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A Secondary Isotope Effect Study of Equine Serum Butyrylcholinesterase-Catalyzed Hydrolysis of Acetylthiocholine

Kenneth L. Wiley^{a,b}, Jose R. Tormos^{a,c}, and Daniel M. Quinn^{a,*}

^aDepartment of Chemistry, The University of Iowa, Iowa City, IA 52242, USA

Abstract

β -Secondary deuterium isotope effects have been measured for equine serum butyrylcholinesterase-catalyzed hydrolysis of acetyl-L₃-thiocholine (L = H or ²H). The dependencies of initial rates on isotopic substrate concentrations show close adherence to Michaelis-Menten kinetics, and yield the following isotope effects: $D^3k_{cat}/K_m = 0.98 \pm 0.02$ and $D^3k_{cat} = 1.10 \pm 0.02$. The modestly inverse isotope effect on k_{cat}/K_m is consistent with partial rate limitation by a step that converts the sp²-hybridized ester carbonyl of the E + A reactant state into a quasi-tetrahedral transition state in the acylation stage of catalysis. On the other hand, the markedly normal isotope effect on k_{cat} indicates that the Michaelis complex that accumulates at substrate saturation of the active site during catalytic turnover is a tetrahedral intermediate, whose decomposition is the rate-limiting step. These results compliment a previous report [J. R. Tormos *et al.*, *J. Am. Chem. Soc.* 127 (2005) 14538–14539] that showed that substrate-activated hydrolysis of acetylthiocholine, catalyzed by recombinant human butyrylcholinesterase, is also rate limited by decomposition of an accumulating tetrahedral intermediate.

Keywords

butyrylcholinesterase; catalytic mechanism; transition state structure; isotope effects

1. Introduction

The cholinesterases comprise a family of enzymes that are noted for their catalytic power [1,2]. Acetylcholinesterase (AChE) catalyzes the hydrolysis of acetylcholine and acetylthiocholine (ATCh) with rate constants that approach diffusion control at low substrate concentrations, and accelerates the hydrolysis of acetylcholine by 10¹³-fold. Though an order of magnitude slower than AChE, BuChE nonetheless effects catalytic turnover of ATCh and butyrylthiocholine (BuTCh) with admirable efficiency [3]. Elucidating the molecular origins of the high catalytic activity of cholinesterases is therefore an endeavor of fundamental importance. However, due to the very high catalytic activities of cholinesterases ($k_{cat} \sim 10^4 \text{ s}^{-1}$ for AChE-catalyzed hydrolysis of ATCh [1,2]; $k_{cat} \sim 10^3 \text{ s}^{-1}$ for BuChE-catalyzed hydrolysis of BuTCh [3]), characterization of the structural changes in the substrate that accompany catalytic turnover can be challenging.

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*To whom correspondence should be addressed. daniel-quinn@uiowa.edu; Phone: 1-319-335-1335; Fax: 1-319-335-1270.

^bCurrent address: FDA National Center for Toxicological Research, 3900 NCTR Road, Jefferson, AR 72079, USA

^cCurrent address: Department of Biochemistry, University of Texas Health Science Center, San Antonio, TX 78229, USA

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Kinetic secondary isotope effects provide probes of chemical reaction mechanisms, including those that occur in the active sites of enzymes, that report on structural changes as the substrate is converted from the reactant state to the transition state. Consider, for example, the conversion of acetylthiocholine to a tetrahedral intermediate (one of the several steps in acyl transfer reactions [4]) in the acylation stage of catalysis, as outlined in Figure 1. Nucleophilic attack of the active site serine on the substrate converts the carbonyl carbon from sp^2 hybridization in the reactant state to sp^3 hybridization in the tetrahedral intermediate. In the reactant state, the carbonyl C=O has a marked bond dipole that is δ^+ at the carbonyl carbon. This and the π -system of C=O allow for resonance delocalization of β -CL (L = H or ^2H) σ -electron density into the carbonyl system by hyperconjugation [5]. This electron delocalization decreases electron density in the β -CL bonds. In the tetrahedral intermediate the π -system is no longer available, hyperconjugation is not possible, and electron density is returned to β -CL bonds, which increases the strength (stiffness) of the bonds. The increased stiffness of β -CL bonds in the tetrahedral intermediate increases the curvature of the vibrational potential in the tetrahedral intermediate versus the reactant state, as shown in Figure 2. This renders the isotopic difference in β -CL zero point energies larger in the tetrahedral intermediate than in the reactant state, which in turn necessitates that $\Delta E^{\text{H}3} > \Delta E^{\text{D}3}$, and therefore $^{\text{D}3}\text{K} \equiv K_{\text{H}3}/K_{\text{D}3} = 0.8$. Correspondingly, the kinetic β -secondary deuterium isotope effect $^{\text{D}3}\text{k} \equiv k_{\text{H}3}/k_{\text{D}3}$ will be increasingly inverse (i.e. will more closely approach the equilibrium isotope effect $^{\text{D}3}\text{K} = 0.8$) the more the transition state resembles the tetrahedral intermediate. Therefore, inverse kinetic β -secondary deuterium isotope effects for acyl transfer reactions not only indicate that the sp^2 -hybridized reactant state is being converted to a sp^3 -hybridized tetrahedral intermediate, but also provide a measure of how far the reaction assembly has progressed from sp^2 to sp^3 hybridization in the transition state [5,6].

