# Effect of Pressure on Deuterium Isotope Effects of Yeast Alcohol Dehydrogenase: Evidence for Mechanical Models of Catalysis†

Dexter B. Northrop*,‡ and Yong-Kweon Cho§

*Division of Pharmaceutical Sciences, School of Pharmacy, 425 North Charter Street, University of Wisconsin−Madison, Madison, Wisconsin 53706, and Department of Biochemistry, Changwon National University, Changwon City, Kyungnam, South Korea 641-773*

*Received November 3, 1999*

ABSTRACT: Moderate pressure accelerates hydride transfer catalyzed by yeast alcohol dehydrogenase, indicative of a large negative volume of activation [Cho and Northrop (1999) *Biochemistry 38*, 7470−7475]. A comparison of the effects of pressure on the oxidation of normal versus dideuteriobenzyl alcohol generates a monophasic decrease in the intrinsic isotope effect; therefore, the volume of activation for the transition-state of deuteride transfer must be even more negative, by 10.4 mL/mol. This finding appears consistent with hydrogen tunneling previously proposed for this dehydrogenase [Cha, Y., Murray, C. J., and Klinman, J. P. (1989) *Science 243*, 1325−1330]. However, a global fit of the primary data shows that the entire isotope effect arises from a transition-state phenomenon, unlike normal isotope effects, which arise from different vibrational frequencies in reactant states, and tunneling isotope effects, which arise from a mixture of both states. Assuming the phenomenon is tunneling, the isotopic data are consistent with a Bell tunneling correction factor of $Q_H = 12$ and an imaginary frequency of $\nu_H^{\ddagger} = 1220$ cm$^{-1}$, the first so calculated from experimental enzymatic data. This excessively large correction factor and the large difference in the isotopic activation volumes, plus the low isotope effects at extrapolated pressures, challenge traditional applications of physical organic chemistry and transition-state theory to enzymatic catalysis. They suggest instead that something other than transition-state stabilization or tunneling is responsible for the rate acceleration, something unique to the enzymatic transition state that does not occur in nonenzymatic reactions. Arguments for the vibrational model of coupled atomic motions and the fluctuating enzyme model of protein domain motion are put forward as possible interpretations.

For a variety of reasons,[1] the effects of high hydrostatic pressure on the kinetics of enzymatic reactions have not been explored as much as one might have expected, given the parallel developments of isotope effects and pH effects. The major caveat is whether protein denaturation and subunit dissociation might occur coincidentally with, but independently of, changes in rate constants contributing to catalytic turnovers and thereby bring ambiguity to the interpretation of pressure effects on enzymes. Denaturation causes *removal* of enzyme from catalytic turnovers and has no mechanistic significance; in contrast, changes in rate constants cause *modulation* of catalytic turnovers, which may contain highly specific information about chemical and kinetic mechanisms that could be of great mechanistic value—if multiple effects could be deconvoluted and their activation volumes properly assigned. The first problem is to find a means to distinguish between pressure-dependent *removal* and *modulation*. Once that is resolved, the second problem of assigning activation volumes to specific rate constants can be engaged.

A problem similar to the first was addressed by Parmentier et al. (*7*), who used isotope effects to distinguish between alternative models of allosteric regulation: the two-state concerted model of Monod et al. (*8*) and the multistate sequential model of Koshland et al. (*9*). Because an isotope effect reports back only from active enzyme, it can distinguish between a loss of activity due to *removal* of enzyme from participation in catalytic turnovers (the Monod model, which will not change the isotope effect on the remaining activity), and a loss due to *modulation* of rate constants contributing to catalytic turnovers [the Koshland model, which will change *commitments to catalysis* (*10*), which in turn may change the expression of intrinsic isotope effects]. Accordingly, it seemed reasonable to extend the experimental design of Parmentier et al. (*7*) to the first pressure problem.

The oxidation of benzyl alcohol by yeast alcohol dehydrogenase (YADH)[2] was chosen as a test system because Cha et al. (*11*) had demonstrated that an intrinsic deuterium isotope effect arising from dideuteriobenzyl alcohol was fully expressed on the kinetic parameter, *V/K*, which measures substrate *capture* (*12*). Therefore, at low to moderate pressures, changes in capture of benzyl alcohol were expected to be directly proportional to changes in the rate constant for hydride transfer, which proved to be the case (*13*). Isotopic experiments were begun with the expectation that the intrinsic isotope effect would be independent of pressure,

† This project was supported by NSF Grant MCB-9219042, NIH Grant GM-46695, and the Graduate School of the University of Wisconsin. A preliminary report has appeared (*1*).

* To whom correspondence should be addressed: Phone (608) 263-2519; FAX (608) 262-3397; e-mail DBNORTHROP@PHARMACY.WISC.EDU.

‡ University of Wisconsin−Madison.

§ Changwon National University.

10.1021/bi992537z CCC: $19.00 
Published on Web 02/09/2000

but as will be seen, this proved not to be the case. Nevertheless, despite this added complexity, real versus apparent changes in isotope effects still allow a clear distinction between removal and modulation of active enzyme, because the various combinations of volume changes and reaction mechanisms display very different pressure dependencies. These differences provide a first solution to the second pressure problem as well, and allow an assignment of an activation volume to a specific step within a kinetic mechanism. This success suggests that other pressure studies with other enzymes will likely lead to other activation volumes being assigned to other specific steps as well.

