Biochemical Thermodynamics and Rapid-Equilibrium Enzyme Kinetics

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Biochemical thermodynamics is based on the chemical thermodynamics of aqueous solutions, but it is quite different because pH is used as an independent variable. A transformed Gibbs energy G' is used, and that leads to transformed enthalpies H' and transformed entropies S'. Equilibrium constants for enzyme-catalyzed reactions are referred to as apparent equilibrium constants K' to indicate that they are functions of pH in addition to temperature and ionic strength. Despite this, the most useful way to store basic thermodynamic data on enzyme-catalyzed reactions is to give standard Gibbs energies of formation, standard enthalpies of formation, electric charges, and numbers of hydrogen atoms in species of biochemical reactants like ATP. This makes it possible to calculate standard transformed Gibbs energies of formation, standard transformed enthalpies of formation of reactants (sums of species), and apparent equilibrium constants at desired temperatures, pHs, and ionic strengths. These calculations are complicated, and therefore, a mathematical application in a computer is needed. Rapid-equilibrium enzyme kinetics is based on biochemical thermodynamics because all reactions in the mechanism prior to the rate-determining reaction are at equilibrium. The expression for the equilibrium concentration of the enzyme−substrate complex that yields products can be derived by applying Solve in a computer to the expressions for the equilibrium constants in the mechanism and the conservation equation for enzymatic sites. In 1979, Duggleby pointed out that the minimum number of velocities of enzyme-catalyzed reactions required to estimate the values of the kinetic parameters is equal to the number of kinetic parameters. Solve can be used to do this with steady-state rate equations as well as rapid-equilibrium rate equations, provided that the rate equation is a polynomial. Rapid-equilibrium rate equations can be derived for complicated mechanisms that involve several reactants and various types of inhibitors, activators, and moderators.

Biochemical Thermodynamics

Physical chemists have studied the thermodynamics and kinetics of chemical reactions in aqueous solutions over a long period of time, and we know that the reactions in enzyme catalysis must involve reactions between ions and molecular species. However, the thermodynamics and kinetics of enzyme-catalyzed reactions are actually quite different from the classical physical chemistry of reactions in aqueous solutions. This article is about these differences.

Chemical Thermodynamics in Dilute Aqueous Solutions. The thermodynamics of chemical reactions in aqueous solutions is primarily based on the Gibbs energy G that provides the criterion for spontaneous chemical reaction at specified temperature and pressure, $(dG)_{T,P} \leq 0$. The fundamental equation for the Gibbs energy G of a chemical reaction system is

$$dG = -S\,dT + V\,dP + \sum_{j=1}^{N} \mu_j\,dn_j \qquad (1)$$

S is the entropy of the system, N is the number of different species, μ_j is the chemical potential of species j, and n_j is the amount of species j. The chemical potentials of species can be replaced with Gibbs energies of formation of species, $\Delta_f G_j$.

$$dG = -S\,dT + V\,dP + \sum_{j=1}^{N} \Delta_f G_j\,dn_j \qquad (2)$$

Chemical reactions in aqueous solutions balance atoms and electric charges. Physical chemists express equilibrium constants K in terms of the activities of the reacting species. For chemical reactions in dilute aqueous solutions, the extended Debye−Hückel equation can be used to calculate the Gibbs energy of formation of species j at concentration c_j using

$$\Delta_f G_j = \Delta_f G_j^\circ + RT \ln \gamma_j c_j = \Delta_f G_j^\circ + RT \ln \gamma_j + RT \ln c_j \qquad (3)$$

where $\Delta_f G_j^\circ$ is the standard Gibbs energy of formation of ion j at zero ionic strength and γ_j is the activity coefficient of ion j. The standard Gibbs energy of formation $\Delta_f G_j^\circ$ of species j is relative to the elements that it contains, each taken as $\Delta_f G_j^\circ(\text{element}) = 0$ for a reference form. Neutral species at low concentrations in aqueous solution are not significantly affected by the ionic strength, but activity coefficients γ_j of ions in the physiological range of ionic strength can be calculated using the extended Debye−Hückel equation.[1]

$$\ln \gamma_j = -\alpha z_j^2 I^{1/2}/(1 + BI^{1/2}) \qquad (4)$$

I is the ionic strength, z_j is the charge on the ion, α is the Debye−Hückel constant that is a function of temperature, and

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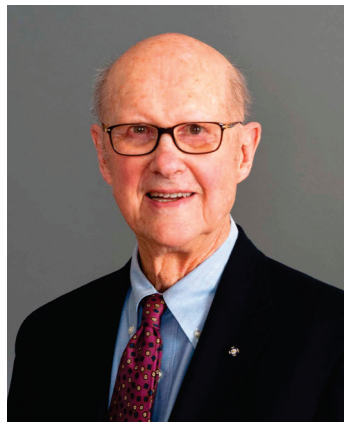

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$B = 1.5\ \mathrm{L}^{1/2}\ \mathrm{mol}^{-1/2}$ is an empirical constant that is taken to be independent of temperature. This equation does not yield very accurate values above about 0.35 M. Substituting eq 4 into eq 3 yields the Gibbs energy of formation of species at ionic strength I.

$$\Delta_f G_j(I) = \Delta_f G_j^\circ(I = 0) - RT\alpha z_j^2 I^{1/2}/(1 + BI^{1/2}) + RT \ln c_j \quad (5)$$

The NBS Tables of Chemical Thermodynamic Properties[2] give values of $\Delta_f G_j^\circ$, $\Delta_f H_j^\circ$, and $\bar{S}_j^\circ$ at zero ionic strength.

Biochemical Thermodynamics of Enzyme-Catalyzed Reactions. Biochemists do not deal directly with species like physical chemists do. They deal with reactants like ATP, which is made up of ATP^{4-}, $HATP^{3-}$, and H_2ATP^{2-} in the pH range of 5−9. Their analytical methods yield the concentration of ATP rather than the concentrations of species. The pH is considered to be an independent variable that is controlled by a buffer. Apparent equilibrium constants K' are written in terms of concentrations of reactants (sums of species), and the consumption or production of hydrogen ions is not included in writing biochemical equations like ATP + H_2O = ADP + P_i. Biochemical equations do not balance hydrogen atoms or electric charges, but they do balance atoms of other elements. When water is a reactant, its concentration is not used in the expression for the apparent equilibrium constant, but its standard Gibbs energy of formation is used in the calculation of the apparent equilibrium constant.

