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A Compelling Experimental Test of the Hypothesis That Enzymes Have Evolved To Enhance Quantum Mechanical Tunneling in Hydrogen Transfer Reactions: The β-Neopentylcobalamin System Combined with Prior Adocobalamin Data

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An intriguing but controversial hypothesis has appeared that "The optimization of enzyme catalysis may entail the evolutionary implementation of chemical strategies that increase the probability of tunneling and thereby accelerate the reaction rate" (Kohen, A.; Klinman, J. P. *Acc. Chem. Res.* **1998**, *31*, 397). Restated, enzymes *may* have evolved to enhance quantum mechanical tunneling by coupling to protein low ν modes that squeeze the reacting centers together in, for example, their $H^{\bullet}$ atom abstraction reactions. Such a putative "protein squeezing" mechanism would enhance hydrogen quantum mechanical tunneling by reducing the barrier width. An alternative hypothesis is that enzymes do not enhance tunneling, but simply exploit the same amount of tunneling present in their enzyme-free solution reactions, if those reactions occur. A third, conceivable hypothesis is that enzymes might even inadvertently decrease the amount of tunneling as an undesired result of increasing the barrier width while reducing the barrier height. Testing these hypotheses *experimentally* requires the extremely rare event of being able to measure the amount of tunneling both in the enzyme system *and* in a very similar if not identical reaction in enzyme-free solution. This has been accomplished experimentally in only one prior case, our recent study of AdoCbl (coenzyme B_{12}) and 8-Meo-AdoCbl undergoing enzyme-like $H^{\bullet}$ abstraction reactions (Doll, K. M.; Bender, B. R.; Finke, R. G. to *J. Am. Chem. Soc.* **2003**, in press). The data there reveal *no change* in the level of tunneling within or outside of the enzyme in comparison to the best literature data for an AdoCbl-dependent enzyme, methylmalonyl-CoA mutase. However, that first system suffers from two limitations: the measurement of the KIE (kinetic isotope effect) data in a nonenzymic 80−110 °C temperature range; and lower precision data than desired due to the HPLC−MS method required for one of the KIE analyses. These limitations have now been overcome by the synthesis, then thermolysis and KIE study vs temperature of the $H^{\bullet}$ abstraction reaction of β-neopentylcobalamin (β-NpCbl) in ethylene glycol-d_0 and ethylene glycol-d_4. This is the first experimental test of Klinman's hypothesis using KIE data obtained *at enzyme-relevant temperatures*. The key data obtained are as follows: deuterium KIEs of 23.1 ± 3.0 at 40 °C to 39.0 ± 2.3 at 10 °C; an activation energy difference $E_D - E_H$ of 3.1 ± 0.3 kcal mol^{-1}; and a pre-exponential factor ratio A_H/A_D of 0.14 ± 0.07. Moreover, our now three sets of data (NpCbl; AdoCbl; 8-MeOAdoCbl) are shown to lie on the same ln KIE vs $1/T$ linear plot yielding a set of enzyme-temperature-relevant, high-precision KIE, $E_D - E_H$, and A_H/A_D data over a relatively large, 110 °C temperature range. Significantly, the enzyme-free solution KIE, $E_D - E_H$, and A_H/A_D are identical within experimental error to those for methylmalonyl-CoA mutase. This finding leads to the conclusion that there is *no* enzymic enhancement of the tunneling in at least this B_{12}-dependent enzyme. This B_{12} enzyme does, however, exploit the same (unchanged) level of tunneling measured for the nonenzymic, $Ado^{\bullet}$ solution $H^{\bullet}$ abstraction reaction. A discussion is presented of the still open question of if this first experimental finding, of "no enzymic enhancement of tunneling" in one B_{12}-dependent enzymic system, is likely to prove more general or not.

Introduction

There is considerable interest in the study and understanding of quantum mechanical (QM) tunneling in enzymatic hydrogen transfer reactions[1−20] including in AdoCbl- (coenzyme B_{12}-) dependent reactions.[21,22] An intriguing but controversial[1,23−28] hypothesis is that "The optimization of enzyme catalysis may entail the evolutionary implementa-

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tion of chemical strategies that increase the probability of tunneling and thereby accelerate the reaction rate".[2] Restated, enzymes may have evolved to enhance QM tunneling in, for example, the H•-atom abstraction reactions catalyzed by enzymes such as the AdoCbl-dependent enzymes.[21,29−34] This hypothesis is often cited as reasonable[1,16,20,35−37] since enzymes have many low-frequency motions[38−47] that might well couple to a R•···•H reaction coordinate, making the R•···•H distance smaller, thereby reducing the barrier width and enhancing tunneling.[1,20,36,37]

The criteria often used to identify QM tunneling in hydrogen-transfer reactions were established by Kreevoy[48,49] in 1992. The three Kreevoy criteria diagnostic of tunneling are (i) a deuterium kinetic isotope effect (k_H/k_D; KIE) significantly larger than the ground-state zero-point energy (GS-ZPE) KIE maximum (6.4 at 20 °C, or 8.9 at 20 °C if secondary isotope effects are included); (ii) an activation energy difference ($E_D - E_H$) greater than 1.2 kcal mol^{-1}; and (iii) a ratio of pre-exponential factors (A_H/A_D) less than 0.7. The first criterion simply requires the measurement of the KIE while the second two criteria can be obtained from the measurement of the isotope effect as a function of temperature and, then, an Arrhenius plot of ln KIE vs $1/T$. The value $E_D - E_H$ is obtained from the slope while A_H/A_D is obtained from the intercept (see section S-1 in the Supporting Information). Although hydrogen tunneling is of course a quantum mechanical phenomenon better treated by crossing anharmonic potentials, the so-called semiclassical treatment behind the Kreevoy criteria (i.e., transition-state theory patched up by the addition of tunneling) is what has been used historically to quantitate tunneling in the prior enzyme literature; hence, by necessity the Kreevoy criteria are also employed herein. We will return to the more rigorous treatment of hydrogen quantum mechanical tunneling at the end of this paper, notably important work in a series of papers by Siebrand and co-workers (vide infra).