## THEORY

The effect of removal of active enzyme from catalytic turnovers can be described within the conventions of an isomechanism (*14*). Consider the enzymatic reaction sequence shown in Scheme 1, where G represents an inactive isomer of free enzyme,[3] E an active form, and EX ⇌ FY a single isotopically sensitive step.[4] Not shown is the conversion of enzyme form F back to G to complete a catalytic cycle, which could be the release of a second product or the second half of a ping-pong reaction, for example, but is not a part of substrate capture (*12*). A general expression for the effect of pressure on substrate capture can be written as (*13*)

Scheme 1

$$G + S \rightleftharpoons E + S \underset{k_2}{\overset{k_1}{\rightleftharpoons}} ES \underset{k_4}{\overset{k_3}{\rightleftharpoons}} EX \underset{k_6}{\overset{k_5}{\rightleftharpoons}} FY \underset{k_8}{\overset{k_7}{\rightleftharpoons}} FP \xrightarrow{k_9} F + P$$

$$|V/K|_p = \left(\frac{k_1}{1 + K_{G/E}e^{-\Delta V_{G/E}p/RT}}\right) \times \left(\frac{R_0 e^{-\Delta V^{\ddagger}p/RT}}{1 + C_f e^{-\Delta V^{\ddagger}p/RT} + C_r e^{-(\Delta V^{\ddagger} - \Delta V_{eq})p/RT}}\right) \quad (1)$$

where $R_0$ is the product ratio of forward and reverse enzymatic rate constants up to and including the isotopically sensitive step, e.g., $k_3k_5/k_2k_4$ in Scheme 1, $\Delta V^{\ddagger}$ is the volume of activation between reactants E + S and the transition state of the isotopically sensitive step in milliliters per mole,[5] $p$ is the pressure in bar (0.98692 standard atmosphere), $R$ is the gas constant at 82.0578 mL·bar/(mol·$K$), $T$ is the temperature in kelvins, $K_{G/E}$ is the equilibrium constant between free enzyme forms E and G, $\Delta V_{G/E}$ is the volume difference between the forms, $C_f$ and $C_r$ are the forward and reverse commitments to catalysis (*10*), $k_5/k_4(1 + k_3/k_2)$ and

[1] In 1980, Kenneth Neet addressed a meeting of the International Union of Biochemistry in Toronto, Canada, and opened his address on hysteretic enzymes with these words: "All scientists worship at the alter of the straight line." His point was that linearization of natural functions in the form of Lineweaver−Burk plots, Scatchard plots, Arrhenius plots, etc., was necessary in the days of graphical analyses of data, but with the advent of the digital computer we could now analyze curves—and ask new questions. Most fields of science made the transition after Neet's remark; pressure effects did not. The tradition of plotting the logarithm of functions versus pressure remains very strong, and it confounds those who do so and obtain something other than the expected straight line. The majority of high-pressure studies that generated curved logarithmic plots have been misinterpreted, in that the atavistic straight line is frequently drawn not once but twice. Pairs of lines can be found passing through data points in linear portions of the curves, one at low pressure and one at high pressure, and volume changes have been extracted from the slopes of the paired lines. These volume changes are in error. The appropriate lines accompanying such graphical analysis are asymptotes, and asymptotes to logarithmic functions of pressure usually lie considerably apart from the data points (for example, see Figure 15.15 in ref *1*). But graphical analysis, even if done correctly, cannot deal adequately with logarithmic curves because there is no way to take the antilog of a curve; there is no way to collect a pair of volume changes determined graphically and return to the complete mathematical description of the physical process from which the logarithmic data were generated. In some cases, curved logarithmic data have been fit to an arbitrary quadratic equation, but that approach has been discredited also (*2*). Within enzymology, most-high pressure studies have employed an overly simplistic paradigm for enzyme kinetics, namely, the classical textbook derivation of the Michaelis−Menten rate equation using a mechanism containing a single so-called "rate-limiting step", which was discredited shortly after Neet's remark (*3*−*5*). This paradigm generates linear log ($k_{cat}$) plots, but nevertheless predicts curved plots for log ($K_M$) and log ($k_{cat}/K_M$) vs pressure. When multiple steps were addressed, a "weighted mean" of the two activation volumes was sometimes computed to give a "composite volume", which could then be addressed in a traditional logarithmic equation of pressure dependence (*6*). The composite volume was shown recently to be the tangent to the resulting biphasic curve at zero pressure (*1*), and as with asymptotes, there is no way back from the slope of this tangent to the rate equation nor even to values for either of the two volumes generating the curve. With the advent of computers and nonlinear regression analyses, straight lines and logarithmic plots are no longer needed; we can analyze nonlinear *exponential* functions directly. Moreover, computer fitting to pressure dependencies is not limited to only two paired volume changes as with logarithmic plots but may originate from multiple thermodynamic and kinetic volume changes. For example, eq 7 has four volume changes acting on five separate parameters, and the fitted data in Table 1 contain three distinct but well-determined volume changes, one of them thermodynamic and two kinetic in origin. Pressure effects can now join isotope effects and pH effects as a sophisticated tool for probing enzymatic catalysis.

[2] Abbreviations: YADH, yeast alcohol dehydrogenase (EC 1.1.1.1): $NAD^+$, niconamide adenine dinucleotide.

[3] Free enzyme as represented by G in Scheme 1 was originally conceived within the kinetic theory of isomechanisms as being an intermediate along the cyclic pathway of catalysis for a single-substrate reaction that generated a single product (*14*). For multisubstrate enzymatic reactions, the kinetics of capture for one substrate are defined as occurring under saturating concentrations of all others; consequently, for YADH when S is the alcohol, "free G" is E-$NAD^+$; "free E" is E*-$NAD^+$, and F is E*-NADH. Moreover, because [S] is extrapolated to zero in the kinetics of capture, G is at equilibrium not only with E but also with any and all other forms of enzyme that are incapable of participating in capture. These include subtle conformational changes that may be physically undetectable by such techniques as fluorescence spectral analysis, frequently so employed, plus the more drastic changes of protein denaturation and subunit dissociation. In this way, the kinetics of capture formulated within an isomechanism obviates the major caveat of studying enzymatic reactions at high pressure.