The fact that pH is considered to be an independent variable in biochemical thermodynamics is very important because that means that $(dG)_{T,P} < \_0$ does not provide the criterion for spontaneous change and equilibrium. Because a new constraint is added in studying enzyme-catalyzed reactions, a new thermodynamic potential has to be defined. The way to do this is well-known, and it is called a Legendre transform.[3] A new thermodynamic potential is defined by subtracting from an existing thermodynamic potential the product of an extensive property and the corresponding intensive property. There are a number of different applications of transformed thermodynamic properties in chemistry.[4] Alberty and Oppenheim[5] used Legendre transformed thermodynamic properties for complex gas systems at a specified partial pressure of a reactant like H_2 or C_2H_4.

The transformed Gibbs energy G' for a system of biochemical reactions at a specified pH is defined by subtracting the product of the chemical potential of hydrogen ions $\mu(H^+)$ and the amount of the hydrogen component in the system $n_c(H)$ from the Gibbs energy G of the system. Components are the things that are conserved in a reaction system. The amount of hydrogen component $n_c(H)$ in a biochemical reaction system is given by

$$n_c(H) = \sum_{j=1}^{N} N_H(j) n_j \quad (6)$$

$N_H(j)$is the number of hydrogen atoms in species j, and N is the number of different species in the system. The index number for species is represented by j, so that the index number introduced later for reactants (sums of species differing in the numbers of hydrogen atoms) can be represented by i.

The transformed Gibbs energy G' that has the chemical potential of hydrogen ions as a natural variable is defined by the following Legendre transform[6−8]

$$G' = G - n_c(H)\mu(H^+) \quad (7)$$

The differential of the transformed Gibbs energy is

$$dG' = dG - n_c(H)\, d\mu(H^+) - \mu(H^+)\, dn_c(H) \quad (8)$$

Substituting eq 1 for dG in eq 8 yields the fundamental equation for the transformed Gibbs energy G'.

$$dG' = -S\, dT + V\, dP + \sum_{j=1}^{N} \mu_j\, dn_j - n_c(H)\, d\mu(H^+) - \mu(H^+)\, dn_c(H) \quad (9)$$

The differential of the amount of the hydrogen component can be derived from eq 6.

$$dn_c(H) = \sum_{j=1}^{N} N_H(j)\, dn_j \quad (10)$$

Substituting eq 10 into eq 9 yields the fundamental equation for G'.

$$dG' = -S\, dT + V\, dP + \sum_{j-1}^{N-1} \mu_j'\, dn_j - n_c(H)\, d\mu(H^+) \quad (11)$$

The transformed chemical potential of the jth species is given by

$$\mu_j' = \mu_j - N_H(j)\mu(H^+) \quad (12)$$

Notice that the term for H^+ in the summation in eq 11 is equal to 0 because

$$\mu(H^+) - N_H(H^+)\mu(H^+) = 0$$

Equation 11 can be used to derive the expression for the apparent equilibrium K' constant of an enzyme-catalyzed reaction. This involves a number of steps. More information is available in two books,[9,10] a draft IUBMB/IUPAC report[11] and a physical chemistry textbook.[12] The second book was written in *Mathematica*, and it contains a CD that provides *Mathematica* programs and a database for biochemical thermodynamics.

Equation 11 shows that the transformed Gibbs energy has the natural variables T, P, and $\mu(H^+)$, rather than just T and P. However, $\mu(H^+)$ is not a very convenient independent variable because it is a function of both the temperature and the concentration of hydrogen ions. The differential of the chemical potential of hydrogen ions can be made an independent variable by use of the expression for the differential of $\mu(H^+)$.

$$d\mu(H^+) = \frac{\partial \mu(H^+)}{\partial T} dT + \frac{\partial \mu(H^+)}{\partial [H^+]} d[H^+] = -\bar{S}(H^+)\, dT - RT \ln(10)\, dpH \quad (13)$$

where $\bar{S}(H^+)$ is the molar entropy of hydrogen ions.

Substituting the second form of eq 13 into eq 11 yields

$$dG' = -S'\, dT + V\, dP + \sum_{j=1}^{N-1} \mu_j'\, dn_j + RT \ln(10) n_c(H)\, dpH \quad (14)$$

The summation is over species other than H^+. The term for H^+ in the summation in eq 14 is equal to 0 because $\mu(H^+) - N_H(H)\mu(H^+) = 0$. The transformed entropy S' of the system at a specified pH is given by $S' = S - n_c(H)\bar{S}(H^+)$. Equation 14 shows that the natural variables in biochemical thermodynamics are T, P, and pH rather than the natural variables T and P in chemical thermodynamics. Therefore, the criterion for spontaneous change in biochemical thermodynamics is $(dG')_{T,P,pH} \leq 0$. The Legendre transform has introduced a new type of term, $RT \ln(10) n_c(H)$ dpH in eq 14, where $n_c(H)$ is the number of hydrogen atoms in the system.

When the pH is specified, some of the $N - 1$ terms in eq 14 can be aggregated, for example, ATP^{4-}, $HATP^{3-}$, and H_2ATP^{2-}, which are pseudoisomers at a specified pH. At equilibrium at a specified pH, these pseudoisomers have the same transformed Gibbs energy of formation $\Delta_f G_j'$ because they are in equilibrium with each other.

$$dG' = -S'\, dT + V\, dP + \sum_{i=1}^{N'} \mu_i'\, dn_i + RT \ln(10) n_c(H)\, dpH \quad (15)$$

N' is the number of biochemical reactants in the system, which may be considerably less than $N - 1$. Note that the subscripts have been changed to i, which applies to reactants rather than species.