The three Kreevoy criteria have been used successfully by Klinman[7,8,15] in eight different enzyme systems. In addition, there is strong experimental evidence for QM tunneling in four AdoCbl-dependent enzymes, diol-dehydratase,[29−32] ethanolamine ammonia lyase,[33,34] glutamate mutase,[50−53] and methylmalonyl-CoA mutase.[21] The most definitive results are in a report by Banerjee and co-workers[21] studying QM tunneling in methylmalonyl CoA mutase (MMCo-A)[21] by Kreevoy's criteria.[48,49] Their key results are a KIE of 35.6 at 20 °C, an activation energy difference of ~3.4 kcal mol^{-1}, and a pre-exponential factor ratio less than 0.08 (row 4 in Table 1, vide infra). However, the question remains as to whether or not B_{12}-dependent or other enzymes have evolved to enhance hydrogen-transfer QM tunneling.

Upon reflection, we and others[8,28,54] realized that a simple yet definitive test of Klinman's intriguing hypothesis (vide supra) can be accomplished[22,28,55] experimentally *only* by a comparison of the degree of tunneling in a given enzymic system to the amount of tunneling for that same reaction in

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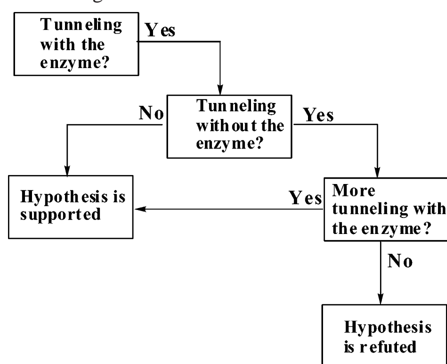
(55) Computational tests of Klinman's hypothesis provide conflicting results: the Paneth[22] group reports that an Arg residue in the enzyme methylmalonyl-CoA mutase is necessary to simulate the observed KIE data, whereas the Karplus[28] group finds that there is no significant tunneling enhancement in the triosephosphate isomerase system when gas, solution, or enzyme systems are simulated. This is one reason an *experimental test* such as the present work was needed and, hence, undertaken.

Table 1. The Observed KIE, Activation Energy Difference, and Pre-Exponential Factor Ratio for the β-NpCbl System, for the Previous Enzyme-Free Systems (AdoCbl and 8-MeOAdoCbl), for the Composite β-NpCbl, AdoCbl, and 8-MeOAdoCbl Data, and for Literature AdoCbl-Dependent Enzyme Systems[a]

	KIE	$E_D - E_H$ (kcal mol^{-1})	A_H/A_D
NpCbl in soln	35.2 ± 1.8 at 19 °C 39.0 ± 2.3 at 10 °C	3.1 ± 0.3	0.14 ± 0.07
AdoCbl in soln[b]	12.4 ± 1.1 at 80 °C ~29.3 at 20 °C[c] ~35.2 at 10 °C[c]	3.0 ± 0.3	0.16 ± 0.07
8-MeOAdoCbl in soln[b]	12.5 ± 0.9 at 80 °C ~21.8 at 20 °C[c] ~24.8 at 10 °C[c]	2.1 ± 0.6[d]	0.5 ± 0.4[d]
composite NpCbl, AdoCbl, and 8-MeOAdoCbl data	**12.4 ± 1.1 at 80 °C** **29 ± 5 at 20 °C** **38 ± 7 at 10 °C**	**3.15 ± 0.08**	**0.13 ± 0.02**
methylmalonyl-CoA mutase[e]	**12.7 ± 2.3 at 80 °C**[f] **35.6 ± 2.4 at 20 °C**[f] **44.2 ± 2.1 at 10 °C**[f]	3.41 ± 0.07 **(3.54 ± 0.19)**[g]	0.078 ± 0.009 **(0.082 ± 0.028)**[g]
ethanolamine ammonia lyase[h]	>10	(3.1 ± 1.1)[i]	(0.038 ± 2.13)[i]
glutamate mutase[j]	28−35 at 10 °C	NA[k]	NA
diol dehydratase[l]	8 and 28.6 at 10 °C	NA	NA
GS-ZPE 1° only	6.4 at 20 °C 6.8 at 10 °C	1.2	1.0
GS-ZPE 1° × 2°	8.9 at 20 °C[m] 9.5 at 10 °C[m]	1.2	1.0

[a] Error bars are shown at 1σ standard deviation. [b] Reference 56. [c] Values are extrapolated from the higher temperature data. [d] The larger error bars for this 8-MeOAdoCbl data set are due to the intrinsically larger errors of the ion-trap HPLC−MS method used to analyze the products.[62,63] [e] Reference 21. [f] This 80 °C value was obtained by extrapolation from the lower temperature data set. Error bars are from our linear regression of this literature ln KIE vs 1/*T* data as detailed in the Supporting Information elsewhere.[56] [g] These are our linear-regression analyses of the literature data set.[21] See the Supporting Information elsewhere[56] for further information (e.g., for why the recalculated error bars shown are in fact the correct ones). [h] References 33 and 34. [i] These numbers were calculated from the data in the literature.[34] See the Supporting Information elsewhere[56] for further information. [j] References 50 and 53. [k] NA = not available in the literature. [l] References 30, 54, 86, and 87. [m] The GS-ZPE 1° and 2° KIE = [(1° KIE) × 1.15 × 1.1^2].