[4] Any of one of three steps in Scheme 1 can be deemed the isotopically sensitive one (and in special cases not addressed here, more than one). The choice simply changes the definitions of the parameters of eq 1, not the form of the equation itself. However, casting the rate equation for capture in this particular form requires that some step be designated an isotopically sensitive one, even in the absence of any isotopic experiments or data, as was the case in Cho and Northrop (*13*).

[5] It should be emphasized that a volume of activation is a property of state, like free energy or entropy, and thus independent of the pathway of the change and of intermittent volume changes that may occur along a particular pathway. In the present case, a volume change may or may not accompany the binding of the substrate, benzyl alcohol, because such a volume change is tacitly incorporated into $\Delta V^{\ddagger}$ as formulated in eq 1, i.e., $\Delta V^{\ddagger} = \Delta V_1 - \Delta V_2 + \Delta V_3 - \Delta V_4 + \Delta V_5$, and $\Delta V^{\ddagger}$ can retain a particular value whether $\Delta V_2$ is the same as $\Delta V_1$ or not, by compensating volume changes associated with the other rate constants.

$k_6/k_7(1 + k_8/k_9)$, respectively, in Scheme 1, and $\Delta V_{eq}$ is formally the volume change between E + S and F + P but is assumed to be between S and P only.

The expression of a deuterium isotope effect requires that a hydrogen form of eq 1 be divided by a deuterium form, during which process the terms within the left set of parentheses will cancel, along with the nonisotopic rate constants of $R_0$. This process is the essence of the experimental design of Parmentier et al. (*7*). The expression of an intrinsic isotope effect, $^{D}k$, on the kinetics of capture is controlled by commitments to catalysis (see eq 16 in ref *10*); it follows that if pressure alters these commitments, the combined pressure effects on substrate capture will take the form

$$|^{D}(V/K)|_p = \frac{^{D}k + C_f e^{-\Delta V^{\ddagger}p/RT} + C_r\,^{D}K_{eq} e^{-(\Delta V^{\ddagger}-\Delta V_{eq})p/RT}}{1 + C_f e^{-\Delta V^{\ddagger}p/RT} + C_r e^{-(\Delta V^{\ddagger}-\Delta V_{eq})p/RT}} \quad (2)$$

where $^{D}K_{eq}$ is the isotope effect on the equilibrium constant, here assumed to be independent of pressure as is $^{D}k$. It may be possible to conduct a successful regression on eq 2 if the volume change of the equilibrium constant is large enough to assist in differentiating the commitments. When not large, this form of equation will impose considerable covariance, leaving an approximate sum of the commitments the best one might hope to resolve:

$$|^{D}(V/K)|_p = \frac{^{D}k + (C_f + C_r)e^{-\Delta V^{\ddagger}p/RT}}{1 + (C_f + C_r)e^{-\Delta V^{\ddagger}p/RT}} \quad (3)$$

To do global fittings of primary isotopic data, however, one must incorporate an expression for the isotopic dependence of sensitive rate constants in the primary equation and not divide one by another. This expression has the following form (*15*):

$$k_i = \frac{k_H}{1 + C_i[^{D}k - 1]} \quad (4)$$

where $C_i$ is the fraction of isotopic labeling, 0 for unlabeled substrates and approaching a value of 1 for labeled substrates. Substituting eq 4 into eq 1 yields the global rate equation for the effects of pressure on *V/K* in the presence and absence of labeled substrate:

$$|V/K|_p = p = \left(\frac{k_1}{1 + K_{G/E}e^{-\Delta V_{G/E}p/RT}}\right) \times \left(\frac{R_0 e^{-\Delta V^{\ddagger}p/RT}}{1 + C_i[^{D}k - 1] + C_f e^{-\Delta V^{\ddagger}p/RT} + C_r e^{-(\Delta V^{\ddagger}-\Delta V_{eq})p/RT}}\right) \quad (5)$$

A pressure-dependent intrinsic isotope effect is described by the expression (*2*)

$$|^{D}k|_p = {}^{D}k(^{d}Q - 1)e^{-\Delta V_Q p/RT} + {}^{D}k \quad (6)$$

where $^{D}k$ is the semiclassical isotope effect originating in the reactant state, $^{D}Q$ is the ratio of Bell tunneling corrections (*16*) and represents the portion of an isotope effect originating in the transition state, and $\Delta V_Q$ is the apparent volume difference between a hydride and deuteride transfer.[6] Substituting eq 6 into eq 5 yields a global rate equation for the effects of pressure on *V/K* when *both* an intrinsic isotope effect itself *and* its expression in the steady state are subject to change as a function of pressure:

$$|V/K|_p = [k_1/(1 + K_{G/E}e^{-\Delta V_{G/E}p/RT})](R_0 e^{-\Delta V^{\ddagger}p/RT}/\{1 + C_i[^{D}k(^{D}Q - 1)e^{-\Delta V_Q p/RT} + {}^{D}k - 1] + C_f e^{-\Delta V^{\ddagger}p/RT} + C_r e^{-(\Delta V^{\ddagger}-\Delta V_{eq})p/RT}\}) \quad (7)$$