Equation 15 can be used to derive the expression for the apparent equilibrium constant for a biochemical reaction $\sum \nu_i' B_i = 0$, where B_i represents a reactant like ATP. Stoichiometric numbers ν_i' are positive for products and negative for reactants. When a single biochemical reaction is catalyzed, the amounts n_i' of the reactants at each stage of the reaction are given by

$$n_i' = (n_i')_0 + \nu_i' \xi' \quad (16)$$

where $(n_i')_0$ is the initial amount of reactant i, ν_i' is the stoichiometric number of reactant i, and ξ' is the extent of reaction. Substituting $dn_i = \nu_i'\, d\xi$ into eq 15 yields

$$dG' = -S'\, dT + V\, dP + \left(\sum_{i=1}^{N'} \nu_i' \mu_i'\right) d\xi' + RT \ln(10) n_c(H)\, dpH \quad (17)$$

so that the transformed Gibbs energy of reaction is given by

$$\Delta_r G' = \left(\frac{\partial G'}{\partial \xi'}\right)_{T,P,pH} = \sum_{i=1}^{N'} \nu_i' \mu_i' \quad (18)$$

At equilibrium, the transformed Gibbs energy of reaction is 0.

In biochemical thermodynamics, the standard Gibbs energies of formation of species $\Delta_f G_j^\circ(I)$ are taken to be functions of ionic strength.

$$\Delta_f G_j^\circ(I) = \Delta_f G_j^\circ(I = 0) - RT\alpha z_j^2 I^{1/2}/(1 + BI^{1/2}) \quad (19)$$

This is in contrast with chemical thermodynamics where standard Gibbs energies of formation of species are always at zero ionic strength (see eq 3). Tables of standard transformed Gibbs energies of formation of species can be prepared for a specified ionic strength (usually the approximately physiological 0.25 M) and can be used at that ionic strength without having to deal with activity coefficients.

Calculation of the Standard Transformed Gibbs Energy of a Reactant That Consists of Two or More Species. There are two ways to calculate the standard transformed Gibbs energy of formation $\Delta_f G_i'^\circ$ of a pseudoisomer group (like ATP^{4-}, $HATP^{3-}$, and H_2ATP^{2-}). This property is equal to the mole fraction weighted-average of the standard transformed Gibbs energies of the three species plus the transformed Gibbs energy of mixing. However, a simpler way to calculate $\Delta_f G'^\circ$ is to use the following partition function[13]

$$\Delta_f G_i'^\circ = -RT \ln\left\{\sum_{j=1}^{N_{iso}} \exp[-\Delta_f G_j'^\circ/RT]\right\} \quad (20)$$

N_{iso} is the number of species in the pseudoisomer group.

The expression for the transformed chemical potential of a reactant (pseudoisomer group) is given by

$$\mu_i' = \mu_i'^\circ + RT \ln[B_i] \quad (21)$$

where $\mu_i'^\circ$ is the standard transformed chemical potential of reactant B_i at a specified temperature, pH, and ionic strength. In making calculations with experimental data, this equation is written in terms of transformed Gibbs energies.

$$\Delta_f G_i' = \Delta_f G_i'^\circ + RT \ln[B_i] \quad (22)$$

It is readily shown that

$$\sum_{i=1}^{N'} \nu_i' \Delta_f G_i'^\circ = -RT \ln K' \quad (23)$$

K' is the apparent equilibrium constant for the enzyme-catalyzed reaction.

Calculation of the Change in Binding of Hydrogen Ions in a Biochemical Reaction. In general, biochemical reactions consume or produce hydrogen ions. Equation 17 can be used to derive the expression for the change in binding of hydrogen ions in a biochemical reaction. When the reactants in a system are involved in a single biochemical reaction, $dn_i' = \nu_i' d\xi'$, where ν_i' is the stoichiometric number of the ith reactant and ξ' is the extent of the biochemical reaction. The change in the transformed Gibbs energy in a single biochemical reaction is given by

$$\Delta_r G' = \sum_{i=1}^{N'} \nu_i' \Delta_f G_i' \quad (24)$$

When there is a single biochemical reaction, the fundamental equation in eq 17 can be written as

$$dG' = -S' dT + V dP + \Delta_r G' d\xi + RT \ln(10) n_c(H) d\text{pH} \quad (25)$$

One of the Maxwell equations (mixed partial derivatives) for this fundamental equation is[9,10]

$$\frac{\partial \Delta_r G'}{\partial \text{pH}} = RT \ln(10) \frac{\partial n_c(H)}{\partial \xi} \quad (26)$$

The derivative on the right-hand side of this equation is the change in binding of hydrogen ions in the reaction, which is represented by $\Delta_r N_H$

$$\Delta_r N_H = \frac{1}{RT \ln(10)} \frac{\partial \Delta_r G'^\circ}{\partial \text{pH}} \quad (27)$$

$\Delta_r G'^\circ$ has been used here because its derivative with respect to pH is the same as that for $\Delta_r G'$. The thermodynamic property $\Delta_r N_H$ can be measured by use of a pHstat. $\Delta_r N_H$ for a biochemical reaction generally varies with the pH, and it is shown below that $\Delta_r N_H = -0.04$ at pH 5 and $\Delta_r N_H = -1$ at pH 9 for ATP + H_2O = ADP + P_i.

There is a simpler way to calculate $\Delta_r N_H$ that yields the same values for a biochemical reaction.[14]

$$\Delta_r N_H = \sum_{i=1}^{N'} \nu_i' \bar{N}_H(i) \quad (28)$$

where $\bar{N}_H(i)$is the average number of hydrogen atoms in reactant i at a specified pH.

