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References under References Page and:

KINETIC ISOTOPE EFFECTS

General

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3. Cleland, O'Leary and Northrop, eds., "Isotope Effects on Enzyme Catalyzed Reactions", Park Press (1977).
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6. Cleland, "Use of I.E.'s to Elucidate Enzyme Mechanisms", *CRC Critical Reviews in Biochemistry* **13**, 385 (1982).
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b) Cha et al., "Hydrogen Tunneling in Enzyme Reactions", *Science* **243**, 1325 (1989).
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Some Specific Applications

1. *Nature of Rate Determining Steps*
 - a) Bush, Shiner and Mahler, "Deuterium I.E. on Initial Rates in Liver Alcohol Dehydrogenase", *Biochemistry* **12**, 4802 (1973).
 - b) Raines et al. "Energetics of a Mutant Triosephosphate Isomerase in Which the Active Site Glu Has Been Changed to Asp", *Biochemistry* **25**, 7142 (1986).
 - c) Sampson and Knowles, "Segmental Motion in Catalysis: Investigation of a Hydrogen Bond Critical for Loop Closure in the Reaction of Triosephosphate Isomerase", *Biochemistry* **31**, 8488 (1992).

2. *Kinetic Order*

- a) Klinman et al., "Deduction of Kinetic Mechanism in Multisubstrate Enzyme Reactions from Tritium Isotope Effects", *J. Biol. Chem.* **255**, 11648 (1980).
- b) Cook et al., "Mechanistic Deductions From Isotope Effects in Multireactant Enzyme Mechanisms", *Biochemistry* **19**, 4853 (1980).

3. *Intrinsic Isotope Effects*

- a) Northrop, "Steady State Analysis of Kinetic Isotope Effects in Enzymic Reactions", *Biochemistry* **14**, 2644 (1975).
- b) Albery and Knowles, "The Determination of the Rate Limiting Step in a Proton Transfer Reaction from the Breakdown of the Swain-Schaad Relation", *J. Am. Chem. Soc.* **99**, 637 (1977).
- c) Miller and Klinman, "Magnitude of Intrinsic Isotope Effects in the Dopamine Hydroxylase Reaction", *Biochemistry* **22**, 3091 (1983).

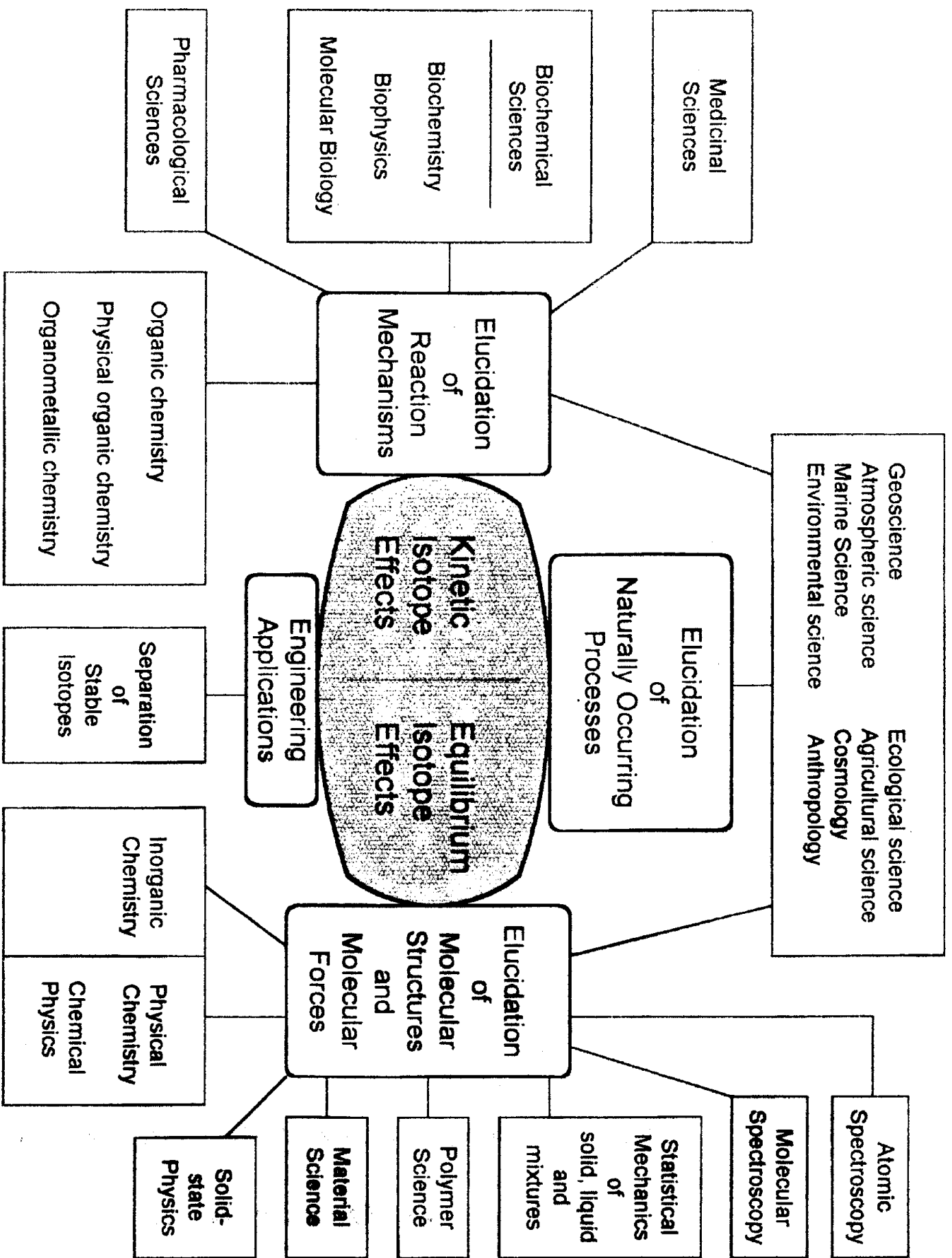
4. *Extraction of Microscopic Constants*