When the substrate in the reactant state is sp^3 hybridized and hybridization is changing toward sp^2 in the transition state, the situation is the reverse of that depicted in Figure 2. In this case the β -CL vibrational potential will be shallower in the transition state than in the reactant state, and the kinetic β -secondary deuterium isotope effect will be normal, i.e. $^{\text{D}3}\text{k} > 1.0$. Moreover, the upper limit for $^{\text{D}3}\text{k}$, i.e. that for equilibrium conversion of the sp^3 reactant state to the sp^2 product should be $1/0.8$, or about 1.25.

What are the expectations for kinetic β -secondary deuterium isotope effects for acyl transfer reactions, of which the elementary step shown in Figure 1 is a component? Most acyl transfer reactions proceed through addition-decomposition mechanisms, in which the nucleophile first attacks the susceptible carbonyl carbon to generate a tetrahedral intermediate, which breaks down with the expulsion of the leaving group to produce the products [4]. Tetrahedral intermediates in nonenzymic acyl transfer reactions are high-energy, steady-state intermediates, less stable than reactants or products by 40–100 kJ mol $^{-1}$ [4]. Therefore, according to the Hammond postulate [7] the transition states for formation and breakdown of the tetrahedral intermediate should resemble the intermediate both in energy and structure. In this situation, kinetic β -secondary deuterium isotope effects will always be inverse, no matter whether formation or breakdown of the intermediate is rate limiting, as illustrated in Figures 1 and 2.

Enzymes achieve their impressive catalytic power, as measured by the degree to which they accelerate erstwhile sluggish nonenzymic reactions, by lowering the energy of the transition state(s), which in turn lowers the free energy of activation [8]. Understanding how enzymes do this is the crux of understanding their catalytic function. A way in which cholinesterases, and other enzymes that catalyze acyl transfer reactions, may realize a substantial portion of their catalytic accelerations is to stabilize high-energy tetrahedral intermediates, which in turn will stabilize the transition states for their formation and decomposition. In this paper,

results of isotope effect experiments are described that show that equine serum BuChE stabilizes a tetrahedral intermediate to such an extent that the intermediate is an accumulating Michaelis complex (i.e. reactant state) during catalytic turnover. Results for equine serum BuChE are compared to those in the literature for human BuChE catalysis [9], and are discussed in light of observations of tetrahedral intermediates in x-ray structures of the enzyme [10].

2. Materials and Methods

Equine serum BuChE was obtained *gratis* from Dr. B. P. Doctor of the Department of Biochemistry of Walter Reed Army Institute of Research. Acetyl-²H-thiocholine (deuterated ATCh) was synthesized as previously described [6]. ATCh, DTNB and buffer salts, Na₂HPO₄ and NaH₂PO₄, were obtained from Sigma Chemical Co. (St. Louis, MO, USA). SigmaPlot 8.0 (Systat, Chicago, IL, USA) was used for graphical presentation and quantitative analysis of experimental data.