Experimental Data on Apparent Equilibrium Constants and Enthalpies of Biochemical Reactions. Goldberg and Tewari[15−20] have surveyed the literature for experimental measurements of apparent equilibrium constants and heats of reaction for enzyme-catalyzed reactions. They have evaluated these experimental results, put them in a standard form, and ordered them according to the IUBMB classifications (EC numbers). This database is now on the web.[21] The experimental data summarized in these articles can be used to calculate species properties for biochemical reactants. For example, an inverse Legendre transform can be used.[22]

TABLE 1: Species Properties in Aqueous Solution at 298.15 M and 0 Ionic Strength from BasicBiochemData3

	$\Delta_f G°$/kJ mol^{-1}	$\Delta_f H°$/kJ mol^{-1}	z_j	$N_H(j)$
ATP^{4-}	−2768.10	−3619.21	−4	12
$HATP^{3-}$	−2811.48	−3612.91	−3	13
H_2ATP^{2-}	−2838.18	−3627.91	−2	14
ADP^{3-}	−1906.13	−2626.54	−3	12
$HADP^{2-}$	−1947.10	−2620.94	−2	13
H_2ADP^{-}	−1971.98	−2638.54	−1	14
HPO_4^{2-}	−1096.10	−1299.00	−2	1
$H_2PO_4^{-}$	−1137.30	−1302.60	−1	2
H_2O	−237.19	−285.83	0	2

BasicBiochemData3. In chemical thermodynamics in aqueous solution, it was recognized a long time ago that the best way to store thermodynamic information was to store standard Gibbs energies and standard enthalpies of formation of species at 298.15 K and zero ionic strength. Therefore, it should not be a surprise that the best way to store thermodynamic information on enzyme-catalyzed reactions is to store information on standard Gibbs energies of formation and standard enthalpies of formation of species at 298.15 K and zero ionic strength. Two more pieces of information are needed in biochemical thermodynamics; (1) electric charges on species are needed because ionic strength effects almost always have to be calculated. (2) A number of hydrogen atoms in the species are needed because pH effects almost always have to be calculated.

BasicBiochemData3[10,22] contains this information for 199 biochemical reactants. This database summarizes species properties for 199 biochemical reactants in the form of matrices that give $\Delta_f G°$ in kJ mol^{-1}, $\Delta_f H°$ in kJ mol^{-1}, electric charge, and number of hydrogen atoms for a reactant in *Mathematica*. This database can be extended by using more experimental results in the Goldberg/Tewari database.[15−21]

When the apparent equilibrium constant has been measured for an enzyme-catalyzed reaction in which all of the reactants but one have known standard Gibbs energies of formation, it is possible to calculate the standard Gibbs energies of the species of that one reactant.

Calculation of the Apparent Equilibrium Constant for ATP + H_2O = ADP + P_i. BasicBiochemData3 can be used to calculate apparent equilibrium constants, heats of reaction, and changes in binding of hydrogen ions at desired temperatures, pHs, and ionic strengths. Table 1 gives the species properties for the reactants in ATP + H_2O = ADP + P_i. The thermodynamic information in $\Delta_f G°$ and $\Delta_f H°$ is in the differences between these values, and therefore, this many digits have to be retained. This table owes a lot to Prof. Boeiro-Goates and co-workers.[24] In 2001, they determined the entropy of crystalline adenosine by the Third Law method, and with the enthalpy of adenosine from combustion calorimetry, they were able to calculate $\Delta_f G°$ and $\Delta_f H°$ for adenosine (aqueous) with respect to reference states for the elements. This made it possible to calculate $\Delta_f G°$ and $\Delta_f H°$ with respect to the elements for all of the species in the ATP series.

These species' properties make it possible to calculate standard transformed Gibbs energies of formation at 298.15 K, ionic strength 0.25 M, and pHs 5, 6, 7, 8, and 9 for ATP, ADP, P_i, and H_2O. The values in Table 2 were calculated using the *Mathematica* program listGibbsfnpHis.[10]

TABLE 2: Standard Transformed Gibbs Energies of Formation of Reactants in kJ mol^{-1}[a]

	pH 5	pH 6	pH 7	pH 8	pH 9
ATP	−2437.46	−2362.76	−2292.50	−2223.44	−2154.88
ADP	−1569.05	−1495.55	−1424.70	−1355.78	−1287.24
P_i	−1078.49	−1068.49	−1059.49	−1052.97	−1047.17
H_2O	−178.49	−167.07	−155.66	−144.24	−132.83

[a] Conditions: at 298.15 K, ionic strength 0.25 M, and five pHs.

TABLE 3: Standard Transformed Enthalpies of Formation of Reactants in kJ mol^{-1}[a]

	pH 5	pH 6	pH 7	pH 8	pH 9
ATP	−3615.72	−3615.41	−3616.89	−3617.48	−3617.55
ADP	−2625.85	−2625.73	−2627.23	−2727.71	−2627.77
P_i	−1302.90	−1302.05	−1299.39	−1297.99	−1297.79
H_2O	−286.65	−286.65	−286.65	−286.65	−286.65

[a] Conditions: at 298.15 K, ionic strength 0.25 M, and five pHs.

Table 1 also makes it possible to calculate standard transformed enthalpies of formation in kJ mol^{-1} at 298.15 K, ionic strength 0.25 M, and pHs 5, 6, 7, 8, and 9 for these reactants. The values in Table 3 were calculated using the *Mathematica* program listreactantsHT.[10]

Table 1 makes it possible to calculate standard transformed Gibbs energies of reaction, apparent equilibrium constants, and changes in binding of hydrogen ions for ATP + H_2O = ADP + P_i at 298.15 K, ionic strength 0.25 M, and pHs 5, 6, 7, 8, and 9. The values in Table 4 were calculated using *Mathematica* programs.[10]

Rapid-Equilibrium Enzyme Kinetics

There are three types of enzyme kinetic studies, transient-state kinetics, steady-state kinetics, and rapid-equilibrium kinetics. Transient-state kinetics deals with the very rapid reactions that occur when solutions of enzymes and substrates are mixed or disturbed. It requires very special equipment and is not discussed here. Steady-state studies are based on the assumption that the steps in the catalytic mechanism are in steady states. Rapid-equilibrium studies are based on the assumption that the reactions prior to the rate-determining reaction are at equilibrium.