When $\Delta V_{eq}$ is small, eq 7 reduces to:

$$|V/K|_p = \left(\frac{k_1}{1 + K_{G/E}e^{-\Delta V_{G/E}p/RT}}\right) \times \left(\frac{R_0 e^{-\Delta V^{\ddagger}p/RT}}{1 + C_i[^{D}k(^{D}Q - 1)e^{-\Delta V_Q p/RT} + {}^{D}k - 1] + (C_f + C_r)e^{-\Delta V^{\ddagger}p/RT}}\right) \quad (8)$$

On the other hand, when commitments are small, eq 7 reduces to

$$|V/K|_p = \left(\frac{k_1}{1 + K_{G/E}e^{-\Delta V_{G/E}p/RT}}\right) \times \left(\frac{R_0 e^{-\Delta V^{\ddagger}p/RT}}{1 + C_i[^{D}k(^{D}Q - 1)e^{-\Delta V_Q p/RT} + {}^{D}k - 1]}\right) \quad (9)$$

At atmospheric (or zero) pressure, $k_1R_0$ equals the value of *V/K* in eq 9 and these parameters can be combined and replaced by an empirical constant.

## RESULTS AND DISCUSSION

Figure 1A shows the biphasic pressure dependence of the capture of benzyl alcohol by YADH reported by Cho and Northrop (*13*). Also shown are new results obtained with [α,α-$^2H_2$]benzyl alcohol (purchased from Isotec) using the same methods (standard errors averaged less than 7%), where again each data point represents a complete initial velocity kinetic pattern extrapolated to infinite [$NAD^+$] and zero [benzyl alcohol]. The curve is again biphasic but lower in amplitude as a result of the combined primary and secondary deuterium isotope effects, and shifted slightly to the right.

[6] The validity of eq 6 was challenged during the review process because a reviewer thought it lacked a physical justification. To avoid confusion, it should be pointed out that given a single energy barrier, an apparent isotope effect has to be a simple product of reactant-state and transition-state properties, described by Bell as $k_H/k_D \cdot Q_H/Q_D$ but other symbols will do; therefore, the pressure dependency described by eq 6 has to be valid for *any* mix of reactant-state and transition-state phenomena and is not limited to tunneling or to any particular physical origin. An alternative model has been suggested (Richard Schowen, personal communication) in which an isotope effect might *appear* to be pressure-dependent if a reaction proceeded as the sum of two separate pathways with different isotope effects and volumes of activation; pressure would then cause a differential change in their individual energy barriers—but not their individual isotope effects. Attempts were made (unpublished results) to fit data from Figure 1B and ref *2* to this model. The results were decidedly negative, with either a lack of any convergence or, when forced, convergence to extreme parameter values or extreme standard errors together with a lack of agreement between point and line. The reaction in ref *2* can also be eliminated by the downward curvature of logarithmic plots of the primary data because this model requires such plots to curve upwards.

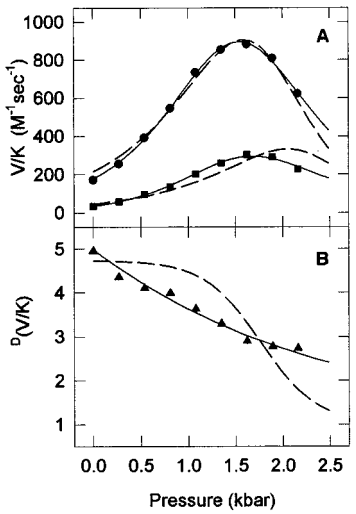


FIGURE 1: Effect of pressure on the capture of benzyl alcohol (●) and dideuteriobenzyl alcohol (■) by yeast alcohol dehydrogenase and on deuterium isotope effects (▲). Values of *V/K* were fit to eqs 8 or 9 to determine the solid lines in both panels, representing pressure effects on hydride transfer, on the availability of enzyme for capture and on the intrinsic isotope effect. The upper curve in panel A reaches a maximum at 1.53 kbar; the lower curve, at 1.67 kbar. The dashed lines show a fit to a truncated eq 5 in which the intrinsic isotope effect is independent of pressure and the apparent decrease in its expression on *V/K* is due to changes in commitments to catalysis.

Figure 1B shows the ratio of the two sets of data points, which takes the form of a monophasic decrease in the sum of isotope effects—a decidedly singular result extracted from nearly 500 awkward enzymatic assays.

The isotopic data of Figure 1B were fitted to eq 6 by use of a BASIC computer program employing the nonlinear regression routine of Duggleby (*17*) to examine whether a pressure-dependent isotope effect might cause the decrease.[7] Good agreement between point and line was obtained along with the following parameters: $^{D}k = 1.1 \pm 1.3$, $^{D}Q = 4.5 \pm 5.1$, and $\Delta V_Q = 10.6 \pm 5.9$ mL/mol. These results were surprising, suggesting that either there is no semiclassical isotope effect whatsoever or, allowing for uncertainty, that the reactant-state origin is smaller than the transition-state origin, suggesting in turn that the latter is something other than hydrogen tunneling. The results were also disappointing because of their large standard errors. A global fit was subsequently applied to the primary data of Figure 1A, using eqs 8 and 9 within the nonlinear regression routine of Duggleby (*17*). The results generated the solid curves shown in both panels and the fitted parameters listed on the left side of Table 1. Virtually identical values and standard errors were obtained in the presence and absence of commitments.[8] Moreover, the primary data constrained the regression analysis to an unprecedented degree, reducing the error of the semiclassical isotope effect from over 100% to less than 3%, despite the very long extrapolation one visualizes in Figure 1B (which dramatically demonstrates the importance of global fitting using Cleland's operator, eq 4). In a reciprocal manner, the isotopic data constrained the regression analysis of the primary curves as well, increasing the precision of $\Delta V_H^{\ddagger}$, $K_{G/E}$, and $\Delta V_{G/E}$ of Cho and Northrop (*13*). The global fit confirms their assignments of the volume changes to (a) the transition state of hydride transfer, causing a rate acceleration at moderate pressure, and to (b) a conformational change of an enzyme−nucleotide complex, causing inhibition at high pressure by removing the complex from participating in the capture of benzyl alcohol.