Initial rates of equine serum BuChE-catalyzed hydrolysis of ATCh were measured by the coupled colorimetric assay described by Ellman *et al.* [11]. Reactions were followed at 27 °C and pH = 7.25 in 0.1 M NaH₂PO₄/Na₂HPO₄ buffer that contained 0.5 mM DTNB as increases in absorbance at 412 nm on a 96-well SPECTRAMax Plus 384 microplate reading UV-visible spectrophotometer. Initial rates were measured in the presence and absence of BuChE, and the nonenzymic rate was subtracted from the total rate measured in the presence of enzyme to give the net enzyme reaction rate. The kinetic parameters V_{\max} and K_m were calculated by fitting data to the Michaelis-Menten equation:

$$V_i = \frac{V_{\max}[A]}{K_m + [A]} \quad (1)$$

When $[A] \ll K_m$, equation 1 reduces to $V_i = (V_{\max}/K_m)[A]$, and first-order kinetics are observed. In this situation reactions were followed for at least three half lives, and pseudo first-order rate constants were calculated by fitting absorbance versus time data to the following equation:

$$A = (A_0 - A_{\infty})e^{-kt} + A_{\infty} \quad (2)$$

In equation 2 A , A_0 and A_{∞} are absorbances at times t , 0 and infinity, respectively, and $k = V_{\max}/K_m$.

Because $V_{\max} = k_{\text{cat}}[E]_T$ and the same enzyme concentration was used to measure initial rates for both isotopic ATCh substrates, isotope effects on V_{\max} are isotope effects on k_{cat} . The nomenclature espoused by Northrop [12] and Cleland [13] for tabulating isotope effects is used herein. Thus, $^{D^3}k_{\text{cat}} = k_{\text{cat}}^{\text{H}^3}/k_{\text{cat}}^{\text{D}^3}$ is the kinetic β -secondary deuterium isotope effect on k_{cat} , which is measured as the ratio of the V_{\max} values of the isotopic substrates. The leading superscript denotes the heavy isotope utilized in the isotope effect determination. Similarly, $^{D^3}k_{\text{cat}}/K_m = (k_{\text{cat}}/K_m)^{\text{H}^3}/(k_{\text{cat}}/K_m)^{\text{D}^3}$, and is measured as the ratio of the V_{\max}/K_m values of the isotopic substrates.

3. Results

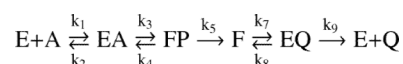
Initial rates were measured for equine serum BuChE-catalyzed hydrolysis of acetyl-³H-thiocholine and acetyl-²H₃-thiocholine as a function of substrate concentration, and the data are plotted in Figure 3. For each of the isotopic substrates, close adherence to Michaelis-

Menten kinetics is observed, as demonstrated by the nonlinear fits to the Michaelis-Menten equation (equation 1 above) in the figure. These fits provide the following results:

acetyl-¹H-thiocholine, $V_{\max}^{\text{H}^3} = 1.24 \pm 0.03 \times 10^{-7} \text{ M s}^{-1}$, $K_m^{\text{H}^3} = 1.2 \pm 0.1 \text{ mM}$; acetyl-²H-thiocholine, $V_{\max}^{\text{D}^3} = 1.14 \pm 0.01 \times 10^{-7} \text{ M s}^{-1}$, $K_m^{\text{D}^3} = 0.93 \pm 0.08 \text{ mM}$. Consequently, these fits provide a measure of the isotope effect on k_{cat} of reasonable precision, $^{D^3}k_{\text{cat}} = 1.10 \pm 0.02$. Additional isotope effects calculated from these data are $^{D^3}K_m = K_m^{\text{H}^3}/K_m^{\text{D}^3} = 1.3 \pm 0.1$ and $^{D^3}k_{\text{cat}}/K_m = 0.84 \pm 0.10$. The sizeable uncertainty in $^{D^3}k_{\text{cat}}/K_m$ suggests that one should not view the value of the isotope effect as reliable. To measure this isotope effect with much greater precision, first-order time courses for turnover of 0.05 mM ($\sim K_m/20$) acetyl-¹H₃-thiocholine and acetyl-²H₃-thiocholine were acquired over greater than three half-lives ($> 88\%$ of hydrolysis of the initial substrate concentration; data not shown). The isotope effect was determined in triplicate, and gave $^{D^3}k_{\text{cat}}/K_m = 0.98 \pm 0.02$.