Early History. The early history of enzyme kinetics has been described by Cornish-Bowden.[25] Henri (1902−1903) assumed that there was equilibrium between the free enzyme and enzyme−substrate complexes, but the enzyme kinetic experiments at that time were very primitive because the logarithmic scale of pH was not introduced by Sorenson until 1909. In 1913, Michaelis and Menten rediscovered the rate equation discussed by Henri and introduced the rate equation

$$v = \frac{k[\mathrm{E}]_t}{1 + \dfrac{K_A}{[\mathrm{A}]}} \tag{29}$$

$[E]_t$ is the total concentration of enzymatic sites and K_A is an equilibrium constant, referred to as a Michaelis constant. In 1925, Briggs and Haldane used the following more general mechanism

$$\mathrm{E} + \mathrm{A} \underset{k_2}{\overset{k_1}{\rightleftharpoons}} \mathrm{X} \xrightarrow{k_3} \mathrm{P} \tag{30}$$

According to this mechanism, the rate of change of the concentration of the enzyme−substrate complex X is given by

$$\frac{\mathrm{d}[\mathrm{X}]}{\mathrm{d}t} = k_1[\mathrm{EA}] - (k_2 + k_3)[\mathrm{X}] \tag{31}$$

After a short transition period, this derivative is equal to 0, and therefore, the steady-state rate equation is

$$v = \frac{k_3[\mathrm{E}]_t}{1 + \dfrac{(k_2 + k_3)}{k_1[\mathrm{A}]}} \tag{32}$$

The limiting velocity is given by $k_3[E]_t$, and the Michaelis constant is given by $(k_2 + k_3)/k_1$. However, k_1 and k_2 cannot be determined from steady-state velocity measurements. Steady-state rate equations always involve more kinetic parameters than can be estimated from velocity measurements. In 1930, Haldane made another important contribution to enzyme kinetics.[26] He showed that when the rate equations for both the forward and reverse enzyme-catalyzed reactions can be studied, the kinetic parameters can be used to calculate the apparent equilibrium constant K' for the reaction that is catalyzed.

The concept of steady-state rate equations can be applied to enzyme-catalyzed reactions with more reactants, and in 1956, King and Altman[27] published a method to derive steady-state rate equations for more complicated mechanisms using linear algebra.

This method and nomenclature were further developed by Cleland[28] in 1963, and it has been widely used.

In 1975, I. H. Segel[29] published "Enzyme Kinetics: Behavoir and Analysis of Rapid-Equilibrium and Steady-State Enzyme Systems". He pointed out that rate equations for more complicated reactions and mechanisms can be obtained by assuming that the reactions in a mechanism up to a rate-determining reaction are at equilibrium. He described the use of the rapid-equilibrium assumption as the simplest and most direct method to obtain rate equations for complex multiligand systems. He pointed out the following: "If the experimental data fit the rapid-equilibrium equation, then we have the simplest kinetic mechanism for the system. If the data do not fit, then we can proceed to more complex models and velocity equations." When rapid-equilibrium rate equations

TABLE 4: Standard Transformed Gibbs Energies of Reaction, Apparent Equilibrium Constants, And Changes in Binding of Hydrogen Ions for ATP + H_2O = ADP + P_i at 298.15 K[a]

	pH 5	pH 6	pH 7	pH 8	pH 9
$\Delta_r G'^\circ$/kJ mol^{-1}	−32.56	−33.22	−36.04	−41.07	−46.70
K'	5.06×10^5	6.61×10^5	2.06×10^6	1.57×10^7	1.52×10^8
$\Delta_r N_H$	−0.04	−0.25	−0.74	−0.96	−1.00

[a] Conditions: ionic strength 0.25 M, and five pHs.

represent the velocity data, the Michaelis constants are all biochemical equilibrium constants.

Estimation of Kinetic Parameters for A → Products. The estimation of kinetic parameters in a rate equation like eq 29 has usually been done by using linear plots. There are three ways to do this, but the most used has been Lineweaver−Burk plots of $1/\nu$ versus 1/[A]. In 1979, Duggleby[30] suggested that kinetic parameters can be estimated by measuring velocities at as many sets of substrate concentrations as there are kinetic parameters. This can be illustrated[31] with two rapid-equilibrium rate equations for A → products, where

$$\nu_1 = \frac{k_f[E]_t}{1 + \dfrac{K_A}{[A]_1}} \quad \text{and} \quad \nu_2 = \frac{k_f[E]_t}{1 + \dfrac{K_A}{[A]_2}} \tag{33}$$

It can be readily shown that the Michaelis constant is given by

$$K_A = \frac{\nu_1 - \nu_2}{\dfrac{\nu_2}{[A]_2} - \dfrac{\nu_1}{[A]_1}} \tag{34}$$

Thus,K_A can be estimated by using two velocity measurements. The limiting velocity $k_f[E]_t$ can then be calculated from either rate equation in eq 33.

When there are more kinetic parameters, Solve in *Mathematica*, *Maple*, or *MatLab* can be used to solve the set of simultaneous rate equations for the kinetic parameters. In *Mathematica*, the Solve operation is described by Solve[eqs, vars,elims], where eqs are the independent equilibrium equations that have to be solved simultaneously (equilibrium expressions and the conservation equation for enzymatic sites), vars is the name of the expression for the concentration of the enzyme−substrate complex to be obtained, and elims are the names of the concentrations to be eliminated. In rapid-equilibrium enzyme kinetics, Solve is used in two ways. First, it is used to derive rapid-equilibrium rate equations. Second, it is used to solve sets of rate equations at several sets of substrate concentrations for the kinetic parameters. The number of rate equations to be solved is equal to the number of kinetic parameters in the rate equation. Solve was developed to solve polynomial equations, and rapid-equilibrium rate equations are always polynomials.

This method has been used in a book[32] about rapid-equilibrium rate equations for seven types of enzyme-catalyzed reactions using *Mathematica*.

Because Michaelis constants in rapid-equilibrium rate equations are equilibrium constants, they are functions of temperature, pH, and ionic strength, and they yield thermodynamic information about a mechanism. Since K_A is an equilibrium constant, the standard transformed Gibbs energy for reaction 35 is given by $\Delta_r G'^\circ = -RT \ln K_A$. This means that the estimation of K_A at two or more temperatures yields $\Delta_r H'^\circ$ and $\Delta_r S'^\circ$. Primes on apparent equilibrium constants are not used in enzyme kinetics.