Table 1: Pressure Effects and Isotope Effects on the Capture of Benzyl Alcohol by YADH[a]

| | | | |
|---|---|---|---|
| $^{D}k$ | $0.99 \pm 0.03$ | $Q_H$ | $11.7 \pm 1.1$ |
| $^{D}Q$ | $4.99 \pm 0.37$ | $Q_D$ | $2.34 \pm 0.04$ |
| $\Delta V_H^{\ddagger}$ | $-38 \pm 1$ mL·mol$^{-1}$ | $Q_T$ | $1.70 \pm 0.02$ |
| $\Delta V_Q$ | $10.4 \pm 1.5$ mL·mol$^{-1}$ | $\nu_H^{\ddagger}$ | $1220 \pm 11$ cm$^{-1}$ |
| $K_{G/E}$ | $0.012 \pm 0.002$ | $\Delta V_D^{\ddagger}$ | $-49 \pm 1$ mL· mol$^{-1}$ |
| $\Delta V_{G/E}$ | $-72.5 \pm 1.4$ mL·mol$^{-1}$ | | |

[a] Data on the left were obtained by a fit of Figure 1A to either eq 8 or 9.[8] Data on the right (except for $\Delta V_D^{\ddagger}$) were derived from those on the left by use of eq 10. Only the tritium activation energy of ref *14* was used, under the assumption that tritium tunnels the least and its activation energy is closest to the true barrier height. When a finite and significant value was obtained for tritium tunneling, the activation energy was adjusted upward according to the relationship $E = -2.3RT(\log k/Q_t)$ and the iteration was repeated. The small adjustment raised the activation energy from 70.9 to 72.2 kJ·mol$^{-1}$ and lowered $Q_H$ from 11.73 to 11.69. $\Delta V_D^{\ddagger}$ was obtained from an alternative fit to a modified form of eq 9 in which $\Delta V_Q$ was replaced by $\Delta V_H^{\ddagger} - \Delta V_D^{\ddagger}$.

As part of model discrimination, the isotopic data were also fit to eq 3 to examine what the function would look like if an increase in the commitments had modulated the expression of a the intrinsic isotope effect, causing it only to appear to decrease. The result generated a good agreement between point and line (not shown) but standard errors were very large and the results themselves were nonsensical, with $^{D}k = 33 \pm 195$ and $C_f + C_r = 7 \pm 49$. Nevertheless, a global fit to further test the modulation model was applied to the primary data from Figure 1A using a truncated form of eq 5 with commitments combined as in eq 3. It generated the curves shown as dashed lines in Figure 1 together with large standard errors. Clearly, there is a severe lack of agreement between point and line in both panels that rules out the modulation model.

With a precise estimate of $^{D}Q$ in hand, it becomes possible for the first time to fit experimental data directly to the Bell tunneling correction equation for a real enzymatic reaction suspected of tunneling, to supplement computer modelings such as those of Huskey and Schowen (*18*) and by Rucker

[7] Actually, the data in Figure 1B were first fitted to a simple curve of analytical geometry, an inverse hyperbola, which is independent of the assumptions underlying Bell's model of hydrogen tunneling or the pressure-dependent intrinsic isotope effect described by eq 6. A curve was generated that appeared indistinguishable from the solid line shown, with an amplitude that is 97% ± 32% of the difference between the intercept and an asymptote of 1. This means that at least 65% of the isotope effect is abolished at high pressure, a conclusion that is model-independent and unprecedented in the chemical literature of pressure effects on isotope effects.

[8] The product of $k_1R_0$ was fixed at 170.9 M$^{-1}$ s$^{-1}$, the experimental value of *V/K* for normal benzyl alcohol measured at atmospheric pressure, after a regression to eq 8 returned a value of 170.5 M$^{-1}$ s$^{-1}$, verifying the assertion of Cha et al. (*11*) that the intrinsic isotope effect was fully expressed. In the fit to eq 8, $C_f + C_r < 0.001 \pm 10$, which is generally interpreted as not significantly different from zero. When $\Delta V^{\ddagger}$ was masked at −38 mL/mol and the regression repeated, $C_f + C_r < 0.001 \pm 0.012$. In separate and independent experiments involving the effects of pressure on solvent isotope effects of YADH, global regression returned a value for $C_f + C_r$ of $<0.001 \pm 0.0014$ and the upper limit of $(C_f + C_r)^{-\Delta V^{\ddagger}p/RT}$ did not exceed 0.05 at 2.5 kbar. (Northrop and Cho, submitted for publication).

and Klinman (*19*, see below). Bell (*16*) assumed a truncated parabolic energy barrier and derived the following series function:

$$Q_t = \frac{^{1/2}u_t}{\sin(^{1/2}u_t)} - \sum_{n=1}^{\infty}(-1)^n \frac{\exp\left(\dfrac{u_t - 2n\pi}{u_t}\alpha\right)}{\dfrac{u_t - 2n\pi}{u_t}} \quad (10)$$

where $u_t$ equals $h\nu^{\ddagger}c/k_BT$, $\alpha$ equals $E/k_BTN$, $h$ is Planck's constant, $\nu^{\ddagger}$ is the imaginary frequency, $c$ is the speed of light, $k_B$ is Boltzmann's constant, $T$ is temperature, $E$ is the activation energy, and $N$ is Avogadro's number (*20*). Using 70.9 kJ/mol as the value of the energy of activation (*21*) and following the iterative procedure of Northrop (*2*), the tunneling corrections shown on the right side of Table 1 were obtained.