- a) Palcic and Klinman, "Isotopic Probes Yield Microscopic Constants", *Biochemistry* **22**, 5957 (1983).
- b) Farnum et al., "pH Dependence of Deuterium Isotope Effects and Tritium Exchange in the Bovine Plasma Amine Oxidase Reaction", *Biochemistry* **25**, 1898 (1986).
- c) Klinman and Matthews, "Calculation of Substrate Dissociation Constants from Steady State Isotope Effects in Enzyme Catalyzed Reactions", *J. Am. Chem. Soc.* **107**, 1058 (1985).

5. *Mechanistic features*

Hess, R. A., Hengge, A. C. & Cleland, W. W. Isotope effects on enzyme-catalyzed acyl transfer from p-nitrophenyl acetate: Concerted mechanisms and increased hyperconjugation in the transition state. *J. Am. Chem. Soc.* **120**, 2703-2709 (1998).

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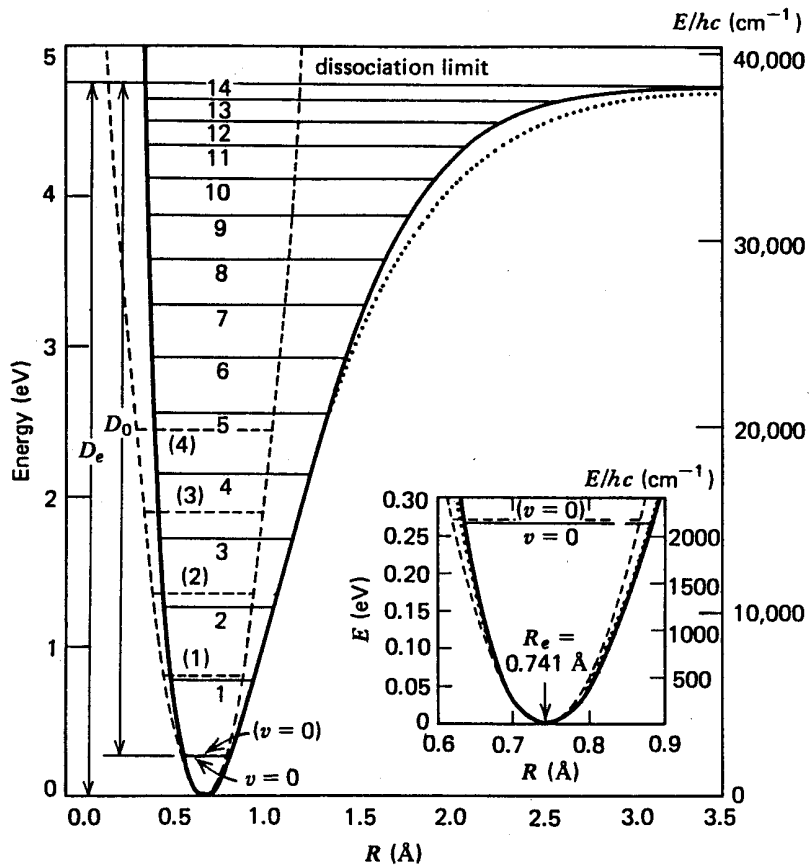