Scheme 1. A Conceptually Simple Three-Step Procedure for Testing the Hypothesis[7] That Enzymes Have Evolved To Enhance Quantum-Mechanical Tunneling



the enzyme-free reaction, Scheme 1. However, the *problem* in applying the methodology in Scheme 1 lies in finding enzymatic reactions where the identical or nearly identical reaction can be studied *without* the enzyme.[8] Extremely slow rates, or reactions that simply do not occur when the enzyme is absent, are among the reasons why the ostensibly simple and definitive test in Scheme 1 has not been previously applied *experimentally* save in one recent case.[56] The one exception is our recent experimental test[56] of the enzyme-enhanced tunneling hypothesis using AdoCbl-dependent H• abstraction reactions from ethylene glycol.[57−60] Specifically, AdoCbl and, separately, 8-MeOAdoCbl were thermolyzed in a mixture of ethylene glycol-d_0 and deuterated ethylene glycol-d_4 at temperatures from 80 to 120 °C. This allowed us to measure Kreevoy's three criteria[48,49] diagnostic of QM tunneling using HPLC in the AdoCbl system and using HPLC−MS in the 8-MeOAdoCbl system. The results indicated *no enhancement* of QM tunneling in Ado•-mediated H• abstraction reactions in solution in comparison to Banerjee's enzymic-Ado•-mediated H• abstraction reactions.[56] However, because our solution reaction has a measurable rate only from 80 to 120 °C, the KIEs measured in our report had to be *extrapolated* to lower temperatures for comparison to the enzymic KIEs measured between 5 and 40 °C. This is a potential source of error since the ln KIE vs 1/*T* plots are not truly linear, at least over a large temperature ranges in systems which display tunneling.[48,61] Another problem is that the *HPLC*−MS method required to obtain the KIE data did not provide data as precise as needed to compellingly rule out any enzymic enhancement of tunneling, a difficulty which has been reported by others in the literature.[62,63] A third issue unanswered in the Ado•-based system is whether simple alkyl radicals, R•, display a different level of tunneling vs the biological Ado•: that is, has Ado• been selected by evolution due to any special tunneling ability in its H•-atom abstraction reactions?

Herein we overcome the primary limitations of the AdoCbl system[56] with a study of β-neopentylcobalamin (β-NpCbl), Figure 1, in combination with our prior data sets for AdoCbl and 8-MeoAdoCbl. The β-NpCbl system is special

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(57) The enzymic and non enzyme B_{12} dependent reactions compared herein, as well as in our prior report,[56] are very similar but not absolutely identical. The three Kreevoy criteria for the enzyme-free, solution reaction of Ado• abstracting a H• from ethylene glycol (the solution version of the AdoCbl-dependent diol dehydratase reaction) has been compared to the Ado• abstraction of a H• from the methyl group of methylmalonyl-CoA, the AdoCbl-dependent methylmalonyl-CoA mutase, enzyme-catalyzed system. *However*, the literature for H• abstractions makes it clear that little difference is expected in the KIEs for such reactions of similar bond energy C−H bonds of 91.1−95 kcal/mol in ethylene glycol[58,60] and estimated 92−101 kcal/mol in methylmalonyl-CoA.[59,60] Convincing *experimental support* for the validity of comparing these H• abstractions is found in the fact that all three enzyme-free systems studied, NpCbl, AdoCbl, and 8-MeOCbl, give results *identical within experimental error to the AdoCbl-dependent methylmalonyl-CoA mutase system;* that is, the results themselves strongly support, ex post facto, the comparison of the slightly different enzyme-based and enzyme-free solution systems.
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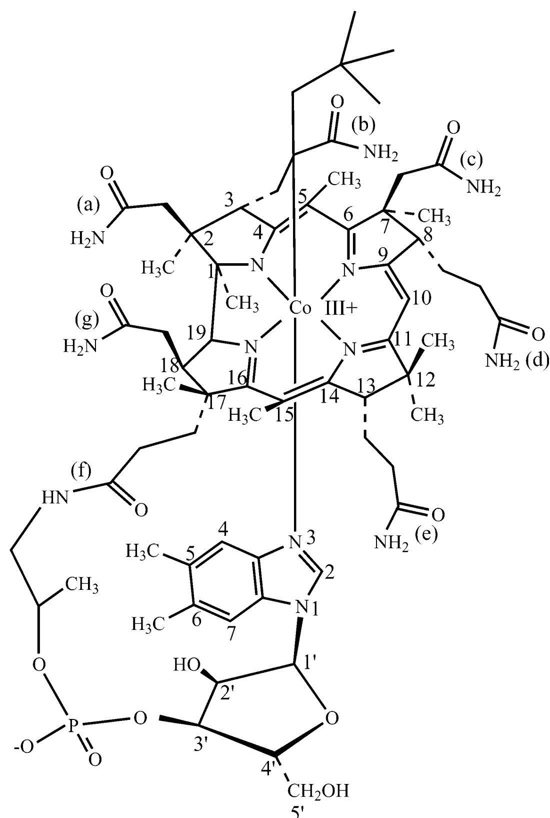
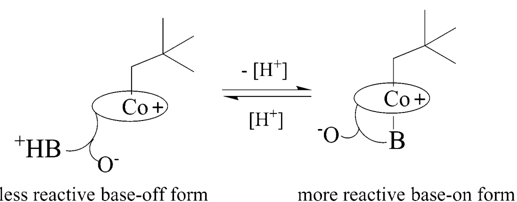


Figure 1. The structure of β-neopentylcobalamin (β-NpCbl) including the IUPAC numbering system of the corrin ring.

in that (i) its thermolysis has a measurable rate at temperatures from 10 to 40 °C, thereby allowing us to compare directly KIEs with Banerjee's precise enzyme values[21] measured in a similar temperature range (5−40 °C); and (ii) the neopentane (Np−H[D]) product can be followed by *GC*−MS, which should provide data of higher precision (β-NpCbl thermolysis and Co−C bond scission produces $Co^{II}Cbl^{\bullet}$ and the neopentyl radical ($Np^{\bullet}$); the $Np^{\bullet}$ then abstracts a $H^{\bullet}$ atom from the ethylene glycol-d_0 or -d_4 substrate producing Np−H(D)). An additional special feature of the β-NpCbl system is that (iii) it allows us to test whether a simple alkyl radical, $Np^{\bullet}$, displays tunneling similar to that observed for the electronically different, more electron deficient $Ado^{\bullet}$ radical.[64−66]

Overall, the β-NpCbl system examined herein is the first experimental test of the enhanced-tunneling hypothesis with enzyme-free data obtained at enzyme-relevant temperatures. The results of combining the NpCbl data with our earlier data sets for AdoCbl and 8-MeOAdoCbl are of considerable significance in that the enhanced-tunneling hypothesis is refuted, at least for the AdoCbl-dependent methylmalonyl-CoA mutase enzyme used in the comparison:[21] QM tunneling of this enzyme's $H^{\bullet}$ abstraction reactions *is not* enhanced within relatively small error bar limits. Instead, this enzyme simply exploits the existing, *unchanged* level of QM tunneling that we detect in the analogous enzyme-free, solution $H^{\bullet}$-atom abstraction reaction.