The finite Bell tunneling corrections confirm quantitatively the proposal of Cha et al. (*11*) that the intrinsic deuterium isotope effect of YADH has a transition-state component, which they ascribed to hydrogen tunneling. Their proposal was largely qualitative because it was based on a breakdown of the Swain−Schaad relationship, which is accompanied by severe error propagation (*22, 23*). Grant and Klinman (*21*) attempted to quantify the degree of tunneling by estimating the Bell tunneling correction that would be consistent with the observed breakdown. They succeeded in defining tunneling corrections as a function of Swain−Schaad breakdown but unfortunately found two corrections coincident with some breakdowns and were unable to resolve the resulting ambiguity. The corrections in Table 1 also verify the calculations of Huskey and Schowen (*18*), who performed computer modeling of the interplay between primary and secondary isotope effects using vibrational analysis and concluded that, in order to account for data from the alcohol dehydrogenase reaction, the vibrational model had to have 80% of the primary isotope effect coming from hydrogen tunneling, a value that seemed quite excessive at the time. Similarly, Rucker and Klinman (*19*) recently performed a computational study on YADH and concluded that the $\alpha$-secondary isotope effect is primarily due to tunneling, with the semiclassical component highly suppressed by coupled motion to the hydride transfer. Assuming the transition-state phenomenon is tunneling, the isotopic data in Table 1 are consistent with an imaginary frequency of 1220 $cm^{-1}$ and a very large Bell tunneling correction factor of 12 for hydride transfer. Whether the Bell formalism is correct or not, at minimum these values are of interest because they suggest a new type of structure/activity relationship that can focus solely on parameters associated with the transition state. For example, Rucker and Klinman (*19*) calculated a variety of reaction frequences, from 679.9 to 1148.2 $cm^{-1}$ depending upon the choice of some force constants and coupling modes used in their computer model; therefore, the frequency extracted from these pressure studies may be useful in arbitrating those choices. Nevertheless, for YADH, these parameters seem too extreme to accept seriously as real parameters of hydrogen tunneling and perhaps are of only academic interest within the Bell theoretical construction.

The volume changes themselves and the lack of a semiclassical isotope effect originating in differences in zero point energies are another matter. These are not dependent upon a theoretical construction other than that necessary to distinguish between the removal and modulation models. The former is clearly the correct model for YADH data, so we are left with trying to account for (a) the large negative activation volume of hydride transfer, in excess of the space occupied by two water molecules, (b) a volume difference between hydride and deuteride transfer, with the latter even more negative, and (c) the possible origins of an isotope effect arising solely within a transition state. The first question may be dismissed because pressure is known to affect such things as viscosity and the dielectric constant, which in turn can affect reaction rates; hence, apparent activation volumes need not represent an actual "space" as such. Whatever their origin, the fitted volumes return precise measurements on some catalytic property of the active site that is very sensitive to pressure; that in itself is of interest and utility. Even without knowing what that property is, this negative activation volume can be useful in sorting out competing theories of enzyme action. For example, Warshel (*24*) argues that electrostatic stabilization is the primary origin of catalytic power, and it has long been known that one of the largest components of solvent reorganization under pressure is the volume decrease of electrostriction that accompanies charge separations (*25*). But the sign of the activation volume is wrong; pressure drives charges apart and therefore favors electrostatic *destabilization*, not the other way around. Similarly, Cannon and Benkovic (*26*) argue that enzyme-driven solvent reorganization is the major source of catalytic power. But again the sign is wrong. The desolvation accompanying ligand binding generates a positive volume change as water molecules are released from the icelike cages surrounding hydrophobic groups and the electrostriction surrounding ions. Moreover, solvent reorganizations should be expressed in a binding step, not a chemical step, as was found in the capture of $NAD^+$—where it has the correct sign (*13*).

The second question cannot be accounted for spatially, as the volume difference between hydride and deuteride is only 1 mL/mol, nor can it be dismissed by an appeal to nonspatial properties because these should affect hydride and deuteride transfers equally, given the Born−Oppenheimer approximation. Only motion of some kind can be this sensitive to a mass difference. The sensitivity is not without precedent, however. Isaacs (*27*) noted from effects of pressure upon primary isotope effects of eight reactions suspected of tunneling that the activation volume of deuterium was always significantly more negative than that of protium, as is the case in Table 1. Isaacs et al. (*28*) attributed one isotopic volume of activation difference to coupled motion between the hydride and solvent, and indeed the volume difference was determined and later shown to be altered significantly by the change to a more bulky solvent (*2*). The modeling studies of Huskey and Schowen (*18*) require, in addition to tunneling, extensive coupled motion between the transferring hydride and the $\alpha$ proton. Such motion suppresses the primary effect, and without it they estimate the intrinsic isotope effect ought to be $^Dk = 13$ at 80% tunneling. More recently, Antoniou and Schwartz (*29*) have performed modeling studies of the alcohol dehydrogenase reaction that

included variations of the distance between the hydride donor and acceptor and concluded that intramolecular vibrations may strongly influence the reaction rate, enhance tunneling, and contribute to the results of Cha et al. (*11*).