4. Discussion

As discussed in the Introduction, the expectation for acyl transfer reactions is that transition states will resemble high-energy tetrahedral intermediates, and thus will be characterized by inverse kinetic β -secondary deuterium isotope effects. Indeed, the isotope effect $^{D^3}k_{\text{cat}}/K_m = 0.98 \pm 0.02$ is inverse, though modestly so. The isotope effect on k_{cat}/K_m monitors the conversion of the E + A reactant state to the rate-limiting transition state that precedes or is concomitant with the first irreversible step in the mechanism. For cholinesterase reactions that are monitored under initial rate conditions, the first irreversible event is release of the alcohol product that completes the acylation stage of catalysis. This feature is illustrated by considering the following kinetic mechanism for BuChE catalysis:



The various species in the mechanism are E = free enzyme; A = free substrate; EA = initial noncovalent Michaelis complex; FP = complex of acylenzyme intermediate and alcohol product P; F = acylenzyme intermediate; EQ = complex of enzyme and acetate product Q. Note that k_7 is the pseudo first-order rate constant for hydrolysis of the acylenzyme intermediate. The observed rate constants that are obtained from the Michaelis-Menten parameters for this mechanism are given by equations 3:

$$\frac{k_{\text{cat}}}{K_m} = \frac{k_1 k_3 k_5}{k_2(k_4 + k_5) + k_3 k_5} \quad k_{\text{cat}} = \frac{k_3 k_4}{k_4 + k_5} \quad (3)$$

Hence, as alluded to above, k_{cat}/K_m monitors events up to the release of the alcohol product, with rate constant k_5 . Note that k_{cat}/K_m may be rate limited by substrate binding (k_1), by chemical steps that produce the acylenzyme intermediate (k_3), or by product release (k_5). On the other hand, k_{cat} contains the overall rate constants that convert the EA complex to the acylenzyme intermediate F (k_a) and the acylenzyme intermediate to E + Q (k_d). Hence, k_{cat} may be rate limited by either the acylation (k_a) or deacylation (k_d) stage of catalysis. The overall acylation rate constant $k_a = k_3 k_5 / (k_3 + k_4 + k_5)$, and hence the acylation stage of catalysis could be rate limited by chemical catalysis (k_3) or by alcohol product release (k_5). Similarly, the overall deacylation rate constant is $k_d = k_7 k_9 / (k_7 + k_8 + k_9)$, and the deacylation stage of catalysis could be rate limited by chemical catalysis (k_7) or by acetate product release (k_9).

The modest isotope effect $D^3k_{\text{cat}}/K_m = 0.98 \pm 0.02$, which is just at the level of significance, suggests that conversion of the E + A reactant state to the acylenzyme intermediate is rate limited by more than one step. Since in the reactant state the susceptible carbonyl function of the substrate is sp^2 hybridized and in the transition state of the chemical step(s) the carbonyl should be tending toward sp^3 hybridization, one can expect a palpably inverse isotope effect, as outlined in Figures 1 and 2. That the isotope effect is inverse but very near unity suggests that k_{cat}/K_m for horse serum BuChE-catalyzed hydrolysis of ATCh is rate limited by more than one step. A reasonable model is one in which the chemical step, which is susceptible to the isotope effect, is partially rate limiting, and that substrate binding or product release (neither of which will produce an isotope effect), or both, also contribute to rate limitation.

Interpretation of the isotope effect on k_{cat} is more straightforward, though the implications are unusual. The marked normal isotope effect on k_{cat} requires that the rate limiting step is one in which an sp^3 -hybridized carbonyl carbon in the reactant state is being converted to an sp^2 -like transition state. This in turn requires that the accumulating Michaelis complex (reactant state) under conditions of substrate saturation is a tetrahedral intermediate, and that decomposition of this intermediate to an sp^2 -hybridized product is the rate-limiting step for k_{cat} . The normal isotope effect on k_{cat} could arise either from rate-limiting acylation or deacylation (see equation 3 and accompanying discussion), or from partial rate limitation by both stages of catalysis. That is, the isotope effect is consistent with rate limiting decomposition of the tetrahedral intermediate in either acylation or deacylation of equine BuChE.

