximation to the semiclassical barrier height in the event that tunneling occurs near the top of the barrier. Such a model would be in keeping with the Bell correction (1980), which allows for a Boltzmann distribution of particles impinging on the reaction barrier, as well as models put forth by numerous authors [cf. Garrett et al. (1980), Tucker et al. (1985), and Kreevoy et al. (1986)] which are essentially semiclassical models which allow tunneling corrections for crossing of the energy surface below the saddle point.

A very different view has been taken for hydrogen transfer in enzyme-catalyzed reactions by Dogonadze and co-workers (Dogonadze et al., 1977). These authors have proposed that proteins exist in a family of thermally activated states, only some of which achieve the correct distance and internal thermodynamics for hydrogen transfer by quantum mechanical tunneling to occur. This type of model is analogous to the Marcus theory for electron transfer, according to which electron transfer is a temperature-independent quantum mechanical event with temperature dependencies arising from protein reorganization. One of the limitations of Dogonadze's theory is the prediction that, to a first approximation, there should be no difference in the enthalpy of activation for H and D transfer. This property is very different from that seen with reactions studied in solution (Bell, 1980), as well as the limited number of enzyme reactions for which both $E_a(H)$ and $E_a(D)$ have been measured [cf. Sumi et al. (1988)]. As seen in the case of BSAO, the isotope effect on enthalpies of activation is very large, with $E_a(D) - E_a(H) = 9.7$ kJ/mol (Table III). In an effort to address this feature of isotope effect measurements, Bruno and Bialek have recently developed Marcus-like models which incorporate a realistic mass dependence for tunneling, obtaining surprisingly good fits of our data to their model.[6] This result suggests that protein dynamics may play a critical role in the facilitation of ground-state hydrogen tunneling at the BSAO active site.

In conclusion, we have provided several lines of evidence that hydrogen tunneling contributes to the reaction coordinate in the BSAO reaction. This represents the second example of an enzyme reaction characterized by room temperature quantum mechanical effects. It should be noted that the manifestations of tunneling appear very different in the yeast alcohol dehydrogenase reaction (Cha et al., 1989) and in BSAO (this study), with regard to the breakdown of reduced mass considerations, the appearance of coupled motion between the primary and secondary positions of substrate, and isotope effects on Arrhenius plots. One particularly unexpected feature of the BSAO reaction is the compelling evidence for deuterium as well as protium tunneling. In the future, we plan to examine a wide range of enzyme reactions, to establish the generality of room temperature hydrogen tunneling. Additionally, experimental protocols are being pursued with the goal of distinguishing between models for enzyme catalysis which involve protein-assisted ground-state tunneling from those in which tunneling occurs from thermally activated states near the top of a semiclassical reaction barrier.

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On the Effects of Replacing the Carboxylate-Binding Arginine-171 by Hydrophobic Tyrosine or Tryptophan Residues in the L-Lactate Dehydrogenase from *Bacillus stearothermophilus*†

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ABSTRACT: For L-lactate dehydrogenases (LDH's), the interaction of the guanidinium group of their Arg171 residue with the carboxylate group of an α-keto acid is of primary importance in orienting the substrate productively at the active site. LDH's such as that of *Bacillus stearothermophilus* (BSLDH) are of practical importance for the preparation of chiral 2-hydroxy acids used as synthons in asymmetric synthesis but would even be more valuable in this regard if their specificities were broader. With a view to tailoring the specificity of BSLDH toward carbonyl substrates that lack an α-carboxyl group such as ketones, site-directed mutagenesis has been applied to replace Arg171 by the approximately isosteric, but hydrophobic, amino acids Tyr and Trp. The mutant enzymes exhibit remarkably good catalytic activities toward representative α-keto acids RCOCOOH, where R = Me, Et, *n*-Pr, *n*-Bu, and CH_2OH, although for the mutant enzymes the k_{cat}/K_M's are lower by $\sim 10^3$–10^4-fold than those for native BSLDH. Surprisingly, the 171 → Tyr/Trp enzymes are significantly more active than 171 → Lys (Hart et al., 1987a), for which an interaction of a positively charged side chain with substrate COO^- is retained. Preparative-scale 171 → Trp catalyzed reduction of pyruvate gave optically pure L-lactate, showing that L stereospecificity of such LDH enzymes was unaffected by the loss of Arg171. The retention of L stereospecificity is attributed to secondary polar or hydrogen-bonding associations of Arg109 and Thr246, respectively, with the substrate COO^- function that are of sufficient magnitude to maintain "normal" substrate orientation. These "fail-safe" carboxylate-favoring interactions may also be responsible for the failure of the 171 mutant LDH's to accept the representative ketones acetone, methyl pyruvate, and butane-2,3-dione as substrates.

Lactate dehydrogenases (LDH's)[1] are well-documented, NAD/H-dependent enzymes of the oxidoreductase group that catalyze stereospecific C=O ⇌ CH(OH) transformations of the type shown in eq 1 (Holbrook et al., 1975). In addition

$$\underset{\mathbf{1}}{RCOCOOH} + NADH + H^+ \overset{LDH}{\rightleftharpoons} \underset{\mathbf{2}}{RCH(OH)COOH} + NAD^+ \quad (1)$$

a, R = CH_3 **d**, R = $(CH_2)_3CH_3$
b, R = CH_2CH_3 **e**, R = CH_2OH
c, R = $(CH_2)_2CH_3$

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