The third question cannot be answered, nor even addressed, within absolute rate theory as applied usually to enzymatic reactions (*30*) or as modified by semiclassical theory. Moreover, if coupled motion is the answer to the second question, then absolute rate theory is decidedly inadequate, because as pointed out by Lowry and Richardson (*31*), a "weakness of the transition-state theory is the assumption that the reaction coordinate can be separated from the other motions. Detailed calculations for very simple reactions...indicate that the error introduced by the separability assumption into a rate constant calculated from a known potential energy surface is significant." Coupled motion within a tightly bound enzyme-substrate complex is probably extensive; certainly many degrees of vibrational freedom are lost when a substrate binds within a deep active site (and sometimes covered by a flap), as evidenced by spectral changes of both substrates and enzymes, so it follows that some others must be enhanced. If the favored motions are coupled to motion along the reaction coordinate, they could accelerate catalysis.[9] This hypothesis was described by Lumry (*32*) as follows: "In this way several vibrational degrees of freedom contribute their excess energy to satisfy the activation-energy requirement. Moelwyn-Hughes (*33*) has proposed that proteins are good catalysts because they have so many vibrational degrees of freedom which may contribute to the activation energy. That is, the protein provides a good source of activation energy rather than a means for reducing the true activation-energy requirement for forming the activated complex."

Lumry discounted this hypothesis because it predicted curved Arrhenius plots and most known ones at the time were linear (but also very short). However, one of the arguments for hydrogen tunneling is its temperature dependence, which in turn requires that the Arrhenius plot for protium be more curved than plots for deuterium and tritium (see Figure 4 of ref *34*). Hence, if a case can be made to support hydrogen tunneling in the YADH reaction—and it has—then an equal case can be made to support the vibrational model. It was noted above that one form of coupled motion suppresses hydrogen tunneling; this is because the mass of the coupled atom adds to the reduced mass of the transferring hydrogen, which in turn diminishes the isotopic mass difference in the transition state. But that is the case only if the coupled atom is a *drag* on the transfer. In the vibrational model, multiple atomic motions are believed to act in synchrony and *drive* the transfer. If the former reduces a tunneling isotope effect, it seems a reasonable inference that the latter induces a tunneling-like isotope effect.[10] Rucker and Klinman (19) note in their computer modeling studies that coupled motion leads to deviations of the rule of the geometric mean and that "almost paradoxically, coupled motion can both increase and decrease RGM deviations."

Alternatively, once coupled motion is deemed significant and classical transition-state theory is deemed less so, Lumry's fluctuating enzyme model with protein domains in motion becomes a viable mechanism as well (*32*, *37*−*40*). It allows the 40−50 mL that must be accounted for to be spread across the interior matrix of the protein. Moreover, Lumry's model has the right sign, and if applicable generally, then *all* enzymes should have a compressed volume in transition states of chemical steps—which is a testable hypothesis dependent upon the experimental designs of this paper.[11] The fluctuating enzyme nicely addresses the first question and may be reconciled with the second but has problems with the third. Bruno and Bialek (*41*) have done an extensive theoretical analysis of the influence of low-frequency "breathing" vibrations of proteins on hydrogen tunneling and have suggested that reaction rates can be accelerated by fluctuations leading to shorter transfer distances, which they describe as "vibrationally enhanced ground-state tunneling." Similarly, Cannon and Benkovic (*26*) surveyed empirical data and noted that despite 17 orders of magnitude in rate enhancements for a series of enzymatic reactions, $k_{cat}$ values varied within only a few orders of magnitude and did not correlate with tightness of binding within the transition state but did match the frequencies of protein motion such as allosteric transitions and rotation of medium-sized side chains in the matrix. Perhaps the large activation volume *and* pressure-sensitive intrinsic isotope effect are revealing the presence of both types of motion contributing to rate enhancements. Both mechanical models were proposed initially long ago but generally discounted over time in the absence of supporting data; there simply were no experimental designs put forward to test either model. By accident, the present data lend credence to both models and mount serious challenges to the hypothesis that all rate enhancement of enzymatic reactions is due to transition-state stabilization.

## ACKNOWLEDGMENT

This paper is dedicated to Rufus Worth Lumry II in happy anticipation of his approaching 80th birthday. Professor Emeritus Lumry was one of the first to propose a mechanical model of enzymatic catalysis, in his doctoral thesis of 1948. In 1953, with Henry Eyring, he formulated the "rack" mechanism, and for the rest of the century he continued to champion mechanical models over the more popular electronic and protonic devices of physical organic chemistry.

[9] A metaphor for this hypothesis could be the following: a platoon of soldiers marching in step can destroy a bridge if their cadence matches a frequency of the structure. A corollary to this hypothesis suggests that if *dis*favored motions are coupled to motion along the reaction coordinate, they could *de*celerate catalysis, and thus provide a new foundation for extremes of substrate specificity. This mode of *negative* catalysis as coupled to substrate specificity could be equally as important to enzymology as the large rate enhancements brought about by favored motions contributing to the more familiar positive catalysis.

[10] Contemporaneous with this work were reports by Basran et al. (*35*) and Kohen et al. (*36*) on the temperature dependence of isotope effects on methylamine dehydrogenase and a thermophilic alcohol dehydrogenase, respectively. Both present interpretations in support of vibrational motion driving catalysis, along with discussions concerning the inadequacy of classical and semiclassical transition-state theory to account for the experimental results.

[11] These studies have been extended to a second enzyme, formate dehydrogenase (Quirk and Northrop, unpublished results). The volume of activation for hydride transfer is again negative, as predicted, and the entire isotope effect again derives solely from a transition-state phenomenon, only this time the deuterium isotope effect *increases* with increasing pressure—an unprecedented finding that is incompatible with the vibrationally enhanced tunneling model of Bruno and Bialek (*41*; William J. Bruno, personal communication).