FIGURE 7.1

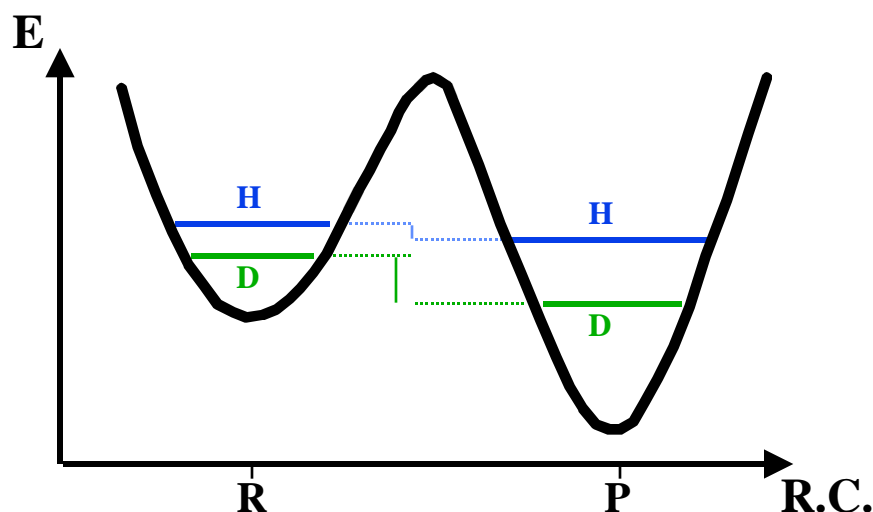
Vibrational energy levels of the H_2 molecule. Solid lines (—) represent the experimental $E(R)$ curve and energy levels; dashed lines (---) give the parabolic approximation described in the text and the first few of the corresponding harmonic oscillator levels; and the dotted line (·····) is a Morse potential fit. The inset shows an enlargement of the region near the minimum. The two "dissociation energies" are indicated: $D_e = 4.748$ eV, $D_0 = 4.477$ eV.

TEST OF HARMONIC OSCILLATOR MODEL WITH VIBRATIONAL CONSTANTS OF H_2 ISOTOPES

(atomic masses: ^1H , 1.00782 amu; D, 2.01410 amu)

| Molecule | Reduced Mass, $\mu/(\text{amu})$ | Vibrational Frequency, $\nu_e \times 10^{-14}(\text{s}^{-1})$ | $\frac{\mu^{1/2} \nu_e}{10^{14} \text{amu}^{1/2} \text{s}^{-1}}$ |
|----------------|----------------------------------|---|--|
| $^1\text{H}_2$ | 0.50391 | 1.3192 | 0.9365 |
| ^1HD | 0.67171 | 1.1429 | 0.9367 |
| D_2 | 1.00705 | 0.9345 | 0.9378 |

Equilibrium Isotope Effect (EIE)



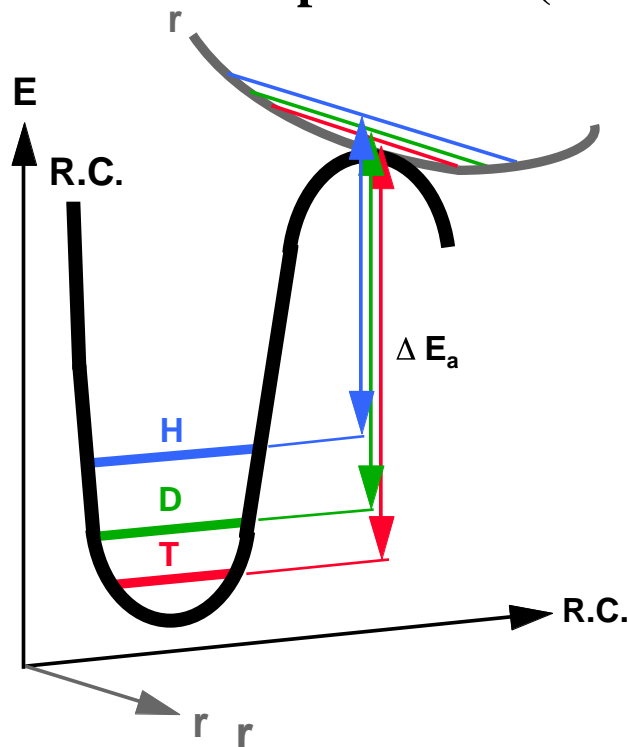
$$\frac{K_H}{K_D} = \exp \frac{E_{D-H}}{RT}$$