Scheme 2. Reaction Scheme for the Equilibrium of the Less Reactive, Base-off β-NpCbl·H^+Cl^- to the More Reactive, Base-on β-NpCbl, Which Then Undergoes Homolysis To Produce $Np^{\bullet}$ and $Co^{II}Cbl^{\bullet}$



Results and Discussion

Synthesis of (β-NpCbl·H^+Cl^-). β-Neopentylcobalamin hydrochloride (β-NpCbl·H^+Cl^-) was synthesized from desalted hydroxocobalamin according to the literature procedure,[67−70] with two precautions. Freshly prepared neopentyl iodide[71] was synthesized specifically to avoid MeI impurities often present in commercial neopentyl iodide.[72−74] The use of neopentyl bromide was also avoided due to its potential to make the α-NpCbl isomer (where the neopentyl group is on the opposite side of the corrin ring).[75−77] The β-NpCbl·H^+Cl^- product was isolated in its stable, base-off form, a synthetically useful, isolable form since it undergoes Co−C bond homolysis at least 100 times slower that the deprotonated, base-on form, Scheme 2.[78]

Thermolysis of β-NpCbl in Ethylene Glycol-d_0 and -d_4. Solutions of β-NpCbl ($\sim 10^{-4}$ M) in a 10% ethylene glycol/ 90% ethylene glycol-d_4 buffered with phosphate (0.02 M NaH_2PO_4, 0.03 M Na_2HPO_4, calculated pH 7.4) were thermolyzed at temperatures ranging from 10 to 40 °C in airtight Schlenk cells. The purpose of the phosphate buffer is to deprotonate the NpCbl·H^+Cl^- (its pK_a is between 4.55 and 5.18),[67,70,79,80] thereby shifting the base-off/base-on equilibrium to the ca. 100-fold more reactive base-on form, Scheme 2.

The Co−C homolysis produces $Co^{II}Cbl^{\bullet}$ (monitored by UV−visible spectroscopy) and $Np^{\bullet}$ (monitored indirectly as Np−H(D) by GC−MS, vide infra). The reactions following Co−C homolysis in β-NpCbl proceed as indicated in Scheme

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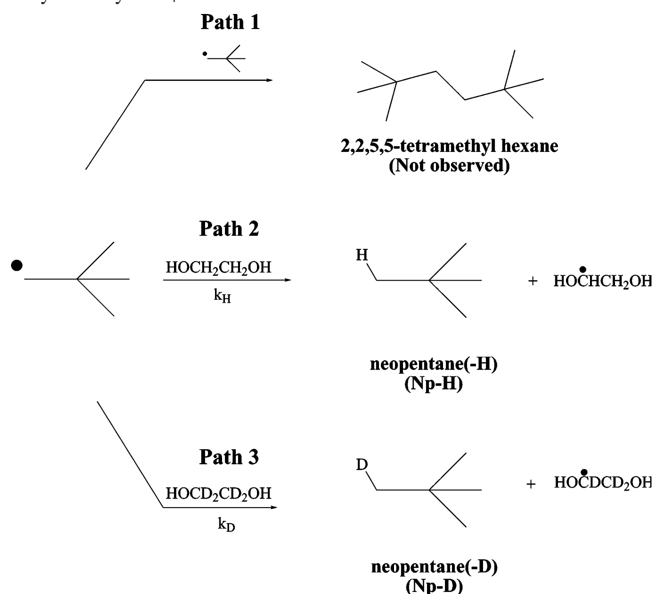
Scheme 3. The Reactions of Np• in a Solution of Ethylene Glycol and Ethylene Glycol-d_4



3 (the rate laws for the appearance of products and the disappearance of β-NpCbl are given in the Supporting Information, section S-2). A control experiment in the presence of the radical trap TEMPO gave results identical to those published earlier (Figure S1 in the Supporting Information).[67] There is no detectable Np• dimerization product, Np−Np, from path 1 (detection limit $\sim 10^{-8}$ M), as the concentration of Np• is never sufficient to allow this bimolecular pathway to effectively compete with the hydrogen/deuterium abstraction reactions shown in paths 2 and 3.

Because ionized Np−H and Np−D are not directly detectable[81,82] by GC−MS, we detected the Me• loss fragment peaks at $m/z = 57$, corresponding to $[(CH_3)_3C]^{\bullet+}$, and at $m/z = 58$, corresponding to $[(CH_3)_2(CH_2D)C]^{\bullet+}$ (see Scheme S1, Supporting Information). The KIE was calculated from these two observable $m/z = 57$ and $m/z = 58$ peaks using eq 1.

$$\mathrm{KIE} = \frac{k_H}{k_D} = \frac{\dfrac{100 - (m/z\text{: } 58 \text{ peak} - 4.6 \times 0.33)}{0.10}}{\dfrac{(m/z\text{: } 58 \text{ peak} - 4.6 \times 1.33)}{0.90}} \quad (1)$$

Note that this equation includes the necessary correction to account for some loss of the deuterium label during the Me• fragmentation (a derivation of this unexceptional equation is available in section S-2 of the Supporting Information.)

As a control, the use of ethylene glycol-d_6 instead of ethylene glycol-d_4 was also performed with no significant changes in the results (KIE = 21.8 at 40 °C). This experiment shows that the hydrogen abstracted is, as anticipated, from a C−H(D) bond of ethylene glycol and not from its O−H(D) bond.[84,85] It also rules out the hypothesis that the Np• is abstracting a hydrogen from the exchangeable hydrogens in the phosphate buffer.

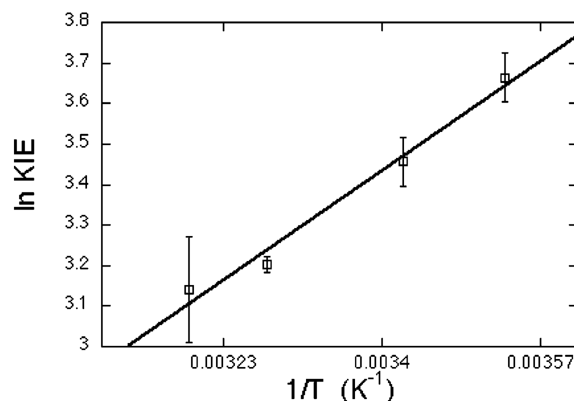


Figure 2. A plot of ln KIE vs $1/T$ for the thermolysis and H•(D•) abstraction reactions of β-NpCbl. From the slope and intercept, the activation energy difference and the preexponential factor ratio given in the text were calculated.