These unusual results are in substantial agreement with x-ray crystallographic studies of human BuChE [10], which show a covalent tetrahedral adduct of the active site serine and a butyrate moiety. This moiety is capable of turnover in the solid state, since soaking crystals with the isosteric 3-bromopropionate resulted in replacement of butyrate in the covalent tetrahedral adduct by 3-bromopropionate. Observations of tetrahedral BuChE adducts in the solid state are in agreement with results herein that show that k_{cat} is rate limited by decomposition of an accumulating tetrahedral intermediate, and also suggest that isotope effects on k_{cat} are monitoring, at least in part, rate-limiting events in the deacylation stage of catalysis.

A previous study of human BuChE catalysis [9] gave results that are consistent with those describe herein. Human BuChE-catalyzed hydrolysis of ATCh showed marked deviation from the relatively simple Michaelis-Menten kinetics for the equine serum enzyme. The close adherence to Michaelis-Menten kinetics of the data for both isotopic substrates in Figure 3 shows that, at high concentrations, neither substrate activation nor inhibition is observed for equine BuChE. However, substrate activation at high substrate concentrations was observed for the human BuChE reaction, and sizeable normal kinetic β -secondary deuterium isotope effects were observed on substrate-activated k_{cat} , but not on k_{cat} itself [9]. These isotope effects were interpreted in terms of allosteric modulation of the active site when a second molecule of substrate binds to the peripheral site of the enzyme. The second molecule of substrate leads to activation via stabilization of the tetrahedral intermediate in the deacylation stage of catalysis, whose breakdown has become the rate-limiting step [9]. Note that the normal isotope effects for human BuChE in reference 9 and those reported herein for equine BuChE do not in themselves establish whether acylation or deacylation is rate limiting for k_{cat} . Indeed, the normal isotope effects for either enzyme could arise from rate limiting decomposition of the tetrahedral intermediate in the acylation stage of catalysis, or in the deacylation stage of catalysis, or from a combination of the two.

Abbreviations

AChE	acetylcholinesterase
BuChE	butyrylcholinesterase
ATCh	acetylthiocholine
BuTCh	butyrylthiocholine
D	^2H
L	the isotopes of hydrogen, H or ^2H
DTNB	5,5'-dithiobis(2-nitrobenzoic acid)
$\text{D}^3\text{k}_{\text{cat}}/\text{K}_{\text{m}}$	β -secondary deuterium isotope effect on $\text{k}_{\text{cat}}/\text{K}_{\text{m}}$
$\text{D}^3\text{k}_{\text{cat}}$	β -secondary deuterium isotope effect on k_{cat} .