Derivation of the Rate Equation for A → Products When the Enzymatic Site and the Enzyme−Substrate Complex Each Have Two p*K*'s. The use of Solve to obtain the rapid-equilibrium rate equation for a more complicated mechanism can be illustrated with the following mechanism in which the enzymatic site and the enzyme−substrate complex each have two acid dissociations.

$$\begin{array}{ccccc} E^- & & EA^- & & \\ \| & & \| & & \\ HE & + A = & HEA & \rightarrow \text{products} & \\ \| & & \| & & \\ H_2E^+ & & H_2EA^+ & & \end{array} \tag{35}$$

The rapid-equilibrium velocity is given by k_f[HEA]. This mechanism involves five equilibrium constants

$$K_{HE} = [H^+][E^-]/[HE] \tag{36}$$

$$K_{H2E} = [H^+][HE]/[H_2E^+] \tag{37}$$

$$K_{HEA} = [H^+][EA^-]/[HEA] \tag{38}$$

$$K_{H2E} = [H^+][HE]/[H_2E^+] \tag{39}$$

$$K_{cHEA} = [HE][A]/[HEA] \tag{40}$$

These five equilibrium constants are chemical equilibrium constants because they are written in terms of species. These equilibrium constants are functions of temperature and ionic strength. The estimation of K_{HE} from velocity measurements makes it possible to calculate the standard Gibbs energy of reaction, $\Delta_r G^\circ(HE = H^+ + E^-) = -RT \ln K_{HE}$. Thus, $\Delta_r G^\circ$, $\Delta_r H^\circ$, and $\Delta_r S^\circ$ for the five equilibria can be determined by making velocity measurements at two or more temperatures. In using Solve, acid dissociations are discussed in terms of acid dissociation constants rather than p*K*'s because Solve was developed to solve sets of polynomial equations. In addition to eqs 36−40, the enzymatic sites have to be conserved.

$$[E]_t = [E^-] + [HE] + [H_2E^+] + [EA^-] + [HEA] + [H_2EA^+] \tag{41}$$

When eqs 36−41 are typed into Solve and [HEA] is designated as the variable to be calculated, the expression for [HEA] is obtained, and multiplying it by k_f yields the expression for the rapid-equilibrium velocity.

$$\nu = ([A]k_f[E]_t[H^+]K_{H2E}K_{H2EA})/([A][H^+]^2K_{H2E} + [H^+]^2K_{cHEA}K_{H2EA} + [A][H^+]K_{H2E}K_{H2EA} + [H^+]K_{cHEA}K_{H2E}K_{H2EA} + K_{cHEA}K_{H2E}K_{H2EA}K_{HE} + [A]K_{H2E}K_{H2EA}K_{HEA}) \tag{42}$$

It is important to understand that the choices of eqs 36−40 for the rate constants to be determined is arbitrary. The equal signs in eq 35 can be put in different places. Thus, the form of the rate equation is arbitrary. The determination of kinetic parameters in eq 42 makes it possible to calculate the kinetic parameters when the equal signs in eq 35 are moved.

An advantage in using a computer to derive rate equations is that the rate equation is obtained in computer-readable form so that very complicated rate equations can be used for calculations without retyping the rate equation. To test the estimation of the six kinetic parameters in this rate equation, the following kinetic parameters are chosen arbitrarily: $K_{HE} = 10^{-8}$, $K_{H2E} = 10^{-6}$, $K_{HEA} = 10^{-7.5}$, $K_{H2EA} = 10^{-5.5}$, $K_{cHEA} = 4$, and $k_f[E]_t = 1$. In

TABLE 5: Values of Kinetic Parameters When H_2E^+ and H_2EA^+ Each Have Two p*K*'s[a]

	$k_f[E]_t$	pK_{HEA}	pK_{HE}	K_{cHEA}	pK_{H2E}	pK_{H2EA}
no errors	1.00	7.50	8.00	4.00	6.00	5.50
$1.05 \times \nu_1$	0.98	7.53	7.98	3.92	6.00	5.49
$1.05 \times \nu_2$	1.00	7.50	8.05	4.04	6.00	5.50
$1.05 \times \nu_3$	1.07	7.47	8.01	4.37	5.99	5.54
$1.05 \times \nu_4$	1.00	7.50	7.96	3.69	6.04	5.50
$1.05 \times \nu_5$	1.00	7.50	8.00	3.99	6.00	5.47
$1.05 \times \nu_6$	1.00	7.50	8.00	4.02	5.79	5.50

[a] Velocities are measured at $\{10^{-9},100\}$, $\{10^{-9},1\}$, $\{10^{-7},100\}$, $\{10^{-7},1\}$, $\{10^{-5},100\}$, and $\{10^{-5},1\}$, and the effects of 5% errors in the measured velocities are calculated, one at a time.

Mathematica, these values are put into the rate equation by using the ReplaceAll operation (/.x→). To estimate the values of the six kinetic parameters, six velocities are calculated at six widely distributed pairs of concentrations $\{[H^+],[A]\}$. The optimum choices of pairs of substrate concentrations for the estimation of rapid-equilibrium kinetic parameters have been discussed in several articles[31,33−35] and a book.[32]

In this test, velocities are calculated at six pairs of concentrations of $\{[H^+],[A]\}$, $\nu\{10^{-9},100\} = 0.03025$, $\nu\{10^{-9},1\} = 0.01305$, $\nu\{10^{-7},100\} = 0.7164$, $\nu\{10^{-7},1\} = 0.1627$, $\nu\{10^{-5},100\} = 0.02171$, and $\nu\{10^{-5},1\} = 0.02076$. These velocities that were calculated using eq 42 are treated like experimental data, and therefore, there are six simultaneous independent equations that can be solved for the six kinetic parameters. An example of the estimation of kinetic parameters from the minimum number of velocity measurements is given in the Appendix. Correct values of the kinetic parameters are obtained, but the effects of experimental errors have to be considered. The way to do this is to change input velocities by 5% one at a time. This yields the values in Table 5.

Although the computer program using Solve is written in terms of $[H^+]$, the table presents p*K*'s. More accurate values of the kinetic parameters can be obtained by raising the 100 to 200 and reducing the 1 to 0.5.