## REFERENCES


1. Northrop, D. B. (1996) in *High-Pressure Effects in Molecular Biology and Enzymology* (Markely, J. A., Royer, C. A., and Northrop, D. B., Eds.) pp 221−224, Oxford University Press, New York.
2. Northrop, D. B. (1999) *J. Am. Chem. Soc. 121*, 3521−3524.
3. Northrop, D. B. (1981) *Biochemistry 20*, 4056−4061.
4. Ray, W. J., Jr. (1983) *Biochemistry 22*, 4625−4637.
5. Laidler, K. J. (1988) *J. Chem. Educ. 65*, 250−254.
6. Laidler, K. J., and Bunting, P. S. (1973) *The Chemical Kinetics of Enzyme Action*, 2nd ed., pp 220−232, Clarendon Press: Oxford, England.
7. Parmentier, L. E., O'Leary, M. H., Schachman, H. K., and Cleland, W. W. (1992) *Biochemistry 31*, 6598−6602.
8. Monod, J., Wyman, J., and Changeux, J.-P. (1965) *J. Mol. Biol. 12*, 88−118.
9. Koshland, D. L., Jr., Nemethy, G., and Filmer, D. (1966) *Biochemistry 5*, 365−385.
10. Northrop, D. B. (1977) in *Isotope Effects on Enzyme-Catalyzed Reactions* (Cleland, W. W., O'Leary, M. H., and Northrop, D. B., Eds.) pp 122−152, University Park Press, Baltimore, MD.
11. Cha, Y., Murray, C. J., and Klinman, J. P. (1989) *Science 243*, 1325−1330.
12. Northrop, D. B. (1998) *J. Chem. Educ. 75*, 1153−1157.
13. Cho, Y.-K., and Northrop, D. B. (1999) *Biochemistry 38*, 7470−7475.
14. Rebholz, K. L., and Northrop, D. B. (1995) *Methods Enzymol. 249*, 211−240.
15. Cleland, W. W. (1977) in *Isotope Effects on Enzyme-Catalyzed Reactions* (Cleland, W. W., O'Leary, M. H., and Northrop, D. B., Eds.) Appendix B, University Park Press, Baltimore, MD.
16. Bell, R. P. (1980) *The Tunnel Effect in Chemistry*, Chapman and Hall, New York.
17. Duggleby, R. G. (1984) *Comput. Biol. Med. 14*, 447−455.
18. Huskey, W. P., and Schowen, R. L. (1983) *J. Am. Chem. Soc. 105*, 5704−5706.
19. Rucker, J., and Klinman, J. P. (1999) *J. Am. Chem. Soc. 121*, 1997−2006.
20. Melander, L., and Saunders, W. H., Jr. (1980) *Reaction Rates of Isotopic Molecules*, p 15, John Wiley & Sons, New York.
21. Grant, K. L, and Klinman, J. P. (1992) *Bioorg. Chem. 20*, 1−7.
22. Saunders, W. H., Jr. (1985) *J. Am. Chem Soc. 107*, 164−169.
23. Northrop, D. B., and Duggleby, R. G. (1990) *Bioorg. Chem. 18*, 435−439.
24. Warshel, A. (1998) *J. Biol. Chem. 273*, 27035−27038.
25. Robinson, C. R., and Sligar, S. G. (1995) *Methods Enzymol. 259*, 395−427.
26. Cannon, W. R., and Benkovic, S. J. (1998) *J. Biol. Chem. 273*, 26257−26260.
27. Isaacs, N. S. (1984) in *Isotope Effects in Organic Chemistry* (Buncel, E., and Lee, C. C., Eds.) Vol. 6, pp 67−105, Elsevier, Amsterdam.
28. Isaacs, N. S., Javaid, K., and Rannala, E. (1978) *J. Chem. Soc., Perkin Trans. 2*, 709−711.
29. Antoniou, D., and Schwartz, S. D. (1997) *Proc. Natl. Acad. Sci. U.S.A. 94*, 12360−12365.
30. Kraut, J. (1988) *Science 242*, 533−540.
31. Lowry, T. H., and Richardson, K. S. (1987) *Mechanism and Theory in Organic Chemistry* (3rd ed.), pp 209−210, Harper & Row, New York.
32. Lumry, R. (1959) *Enzymes*, *2nd Ed. 1*, 157−231.
33. Moelwyn-Hughes, E. O. (1950) *Enzymes 1*, 28−78.
34. Bahnson, B. J., and Klinman, J. P. (1995) *Methods Enzymol. 249*, 373−397.
35. Basran, J., Sutcliffe, M. J., and Scrutton, N. S. (1999) *Biochemistry 38*, 3218−3222.
36. Kohen, A., Cannio, R., Bartolucci, S., and Klinman, J. P. (1999) *Nature 399*, 496−499.
37. Lumry, R. (1995) *Methods Enzymol. 259*, 628−720.
38. Gregory, B. B. (1995) *Protein−Solvent Interactions*, Marcel Dekker, New York.
39. Lumry, R. (1991) in *A Study of Enzymes Vol. 2: Mechanism of Enzyme Action* (Kuby, S. A., Ed.) pp 3−81, CRC Press, Boca Raton, FL.
40. Welch, G. R. (1986) *The Fluctuating Enzyme*, John Wiley & Sons, New York.
41. Bruno, W. J., and Bialek, W. (1992) *Biophys. J. 63*, 689−699.


BI992537Z