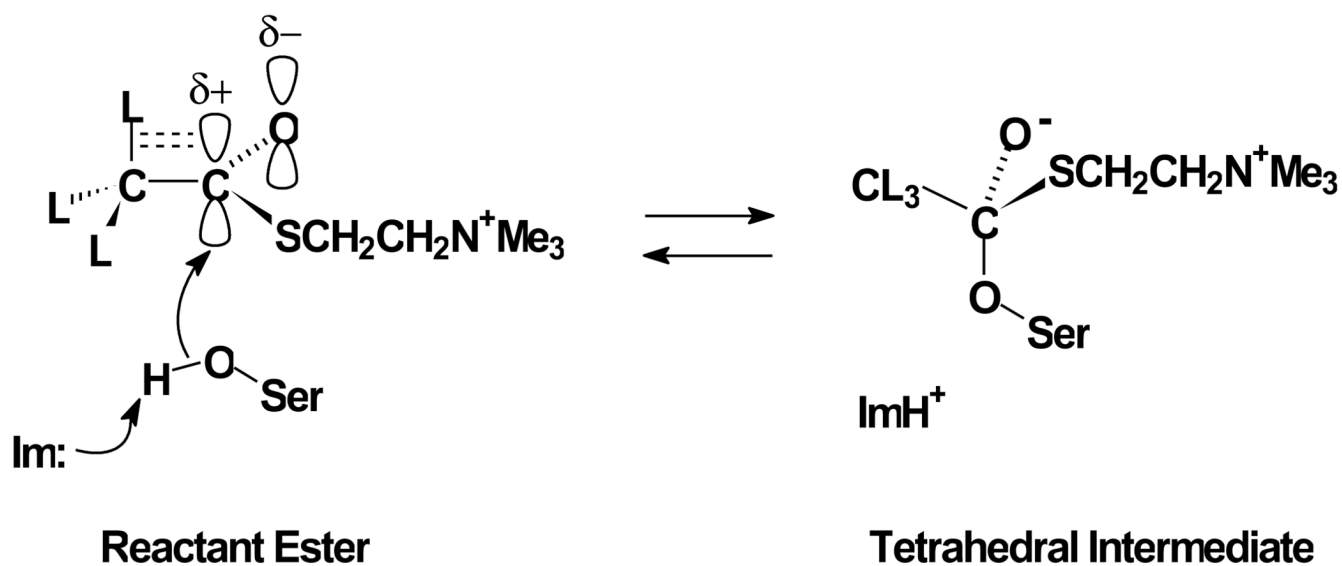
Acknowledgments

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**Fig. 1.**

Structural origins of β -secondary deuterium isotope effects for the formation of the tetrahedral intermediate in the acylation stage BuChE-catalyzed hydrolysis of ATCh. The γ -oxygen of the active site serine covalently adds to the carbonyl carbon of the thioester function of the substrate, with general-base assistance by the imidazole sidechain (Im) of the active site histidine. This nucleophilic addition is marked by change of the hybridization of the carbonyl carbon from sp^2 in the reactant state to sp^3 in the tetrahedral intermediate.

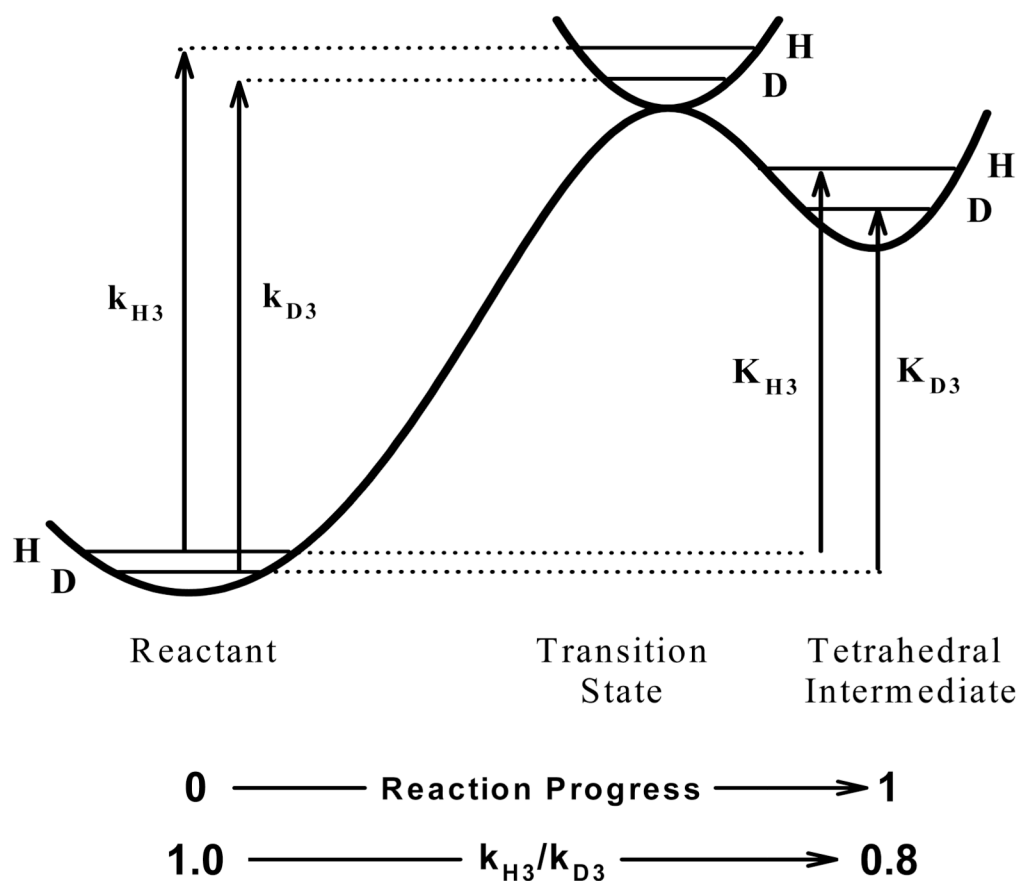


Fig. 2. Energetic origins of β -secondary deuterium isotope effects for the formation of the tetrahedral intermediate in the acylation stage of BuChE-catalyzed hydrolysis of ATCh. Vertical arrows denote values of ΔE_a , and are labeled with the corresponding isotopic reaction rate or equilibrium constant. Interpretation of isotope effects in terms of structural changes in the substrate is discussed in the text.