KIE vs Temperature Results: The Three Kreevoy Criteria. The results yield a large KIE for the hydrogen abstraction of 23 (40 °C) to 39 (10 °C) from the plot of KIE vs temperature, Figure S2 in the Supporting Information. As in the AdoCbl and 8-MeoAdoCbl systems,[56] the KIEs measured in the β-NpCbl system are ~4-fold larger than the predicted maximum GS-ZPE (ground-state zero-point energy) KIEs of 7.9 at 40 °C or 9.5 at 10 °C. (These GS-ZPE maximum KIEs were calculated using a version of the Bigeleisen equation[74] $k_H/k_D \approx e^{[hc(\nu_{CH} - \nu_{CD})]/2k_BT}$, and they include estimated secondary KIEs of 1.15 and 1.1. A C−H stretching frequency of 2891 cm^{-1} and a C−D stretching frequency of 2137 cm^{-1} were used in the calculation.[83]) A plot of ln KIE vs $1/T$ was made, Figure 2, and an activation energy difference ($E_D - E_H$) of 3.0 ± 0.3 kcal mol^{-1} and a ratio of pre-exponential factors (A_H/A_D) of 0.14 ± 0.07 were obtained from the slope and the intercept, respectively.

The values of the KIE (28−39), the activation energy difference ($[E_D - E_H] = 3.1 \pm 0.3$ kcal mol^{-1}), and the pre-exponential factor ratio ($A_H/A_D = 0.14 \pm 0.07$) all signify tunneling by Kreevoy's[48] criteria. A plot of the KIE vs temperature of the three Np•, Ado•, and 8-MeOAdo• data sets on the same graph, Figure 3, strongly suggests that all the data lie on approximately the same curve. Indeed, a comparison of the individual KIE, $[E_D - E_H]$, and A_H/A_D data reveals that these three radicals *display the same tunneling parameters within experimental error*, Table 1. Plotting the ln KIE vs $1/T$ for all three cobalamins, Figure 4, gives a plot that is linear within experimental error over a 110 °C temperature range. A rather precise activation energy difference of 3.15 ± 0.08 kcal mol^{-1} and pre-

(81) We attempted to measure the molecular ions directly by lowering the energy of the electron impact filament, but were not successful as expected since ionized, unfragmented neopentane$^{+\bullet}$ is notoriously difficult to detect.[82]

(82) Silverstein, R. M.; Bassler, G. C.; Morrill, T. C. *Spectrometric Identification of Organic Compounds*, 4th ed.; Wiley and Sons: New York, 1981.

(83) Frei, H.; Ha, T.-K.; Meyer, R.; Guenthard, H. H. *Chem. Phys.* **1977**, *25*, 271.

(84) The H• (or D•) abstraction from the C−H(D) and not the O−H(D) is as expected from Ingold and co-worker's literature: they demonstrated that the abstraction reaction of hydrogen from MeOH and MeOD by methyl radical proceeds at the same rate at 77 K, that is, that C−H abstraction is occurring.[85]

(85) Doba, T.; Ingold, K. U.; Siebrand, W.; Wildman, T. A. *Faraday Discuss. Chem. Soc.* **1984**, *78*, 175.

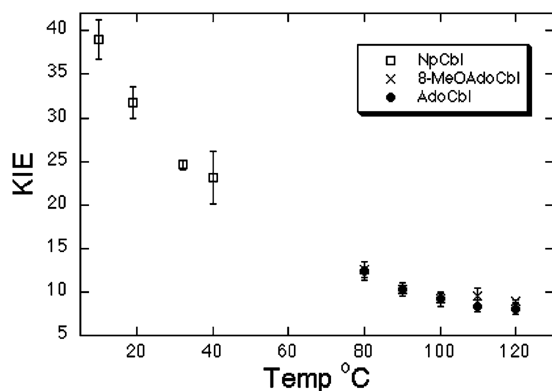


Figure 3. A plot of the observed kinetic isotope effects (KIEs) of the hydrogen abstraction from ethylene glycol vs temperature from this work, Np• (□), and from previous work,[56] Ado• (●) and 8-MeOAdo• (×). The data used to generate this plot is provided in Table S1 of the Supporting Information.

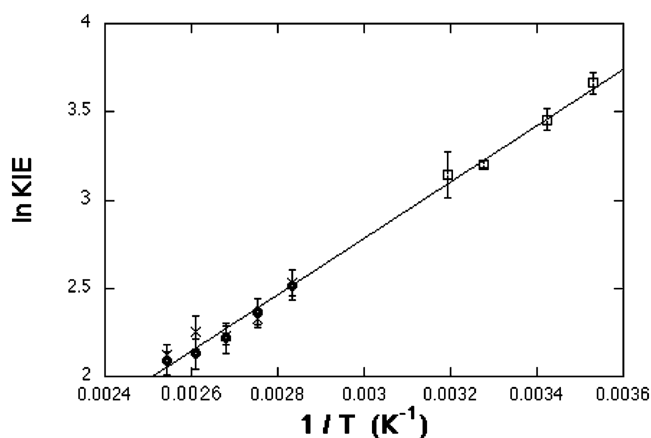


Figure 4. A plot of ln KIE vs $1/T$ using the thermolysis data for all three cobalamin systems, Np• (□), and from previous work,[56] Ado• (●) and 8-MeOAdo• (×). From the slope and intercept, the activation energy difference and the pre-exponential factor ratio given in the text were calculated. The data used to generate this plot is provided in Table S2 of the Supporting Information.

exponential factor ratio of $(A_H/A_D) = 0.13 \pm 0.02$ were calculated using the slope and intercept of this plot as listed in entry 4 in Table 1. The comparison between the composite enzyme-free data and that for methylmalonylCoA mutase, Table 1 entries 4 vs 5 (in bold), *shows no difference in the level of tunneling due to the presence of the enzyme* as measured by the three Kreevoy criteria and within a *relatively* low level of error in both the enzyme-free and enzyme data sets, $\Delta KIE = 6.6 \pm 5.5$ (20 °C), $\Delta\Delta(E_D - E_H) = 0.39 \pm 0.20$ and $\Delta(A_H/A_D) = 0.048 \pm 0.034$ (1σ error bars). The data in Table 1 provide, therefore, a rather stringent test of the enzyme-enhanced tunneling hypothesis. The lack of any detectable difference in the level of tunneling between the simple Np• and the somewhat electron deficient Ado• is also noteworthy.