Derivation of the Rate Equation for Ordered A + B → Products. The mechanism at a specified pH is given by

$$E + A = EA \qquad K_{IA} = [E][A]/[EA] \tag{43}$$

$$EA + B = EAB \qquad K_B = [EA][B]/[EAB] \tag{44}$$

$$EAB + \text{products} \qquad \nu = k_f[EAB] \tag{45}$$

The use of Solve yields the following rate equation[32,36,37] (see Appendix)

$$\nu = \frac{[A][B]k_f[E]_t}{[A][B] + [A]K_B + K_BK_{IA}} \tag{46}$$

To make test calculations, the following values of kinetic parameters at a specified temperature, pH, and ionic strength

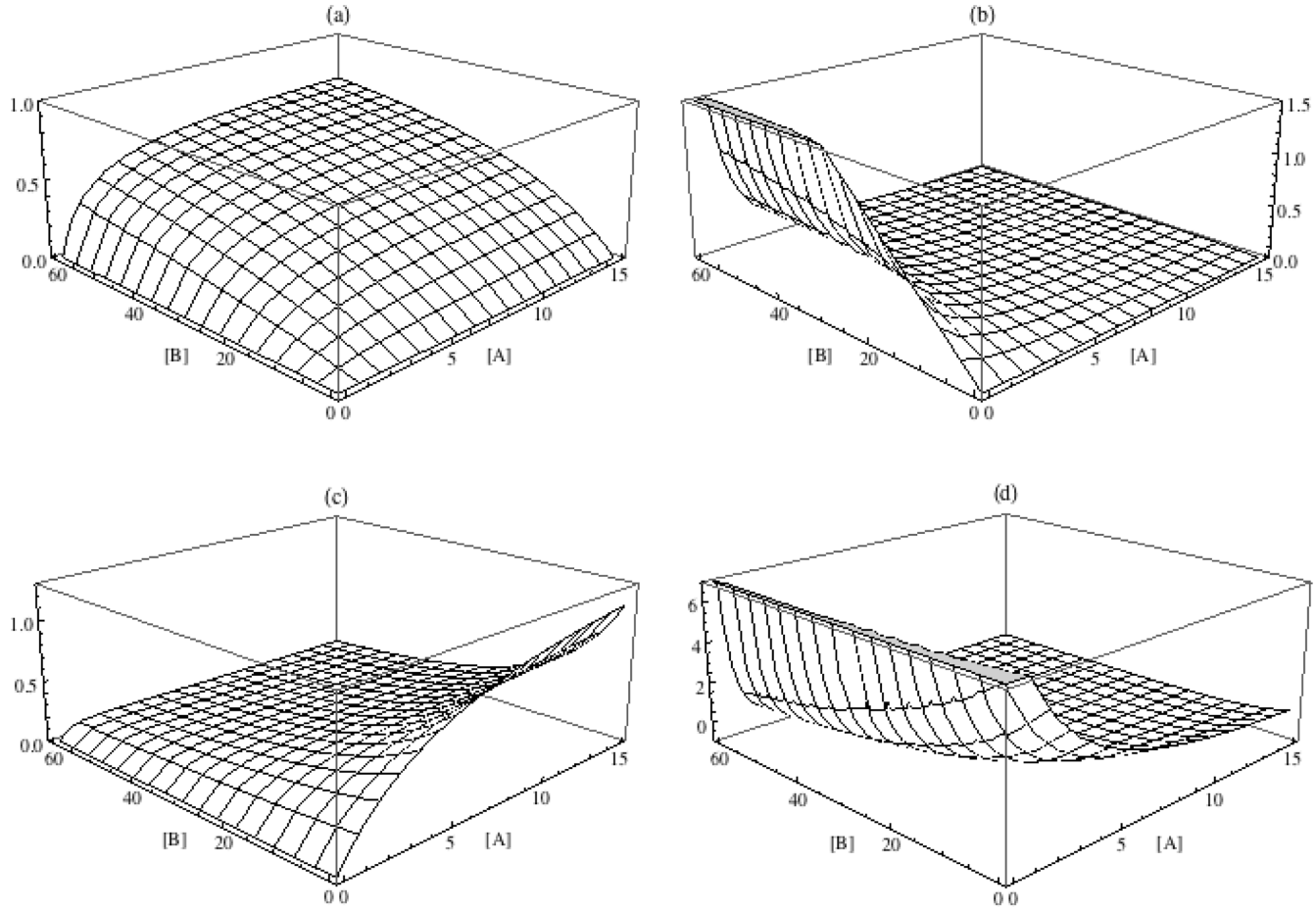

Figure 1. (a) The ν versus [A] and [B] for the ordered A + B → products when $k_f[E]_t = 1.00$, $K_{IA} = 5.00$, and $K_B = 20.00$. (b) 10 dν/d[A]. (c) 10 dν/d[B]. (d) 1000 d(dν/d[A])/d[B].

TABLE 6: Values of Kinetic Parameters for Ordered A + B → Products, Obtained by Using {[A],[B]} = {100,100}, {1,100}, and {1,1} and Testing the Effects of 5% Errors in the Measured Velocities, One at a Time

	$k_f[E]_t$	K_{IA}	K_B
no errors	1.00	5.00	20.00
$1.05 \times \nu_1$	1.06	5.29	21.24
$1.05 \times \nu_2$	1.00	4.45	20.11
$1.05 \times \nu_3$	0.99	5.28	18.74

are chosen arbitrarily: $k_f[E]_t = 1$, $K_{IA} = 5$, and $K_B = 20$. The rapid-equilibrium velocity can be plotted versus [A] and [B], as shown in Figure 1a.

The derivatives of the velocity with respect to [A] and [B] have been calculated to show the pairs of substrate concentrations at which the velocity changes most rapidly with respect to changes in substrate concentrations. Plot (b) shows that the velocity changes most rapidly with changes in [A] at low [A] and high [B]. Plot (c) shows that the velocity changes most rapidly with respect to [B] at high [A] and low [B]. Plot (d) shows that the velocity changes most rapidly with respect to [A] and [B] when both [A] and [B] are as low as practical.

Because there are three kinetic parameters, three velocities have to be measured to estimate the three kinetic parameters. Test calculations can be made at the following pairs of substrate concentrations: {[A],[B]} = {100,100}, {1,100}, and {100,1}. The following three velocities are calculated using the specified kinetic parameters $\nu\{100,100\} = 0.8264$, $\nu\{1,100\} = 0.4545$, and $\nu\{100,1\} = 0.04545$. When the three simultaneous rate equations are solved for the three kinetic parameters, the correct values are obtained, but it is necessary to consider the effects of experimental errors. This is done by introducing 5% errors into the velocities, one at a time. The values of the three kinetic parameters obtained in this way are shown in Table 6.