Table 1: Primary Deuterium Equilibrium Isotope Effects

| dehydrogenase | substrate pair ^a | ^D K _{eq,ox} ^b | K _{eq,ox} ^c |
|--------------------|---------------------------------|--|--|
| secondary alcohols | | | |
| liver alcohol | cyclohexanol-1-d DPNH | 1.18 ± 0.03 ^d | (1.42 ± 0.40) × 10 ⁻⁹ M |
| liver alcohol | cyclohexanol DPND (A-side) | 1.183 ± 0.008 ^d | (1.58 ± 0.39) × 10 ⁻⁹ M |
| lactate | L-lactate DPND (A-side) | 1.19 ± 0.005 | (2.19 ± 0.08) × 10 ⁻¹³ M |
| yeast alcohol | 2-propanol-2-d DPNH | 1.175 ± 0.010 | (4.2 ± 0.2) × 10 ⁻⁹ M |
| malate | L-malate-2-d DPNH | 1.173 ± 0.008 | (2.1 ± 0.8) × 10 ⁻¹³ M |
| isocitrate | 3-oxo-DL-isocitrate-2-d TPNH | 1.168 ± 0.006 | 1.04 ± 0.18 M |
| primary alcohol | | | |
| liver alcohol | ethanol DPND (A-side) | 1.069 ± 0.008 | (6.0 ± 0.1) × 10 ⁻¹³ M |
| amino acid | | | |
| glutamate | L-glutamate-2-d TPNH | 1.14 ± 0.01 | (4.4 ± 0.1) × 10 ⁻¹⁴ M ² |
| hemiacetal | | | |
| glucose-6-P | glucose-1-d DPNH | 1.28 ± 0.02 | (5.2 ± 0.5) × 10 ⁻⁷ M |

^a Reactants between which label is transferred. The labeled molecule actually used in each case is noted. ^b ^DK_{eq,ox} is the deuterium isotope effect (that is, K_{eq,H}/K_{eq,D}) on the equilibrium constant for oxidation by DPN or TPN. All effects were carried out at least in triplicate. ^c Equilibrium constant for oxidation of unlabeled molecule by DPN or TPN, including the concentration of H⁺ when it is a reactant. For isocitrate dehydrogenase, dissolved CO₂ is considered the reactant (i.e., bicarbonate is not included), and, for glutamate dehydrogenase, NH₄⁺ is considered the reactant and the amino group of glutamate is assumed to be protonated. The apparent pK used to calculate CO₂ concentrations from added bicarbonate was 6.4. ^d Since identical values are obtained for equilibrium isotope effects and for equilibrium constants with 100 mM potassium phosphate, pH 8, and cyclohexanol-1-d and with 100 mM Tris-HCl, pH 8, and A-side DPND, Tris apparently does not form Schiff's bases with ketones such as cyclohexanone to any extent at this concentration.

Kinetic Isotope Effects (KIE)



$$\frac{k_H}{k_D} = \exp \frac{hc}{2kT} \left[\sum_{(\text{reactant})}^{3N-6} (\omega_{Hi} - \omega_{Di}) - \sum_{(\text{transition state})}^{3N-7} (\omega_{Hi}^{\ddagger} - \omega_{Di}^{\ddagger}) \right]$$

Maximum Kinetic Isotope Effects Due to the Loss of a Ground-State Stretching Frequency, 25°C

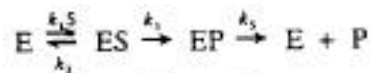
| Bond | $\omega(\text{cm}^{-1})$ | k_H/k_D | k_H/k_T |
|------|--------------------------|-----------|-----------|
| C—H | 3000 | 6.8 | 15.8 |
| N—H | 3100 | 7.5 | 18.2 |
| O—H | 3300 | 8.5 | 21.8 |
| S—H | 2500 | 5.5 | 11.7 |

Maximum Kinetic Heavy Atom Isotope Effects Due to Loss of a Ground State Stretching Frequency, 25°C

| Bond | $\omega (\text{cm}^{-1})$ | k/k' |
|--------------------|---------------------------|---------------------------|
| C- ¹² C | 1129 | $k_{12}/k_{13} = 1.054^*$ |
| C- ¹⁴ N | 1134 | $k_{14}/k_{15} = 1.044$ |
| C- ¹⁶ O | 1113 | $k_{16}/k_{18} = 1.068$ |

* The ¹⁴C isotope effect is calculated to be $(k_{12}/k_{14} - 1) = (1.9)(k_{12}/k_{14} - 1)$ (202).