Conclusions

Herein we have shown (i) that β-NpCbl is a near ideal, low-temperature Co−C thermolysis system (10−40 °C) which allows the acquisition, for the first time, of data in the same temperature range where the KIEs for the B_{12}-dependent methylmalonyl-CoA mutase enzyme system have been measured; (ii) that the resultant GC−MS KIE vs temperature data are of precision that is as good as or better than the precision we obtained previously for the AdoCbl and 8-MeO-AdoCbl systems, respectively;[56] and (iii) that each of the three H• abstraction systems Np•, Ado•, and 8-MeO-Ado• yields identical results within experimental error, thereby strongly suggesting that there is nothing special about—and, specifically there is no enhancement of tunneling by—Ado• over the simple alkyl radical, Np•. Hence, there is no evidence for any evolutionary selection of Ado• due to some special tunneling prowess. Moreover, (iv) we have k_H/k_D isotope effect data over a *110 °C temperature range* for the combination of our three sets of enzyme-free tunneling data, results which allow the calculation of relatively precise $E_D - E_H = 3.15 \pm 0.08$ kcal mol^{-1} and $A_H/A_D = 0.13 \pm 0.02$. Such tunneling data over such a large temperature range have been reported in only a handful of cases.[86,87] Tunneling aficionados will wish to study the experimental work of Ingold[86] and the pioneering theoretical work of Siebrand and co-workers;[87−90] the latter treats the best tunneling data in the literature properly as the quantum mechanical phenomenon it is[87−90] (i.e., rather than patching up transition-state theory by adding on tunneling[48,49,61]). Collaborative efforts are underway to treat the data herein by Professor Siebrand's methods, since only when theory and experiment match can one claim to understand completely reactions involving H tunneling.[90] Finally and most importantly, our results show (v) that within the stated error bars, a direct comparison of the enzyme-free and RCbl (R = Ado, 8-MeOAdo, and Np) systems to the AdoCbl-dependent enzymic data of Banerjee and co-workers *reveals no statistically significant increase in any of the three Kreevoy criteria for tunneling*. This study—the first experimental test of the enzyme-enhanced tunneling hypothesis at enzyme relevant temperatures[59]—strongly suggests that at least B_{12}-dependent methylmalonyl-CoA mutase *has not evolved to enhance QM tunneling in its H• abstraction reactions*, at least in the Occans's Razor interpretation of our data. The enzyme does, however, exploit within experimental error the exact same level of QM tunneling available in the non-enzymatic solution reaction. It remains of interest to try to obtain the Kreevoy criteria for the enzyme B_{12} dependent diol dehydratase[91,92] so that an exact comparison

(86) Brunton, G.; Griller, D.; Barclay, L. R. C.; Ingold, K. U. *J. Am. Chem. Soc.* **1976**, *98*, 6803. This is a classic piece of work that anyone interested in H tunneling will wish to read.
(87) Siebrand, W.; Wildman, T. A.; Zgierski, *J. Am. Chem. Soc.* **1984**, *106*, 4083.
(88) Siebrand, W.; Wildman, T. A.; Zgierski, *J. Am. Chem. Soc.* **1984**, *106*, 4089.
(89) Doba, T.; Ingold, K. U.; Lusztyk, J.; Siebrand, W.; Wildman, T. A. *J. Chem. Phys.* **1993**, *98*, 2962.
(90) (a) Smedarchina, Z.; Siebrand, W.; Fernández-Ramos, A.; Cui, Q. *J. Am. Chem. Soc.* **2003**, *125*, 243. This valuable paper also makes it rather clear that only the proper quantum mechanical treatment of KIEs, and not GS-ZPE corrected transition-state theory, can yield reliable KIEs for comparison to experiment. (b) Siebrand, W., Doll, K. M., Finke, R. G. manuscript in preparation.
(91) Bachovchin, W. W.; Eagar, R. G., Jr.; Moore, K. W.; Richards, J. H. *Biochemistry* **1977**, *16*, 1082.
(92) Bachovchin, W. W.; Moore, K. W.; Richards, J. H. *Biochemistry* **1978**, *17*, 2218.

of enzymic data for the identical reaction[59] ($H^{\bullet}$ abstraction from ethylene glycol) can be made.

Is This "No Enzymic Enhancement of Tunneling" Result More General? It also remains to be seen if similar conclusions can be obtained for other enzymic systems and by the methodology detailed in Scheme 1: such data are certainly needed to verify or refute experimentally the possibly broader generality of the "no-enhancement of tunneling" finding uncovered herein. Such studies are certainly needed since, as discussed elsewhere,[56] little evolutionary pressure appears to have existed to enhance B_{12}-enzyme $H^{\bullet}$ abstraction reactions since the inherent rate of $H^{\bullet}$ abstraction ($\sim 10^3$ s^{-1}) is faster than the turnover rates (≤ 100 s^{-1}) of these relatively slow enzymes.[56] The reader is also referred to a discussion elsewhere[56,87−90] as to whether or not the experimentally derived, "no-enhancement of tunneling" conclusion is likely to prove more general. An important point here is Siebrand's *1984 finding*[88] that a low ν mode at the C−H (or O−H or N−H; i.e., X−H) bond from which the $H^{\bullet}$ abstraction occurs *does couple* to the tunneling mode and *is needed* to account for the observed tunneling data in a number of systems (i.e., to allow the resultant, energetic $C^{\bullet}$ (or $O^{\bullet}$ or $N^{\bullet}$) radical to relax). *However*, that $\sim$120−170 cm^{-1} mode is *largely localized* on the carbon (or O or N) center.[88] Treatment of the tunneling in the fast, yet rate-determining, multiple H^+ transfer involved in carbonic anhydrase reveals that low ν modes are again important and are needed to obtain agreement between measured and calculated KIEs; however, only those *low ν modes at the active site* were needed to reach agreement with experiment; *that is, no protein low ν modes were needed to account for the experimental results*.[90] Even the collective modes in a free-base porphyrin are unable to couple to the resultant radical center.[88] In fact, coupling low ν protein modes would lead to a large temperature dependence of the tunneling, at odds with the weak temperature dependence that is often observed.[93b] These computation results and insights would *seem* to make it likely in the *general* case that collective, low *ν protein* motions will not be able to couple to a H-tunneling reaction site. A recent, in press paper by Siebrand and Smedarchina[93] addresses further the protein-squeezing hypothesis and, in the end, argues strongly against it.[94]