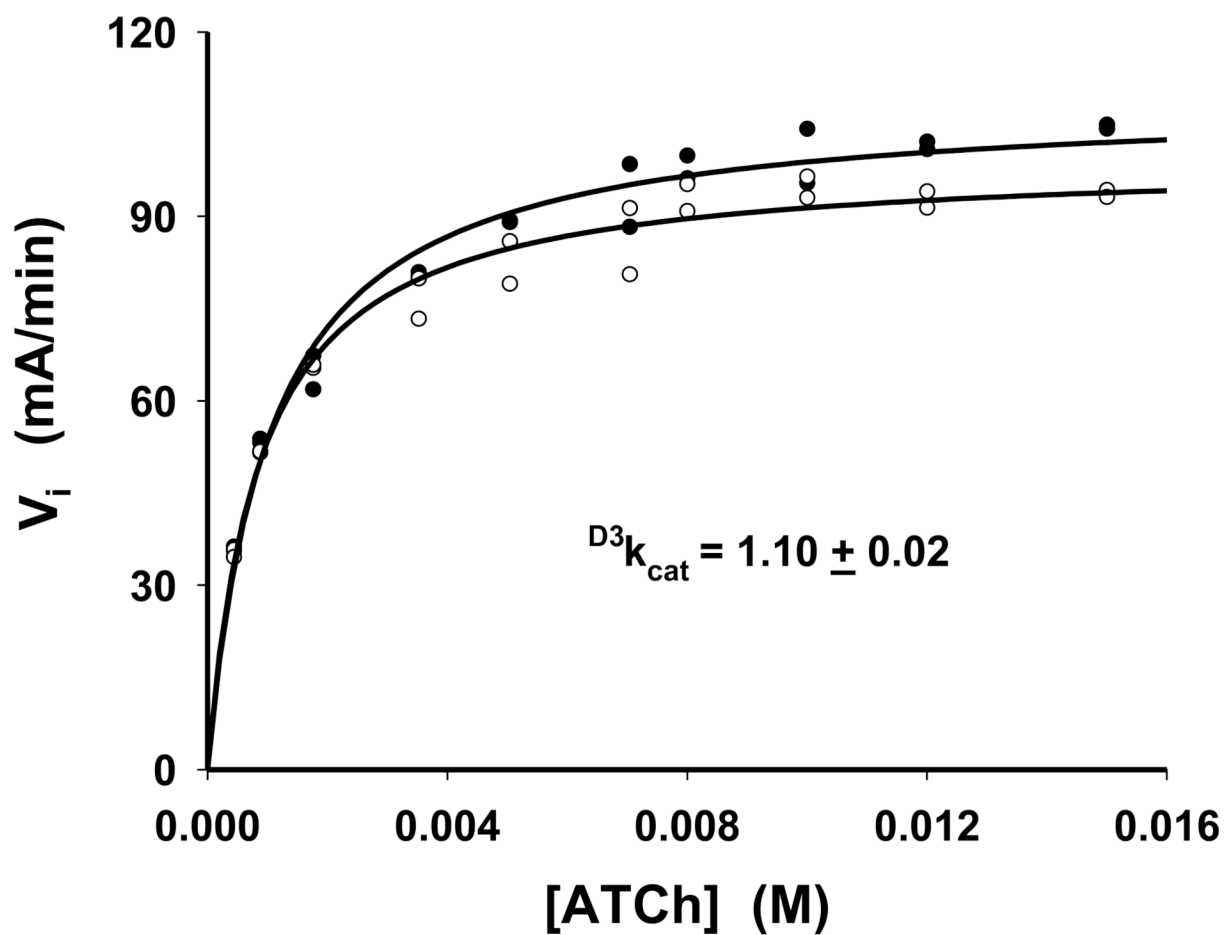


Fig. 3. Dependencies of initial rates on substrate concentrations for equine serum BuChE-catalyzed hydrolysis of acetyl-L₃-thiocholine (L = H or ²H). Solid lines were generated by fitting data to equation 1. Open circles, acetyl-²H₃-thiocholine; closed circles, acetyl-H₃-thiocholine.