Commitments to catalysis



Scheme 1

$$V = \frac{k_2 k_3 E_t}{k_2 + k_3} \quad (1)$$

$$\frac{V}{K} = \frac{k_2 k_3 E_t}{k_2 + k_3} \quad (2)$$

$$K = \frac{k_2(k_2 + k_3)}{k_1(k_2 + k_3)} \quad (3)$$

$$v = \frac{k_1 k_2 k_3 [S] E_t}{k_2(k_2 + k_3) + k_1(k_2 + k_3)[S]} \quad (4)$$

$$\frac{V_H}{V_D} = \frac{k_{3H}/k_{3D} + k_{3H}/k_3}{1 + k_{3H}/k_3}$$

$$\frac{(V/K)_H}{(V/K)_D} = \frac{k_{3H}/k_{3D} + k_{3H}/k_2}{1 + k_{3H}/k_2}$$

$${}^D V = \frac{{}^D k + R}{1 + R}$$

$${}^D(V/K) = \frac{{}^D k + C}{1 + C}$$

Intrinsic isotope effects

$${}^D(V/K) - 1 = \frac{{}^D k + C}{1 + C} - 1 = \frac{{}^D k + C - 1 - C}{1 + C} = \frac{{}^D k - 1}{1 + C}$$

$$\frac{{}^D(V/K) - 1}{{}^T(V/K) - 1} = \frac{\frac{{}^D k - 1}{1 + C}}{\frac{{}^T k - 1}{1 + C}} = \frac{{}^D k - 1}{{}^T k - 1}$$

$$\frac{k_H}{k_T} = \left(\frac{k_H}{k_D} \right) \left[\frac{1/\sqrt{m_1} - 1/\sqrt{m_2}}{1/\sqrt{m_1} - 1/\sqrt{m_2}} \right]$$

$$\frac{k_H}{k_T} = \left(\frac{k_H}{k_D} \right)^{1.442}$$

$$\frac{{}^D(V/K) - 1}{{}^T(V/K) - 1} = \frac{{}^D k - 1}{{}^D k^{1.44} - 1}$$

Swain-Schaad Relationships

I

1. $(k_H/k_D)^{1.442} = k_H/k_T$
2. $(k_D/k_H)^{1.442} = k_T/k_H$
3. $k_H/k_D = (k_H/k_T)^{0.6935}$
4. $k_D/k_H = (k_T/k_H)^{0.6935}$

5. $(k_H/k_D)^{-1.442} = k_T/k_H$
6. $(k_D/k_H)^{-1.442} = k_H/k_T$
7. $k_H/k_D = (k_T/k_H)^{-0.6935}$
8. $k_D/k_H = (k_H/k_T)^{-0.6935}$

II

1. $(k_D/k_T)^{3.263} = k_H/k_T$
2. $(k_T/k_D)^{3.263} = k_T/k_H$
3. $k_D/k_T = (k_H/k_T)^{0.3065}$
4. $k_T/k_D = (k_T/k_H)^{0.3065}$

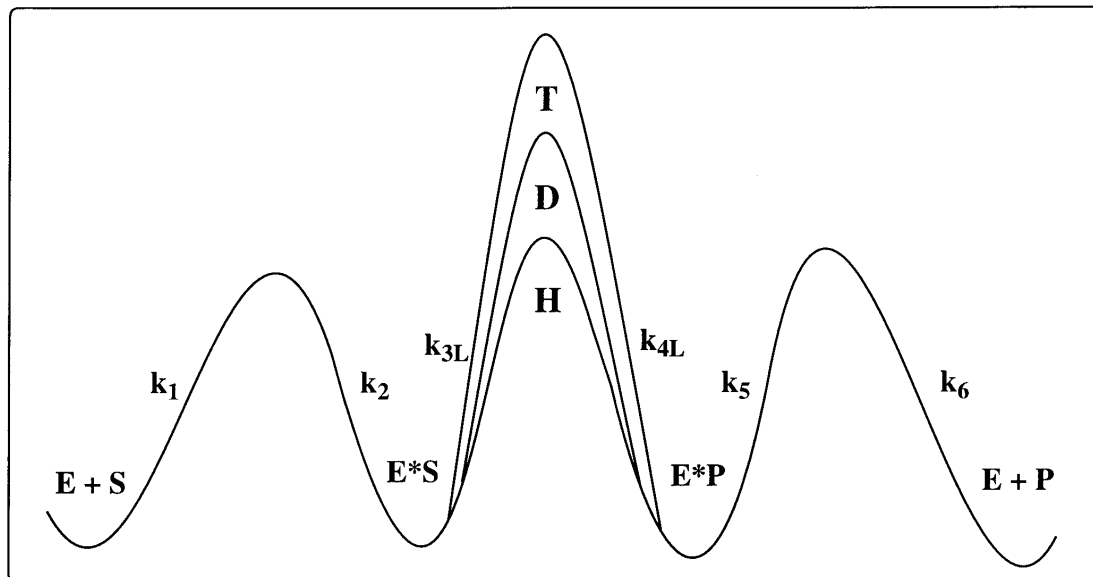
5. $(k_D/k_T)^{-3.263} = k_T/k_H$
6. $(k_T/k_D)^{-3.263} = k_H/k_T$
7. $k_D/k_T = (k_T/k_H)^{-0.3065}$
8. $k_D/k_H = (k_H/k_T)^{-0.3065}$