Nevertheless, more experimental work with additional systems is needed to test the broader generality, *or limitations*, of the findings herein of "no enzyme-enhanced tunneling" for the one B_{12}-dependent enzyme examined. Note that our work also contains a clear prediction about what enzyme systems should be studied: ones with turnover frequencies greater than the inherent $H^{\bullet}$ abstraction rates of $\sim 10^3$ s^{-1} cited above[56] (i.e., with a typical $R^{\bullet}$ as the abstracting species), so that evolution would have had a reason to try to enhance the tunneling of that system. A general experimental problem in such studies (i.e., and with the otherwise conceptually simple approach in Scheme 1) promises to be the inability to obtain precise enough KIE vs temperature data sets, for both the enzymic and enzyme-free systems over large enough temperature ranges, to rule out *any* enzymic contribution to H-transfer tunneling within the desired, very small error bars. Indeed, we believe that it will prove difficult to better, in any future study or system, the relatively small (but still larger than desired) error bars reported for the ΔKIE, $\Delta\Delta(E_D - E_H)$ and $\Delta(A_H/A_D)$ data in the present study. For this reason, theoretical aproaches[90,93] promise to be of special significance in addressing the question of enzymic enhancement of H-tunneling. Significantly, theoretical analysis of the data reported herein by Siebrand's methods[90b] is in full support of the "no enzymic enhancement of tunneling" finding for the present B_{12} system.

Experimental Section

Materials. The following were used as received: neopentyl alcohol (Aldrich, 99%), sodium iodide (Aldrich, 99.999%), calcium hydride (Aldrich 90−95%), phenol (Acros, ACS grade), benzene (Aldrich, HPLC grade), hexane (Fisher Scientific, ACS grade), chloroform (Fisher Scientific, ACS grade), hydrochloric acid (Mallinckrodt, AR grade), ethyl ether (Fisher Scientific, ACS grade, anhydrous), pyridine (Aldrich, 99.8%, anhydrous), tosyl chloride (Aldrich, 99%), TEMPO (Aldrich 99% sublimed), methanol (Fisher Scientific, HPLC grade), argon (General Air), ethylene glycol-d_6 (Cambridge Isotope Labs, 98%), and 2,2,5,5-tetramethylhexane (Chemsampco). Diglyme (Aldrich, 99.5%, anhydrous) was stirred over CaH_2 for 12 h and then distilled under argon. The purities of ethylene glycol-d_0 (Aldrich, 99.8% anhydrous) and ethylene glycol-d_4 (Cambridge Isotope Labs, 98%) were confirmed by GC−MS; hence, these were used as received. Distilled water was filtered through a Barnstead nanopure filtration system.

Instrumentation and Equipment. UV−visible absorption spectra (±1 nm) were recorded on a Hewlett-Packard model 8452A UV−visible diode array spectrophotometer equipped with a thermoelectric Hewlett-Packard 89090A Peltier cell block temperature controller operating at 25.0 ± 0.1 °C. GC−MS was performed on an Agilent 5973N/6890 with a 30 m Agilent HP-5 column. All linear regressions were performed on a Power Macintosh 5400/120 using Microsoft Excel 98.

Thermolyses of β-$NpCbl{\cdot}H^+Cl^-$ were carried out in Schlenk cuvettes[95] prepared by glass blowing PTFE needle valves onto 1 cm path length cuvettes or to 1 mL glass vials. The cuvettes' ability to maintain an oxygen-free environment was tested with air-sensitive

(93) (a) Siebrand, W.; Smedarchina, Z. *J. Phys. Chem.,* submitted. We thank Dr. Siebrand for sharing a preprint of this paper. This paper looks critically at the experimental and theoretical "evidence" for the protein-enhanced tunneling (i.e., the "protein-squeezing") hypothesis.[93] Siebrand and Smedarchina find problems in both the experimental[94] and theoretical work supposedly supporting the protein-squeezing mechanism.[93] They conclude that "... the hypothetical protein squeezing mechanism leads to very short (physically unreasonable) distances combined with low anharmonicities, and is rejected on theoretical and experimental grounds".[93] They also argue that generally flexible proteins are "...ill-equipped to cause strong local compression".[93] They provide an alternative mechanism consisting of charge transfer along with a coupled H^+ transfer and detail how that enhances tunneling by shortening the H^+-transfer distance and increasing the tunneling mode anharmonicity, the two most important parameters in their tunneling model.[93] (b) Private communication from Dr. Siebrand.

(94) Knapp, M. J.; Rickert, K. W.; Klinman, J. P. *J. Am. Chem. Soc.* **2002**, 124, 3865. Siebrand and Smedarchina[93] show that there is no statistically valid difference in the data in this paper for mutant vs wild-type enzyme (due to the too narrow temperature range over which the data could be obtained), thereby negating the claimed support in this paper for the putative protein-squeezing mechanism.

(95) Hay, B. P.; Finke, R. G. *Polyhedron* **1988**, *7*, 1469.

$Co^{II}Cbl^{\bullet}$ (made from the photolysis of AdoCbl in ethylene glycol). No detectable $Co^{II}Cbl^{\bullet}$ decomposition was observed for any of the Schlenk cuvettes employed in this work over the time scale used for our thermolyses (~1 week).