III

1. $(k_D/k_T)^{2.263} = k_H/k_D$
2. $(k_T/k_D)^{2.263} = k_D/k_H$
3. $k_D/k_T = (k_H/k_D)^{0.4419}$
4. $k_T/k_D = (k_D/k_H)^{0.4419}$

5. $(k_D/k_T)^{-2.263} = k_D/k_H$
6. $(k_T/k_D)^{-2.263} = k_H/k_D$
7. $k_T/k_D = (k_H/k_D)^{-0.4419}$
8. $k_D/k_T = (k_D/k_H)^{-0.4419}$

Kinetic complexity



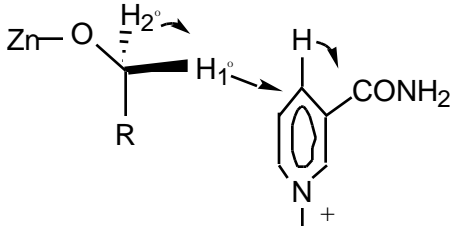
$$\left(\frac{k_L}{k_T}\right)_{\text{obs}} = \frac{\frac{k_L}{k_T} + \frac{k_{3L}}{k_2} + \frac{k_{4L}}{k_5} \left(\frac{k_L}{k_T}\right)}{1 + \frac{k_{3L}}{k_2} + \frac{k_{4L}}{k_5}}$$

$$\left(\frac{k_L}{k_T}\right)_{\text{obs}} < \left(\frac{k_L}{k_T}\right)_{\text{obs}}^{3.26}$$