Thermolysis temperatures were maintained by immersing the cuvettes in a VWR model 1166 thermostated water bath. The temperature of each reaction was verified (±0.2 °C) using a mercury thermometer with gradations in the temperature range of interest.

All samples were prepared in a Vacuum Atmospheres inert atmosphere drybox. An O_2 level of <2 ppm was monitored by a Vacuum Atmospheres model AO 316-C oxygen analyzer.

Alkylcobalamins are very photolabile; hence, all sample preparations done inside the drybox were shielded from light with aluminum foil. The thermolyses were carried out in a dark room with exposure only to photographic quality red light.

***β*-NpCbl·H$^+$Cl$^-$ Synthesis.** *β*-Neopentylcobalamin was synthesized as its HCl salt, *β*-NpCbl·H$^+$Cl-, by the literature method using freshly prepared neopentyl iodide,[67−69,71] yield 37% (literature yield 62% before drying). The product was characterized by UV−visible spectroscopy in buffered ethylene glycol [λ_{max} ($\epsilon \times 10^{-3}$ M cm) 388 nm (9500), 438 nm (8000), 486 nm (6900) within experimental error of literature values[79]]; by LSIMS [found $m/z = 1400.6$ (calcd $m/z = 1400.7$ for [NpCbl − H$^+$])]; and by ^{1}H NMR [δ 8.91 (s, 1H-B2), δ 7.49 (s, 1H-B7), δ 7.38 (s, 1H-B4), δ 6.97 (s, 1H-C-10), δ 6.55 (d, 1H-R1′), in unbuffered D_2O referenced to TSP-d_4], product purity ~90%, results all within experimental error of literature values.[79]

Neopentyl iodide (NpI) was prepared by refluxing neopentyl tosylate (NpOTs) with excess NaI in diglyme.[71] The reaction was followed by ^{1}H NMR until its completion, 155 min. Purification was accomplished by vacuum distillation yielding 20% NpI by ^{1}H NMR in diglyme solution with a purity of >95%. This synthetic route was chosen specifically to avoid the use of MeI, a common impurity in commercial NpI,[96] which, if present, results in the formation of a methylcobalamin (MeCbl) contamination in the final product, *β*-NpCbl·H$^+$Cl$^-$. It is also important to use NpI rather than NpBr to avoid the possible formation of the unwanted α-isomer, α-NpCbl·H$^+$Cl$^-$.[75−77]

Neopentyl tosylate (NpOTs) was synthesized by literature methods[97,98] from neopentyl alcohol and tosyl chloride in pyridine. It was recrystallized from a 17:1 mixture of hexanes/benzene at −15 °C, yield 81% (literature yield 87%);[97] purity >95% by ^{1}H NMR.

***β*-Neopentylcobalamin Thermolysis and Analysis Procedure.** The *β*-neopentylcobalamin thermolysis procedure is similar to our literature thermolysis procedure.[67] *β*-Neopentylcobalamin hydrochloride (~1.0 mg) was weighed out inside a foil-wrapped vial and brought into the drybox. It was dissolved in ethylene glycol buffer solution (0.02 M NaH_2PO_4, 0.03 M Na_2HPO_4, calculated pH 7.4), making a ~2×10^{-4} M *β*-NpCbl·H$^+$Cl$^-$ solution, which was transferred into foil-wrapped, airtight Schlenk cells.[95] The cells that were used in KIE calculations contained 90% ethylene glycol-d_4. The sealed cells were taken into the darkroom and thermolyzed in a temperature-controlled water bath (10.0, 19.0, 32.0, 40.0 °C) for at least 5 half-lives (142, 46, 8, and 2 h). One UV−visible Schlenk cuvette was monitored at each temperature; resultant thermolysis rates were within error of those reported previously.[67] Control experiments were also performed using ethylene glycol-d_6 at 20°C yielding a KIE of 32.8 and at 40 °C yielding a KIE of 20.8. Both of these values are within experimental error of the values measured in the ethylene glycol-d_4 system.

Gas headspace samples (10 μL) were taken from the Schlenk cells and injected into the GC−MS: injector temperature 200 °C; source temperature 160 °C; temperature program, 0 °C for 2.5 min, ramp to 25 °C at 5°C/min, then ramp to 175 °C at 20 °C/min and hold for 1 min until returning to 0 °C; electron energy 70 eV; mass detector set to read $m/z = 35-350$.

In an important control experiment, solutions of 2,2,5,5-tetramethylhexane in ethylene glycol were prepared, and a detection limit of 10^{-8} M was established, showing that if even 1% of the $Np^{\bullet}$ dimerized (path one in Scheme 3), it would have been detected.

Acknowledgment. It is a special pleasure to acknowledge several insightful discussions with Drs. Willem Siebrand and Keith Ingold. Financial support was provided initially by NIH Grant DK26214 and then more recently by PRF 34841-AC3.

Supporting Information Available: Figure S1, the UV−visible spectra of a *β*-NpCbl thermolysis reaction containing excess TEMPO; Figure S2, a plot of KIE vs temperature for the $Np^{\bullet}$ hydrogen-abstraction reactions; Figure S3, a GC−MS trace of a sample from a NpCbl thermolysis reaction; section S-1, a short derivation showing the use of a ln KIE vs 1/*T* plot to diagnose tunneling; section S-2, the kinetic rate laws and the derivation of an expression for the KIE; section S-3, derivation of eq 1; Scheme S1, the reaction scheme for the homolysis and subsequent hydrogen abstractions in ethylene glycol/ethylene glycol-d_4; Scheme S2, the fragmentation reactions of Np−H and Np−D in the mass spectrometer leading to the observed products; Table S1, data used to generate the plot in Figure 3; Table S2, data used to generate the plot in Figure 4. This material is available free of charge via the Internet at http://pubs.acs.org. This Supporting Information has also been published in the Ph.D. dissertation of K. M. Doll (Colorado State University, Spring 2003).

IC0300722

(96) Landauer, S. R.; Rydon, H. N. *J. Chem. Soc.* **1953**, 2224.
(97) Roberts, D. D.; Snyder, R. C., Jr. *J. Org. Chem.* **1980**, *45*, 4052.
(98) Tipson, R. S. *J. Org. Chem.* **1944**, *9*, 235.