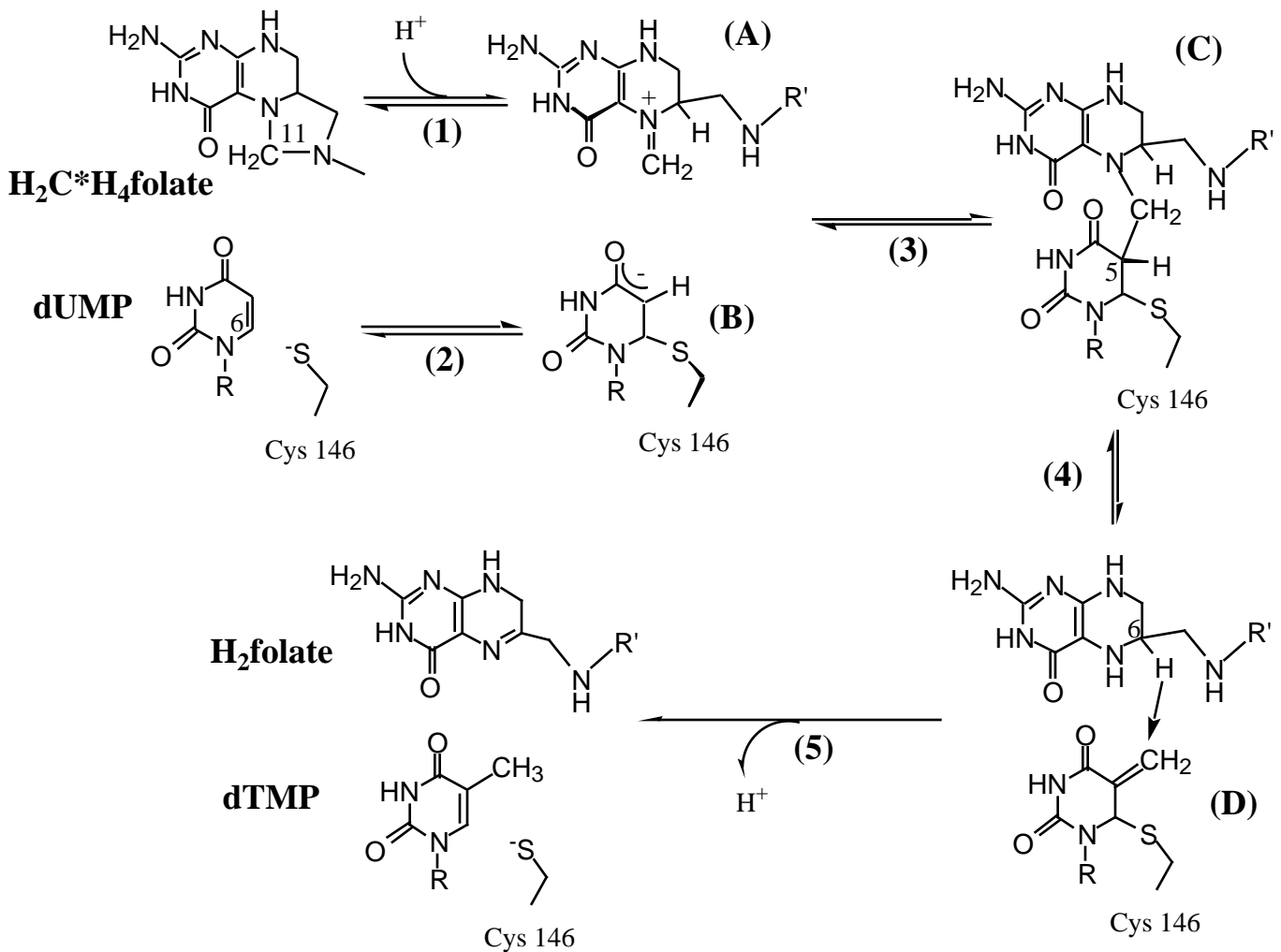
Secondary isotope effects

Isotope effects upon labeling positions which are not being transferred.
 These are due to change in bond order along the reaction coordinat.
 Kinetic secondary effects can vary between unity and the equilibrium effect.

In the ADH catalyzed reaction:



In the TS catalyzed reaction:



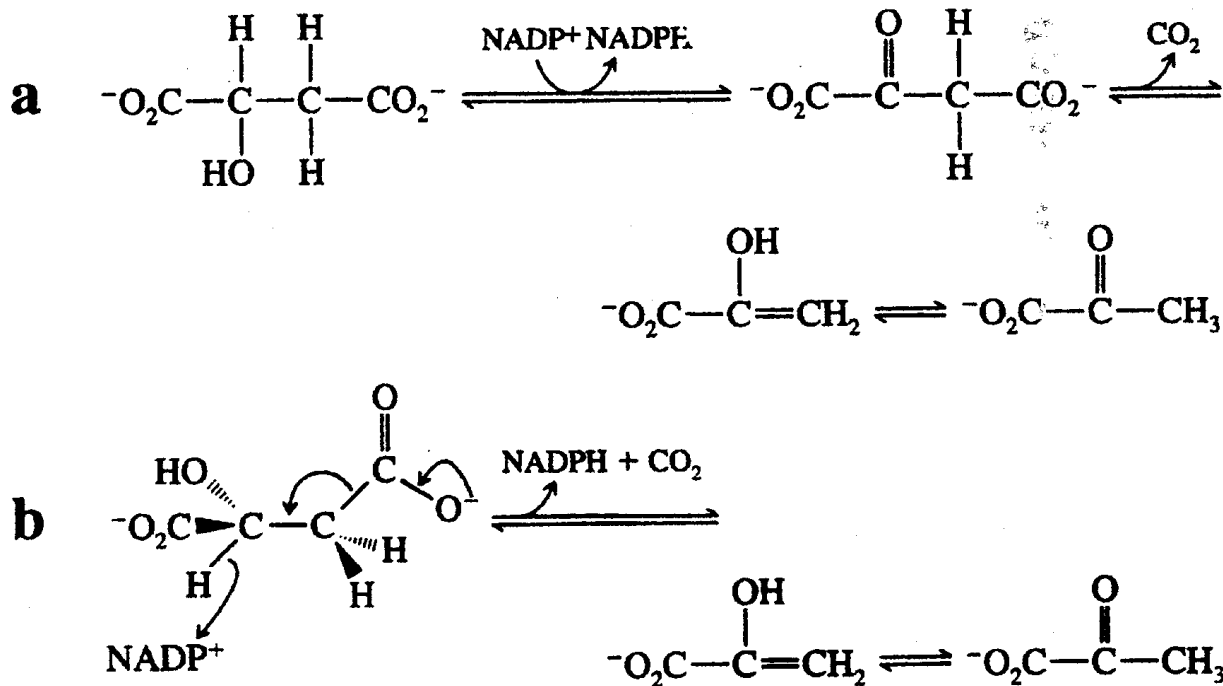
R=2'-deoxyribose-5'-phosphate; R'=(*p*-aminobenzoyl)glutamate

Carreras, C.W. & Santi, D.V. (1994) *Annu. Rev. Biochem.* 64, 721-762.

Spencer, H.T., Villafranca, J.E., & Appleman, J.R. (1977) *Biochem. Biophys. Res. Commun.* 80, 4212-4222.

Multiple isotope effects

Resolving the mechanism of the malic enzyme:
stepwise (a) or concerted (b)?



The mechanism was found to change from stepwise to concerted on changing the cofactor to acetylpyridine-NADP⁺.

Edens, W.A., Urbauer, J.L., & Cleland W.W., *Biochemistry* **1997**, *36*, 1141-1147

Solvent isotope effects

Direct chemical effects (primary IE)

Medium and viscosity effects

(e.g. for malic enzyme: Karsten, W.E, Lai, C.J., & Cook, P.F. *JACS* **1995**, *117*, 5914-5918)

pH vs. pD effects

Solvolysis effects

Protein mass effects (deuterated protein is heavier)

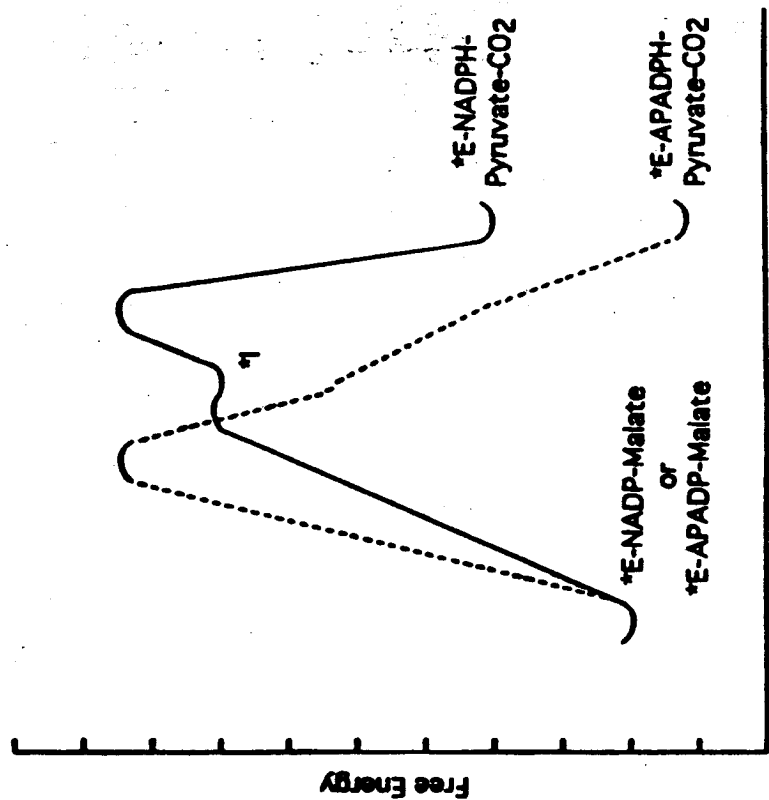
Chemical Mechanism of Malic Enzyme by Isotope Effects

Table 1: ¹³C Kinetic Isotope Effects

| carbon position | malate + NADP ¹³ (V/K) _H | malate-2-d + NADP ¹³ (V/K) _D |
|-----------------|---|---|
| C1 | 1.0011 ± 0.0004 | |
| C2 | 0.9871 ± 0.0021 | 0.9892 ± 0.0013 |
| C3 | 1.0210 ± 0.0005 | 1.0130 ± 0.0006 |
| C4 | 1.0324 ± 0.0003 | 1.0243 ± 0.0004 |

| carbon position | malate + APADP ¹³ (V/K) _H | malate-2-d + APADP ¹³ (V/K) _D |
|-----------------|--|--|
| C1 | 1.0009 ± 0.0007 | |
| C2 | 0.9941 ± 0.0008 | 0.9961 ± 0.0004 |
| C3 | 1.0067 ± 0.0003 | 1.0125 ± 0.0002 |
| C4 | 1.0056 ± 0.0005 | 1.0087 ± 0.0007 |

with hydride transfer preceding decarboxylation. These data agree with those previously obtained by Hermes et al. (1982). When the alternative dinucleotide APADP was substituted into this reaction, however, the C4 kinetic isotope effects for malate and malate-2-d became 1.0056 and 1.0087, respectively. This increase in the C4 isotope effect with malate-2-d suggests a concerted mechanism with the alternative dinucleotide, APADP. The trend of these isotope effects is consistent with those previously observed by Weiss et al. (1991). As stated earlier there are two reasonable explanations for this outcome. First, malic enzyme may change its chemical mechanism with the alternative substrate. Second,



Reaction Coordinate

FIGURE 1: Free energy profiles for the enzymatic reaction of malate to pyruvate and CO₂ with NADP or APADP as the dinucleotide. *E-NADP-malate and *E-APADP-malate represent the activated enzyme-substrate complex, *I is the E-NADPH-oxalacetate intermediate for the stepwise reaction with NADP, *E-NADPH-Pyruvate-CO₂ is the activated enzyme-product complex for the reaction with NADP, and *E-APADPH-Pyruvate-CO₂ is the activated enzyme-product complex for the reaction with APADP.