Velocities at high substrate concentrations are much easier to measure than velocities at low substrate concentrations because the velocity is not changing very much with changes in substrate concentrations. The experimental situation is very different at substrate concentrations below the Michaelis constants. The velocity is much more sensitive to errors in the substrate concentrations, and the velocity decreases more rapidly with extent of reaction. More accurate values of the kinetic parameters can be obtained by using a wider range of substrate concentrations. When velocities are low, replicate measurements can be used, or the enzyme concentration can be increased.

Derivation of the Rate Equation for Random A + B → Products. The mechanism at a specified pH is given by

$$E + A = EA \qquad K_{IA} = [E][A]/[EA] \tag{47}$$

$$E + B = EB \qquad K_{IB} = [E][B]/[EB] \tag{48}$$

$$EA + B = EAB \qquad K_B = [EA][B]/[EAB] \tag{49}$$

$$EAB \rightarrow \text{products} \qquad \nu = k_f[EAB] \tag{50}$$

The use of Solve yields the following rate equation[32,36,37]

$$\nu = \frac{[A][B]k_f[E]_tK_{IB}}{[B]K_BK_{IA} + [A][B]K_{IB} + [A]K_BK_{IB} + K_BK_{IA}K_{IB}} \tag{51}$$

An investigator using this mechanism can report values of K_{IA}, K_{IB}, and K_B. This looks pretty straightforward, but there is another way to write the mechanism.

$$E + A = EA \qquad K_{IA} = [E][A]/[EA] \tag{52}$$

$$E + B = EB \qquad K_{IB} = [E][B]/[EB] \tag{53}$$

$$EB + A = EAB \qquad K_A = [EB][A]/[EAB] \tag{54}$$

$$EAB \rightarrow \text{products} \qquad \nu = k_f[EAB] \tag{55}$$

The use of Solve yields the following rate equation.[32,36,37]

$$\nu = \frac{[A][B]k_f[E]_tK_{IA}}{[B]K_AK_{IA} + [A][B]K_{IA} + [A]K_AK_{IB} + K_AK_{IA}K_{IB}} \tag{56}$$

An investigator using this mechanism can report values of K_{IA}, K_{IB}, and K_A. The two investigators will report different values, but both are right. This kind of problem exists whenever a mechanism involves three sides of a thermodynamic cycle. In this case, the thermodynamic cycle is

$$\begin{array}{ccc} & K_{IA} & \\ E + A & = & EA \\ + & & + \\ B & & B \\ \| K_{IB} & & \| K_B \\ EB + A & = & EAB \\ & K_A & \end{array} \tag{57}$$

This thermodynamic cycle shows that $K_{IA}K_B = K_{IB}K_A$. Thus, both investigators should use this relation to calculate a fourth Michaelis constant.

Derivation of Rate Equations for A + B + C → Products. An example of a more complicated reaction is A + B + C → products.[33] There are five possible mechanisms for this reaction, ranging from the completely ordered mechanism to the completely random mechanism. There are 11 possible reactants and 12 possible reactions in these mechanisms. Actually, only 7 of the 12 reactions are independent.

The completely ordered mechanism I is

$$E + A = EA \tag{58}$$

$$EA + B = EAB \tag{59}$$

$$EAB + C = EABC \tag{60}$$

$$EABC \rightarrow \text{products} \tag{61}$$

The rapid-equilibrium rate equation is

$$v = \frac{[A][B][C]k_f[E]_t}{[A][B][C] + [A][B]K_{ABC} + [A]K_{AB}K_{ABC} + K_AK_{AB}K_{ABC}} \quad (62)$$

There are four kinetic parameters, and therefore, four velocities have to be measured when this is the mechanism. When the kinetic parameters are $V_{exp} = 1$, $K_A = 5$, $K_{AB} = 20$, and $K_{ABC} = 35$, test calculations can be made using the following four triplets of substrate concentrations: {[A],[B],[C]} = {200,200,200}, {0.5,200,200}, {200,0.5,200}, and {200,200,0.5}. These four velocities are $v_1 = 0.8383$, $v_2 = 0.7313$, $v_3 = 0.1198$, and $v_4 = 0.01279$. These velocities yield the correct values for the kinetic parameters, and when there are 5% errors in the velocities, one at a time, the kinetic parameters in Table 7 are obtained.

If the mechanism is completely random, all 13 kinetic parameters can be estimated from 8 velocity measurements because there are 5 thermodynamic cycles.

Effects of the Consumption of Hydrogen Ions in Mechanisms of Enzyme-Catalyzed Reactions. There are four types of pH effects on enzyme-catalyzed reactions.[38] (1) The substrates may have pK's in the pH range of interest. (2) The enzymatic site and enzyme−substrate complexes may have pK's. (3) One or more hydrogen ions may be consumed in the rate-determining reaction. This causes very large effects because it introduces $[H^+]^n$, where n is an integer, into the velocity over the whole range of pH's. (4) Hydrogen ions can also be consumed in reactions prior to the rate-determining reaction. The effects of the consumption of hydrogen ions are of special interest in connection with reductase reactions because of the large increases in the binding of hydrogen ions Δ_rN_H in the reaction that is catalyzed.[36] An example is the ferredoxin−nitrite reductase reaction

$$\text{nitrite} + 6\,\text{ferredoxin}_{red} = \text{ammonia} + 6\,\text{ferredoxin}_{ox} + 2H_2O \quad (63)$$

The values of Δ_rN_H for this reaction range from 7.99 at pH 5 to 7.64 at pH 9. If eight H^+'s are consumed in the rate-determining reaction, the limiting velocity will be given by $k_f[H^+]^8[E]_t$. This sensitivity to the concentration of hydrogen ions will make it very difficult to determine the kinetic parameters, but if hydrogen ions are consumed in reactions prior to the rate-determining reaction, it may be possible to determine the kinetic parameters.[38] Hydrogen ions can also be consumed in reactions prior to the rate-determining reaction. For example, consider ordered A + B → products when one hydrogen ion is consumed by EAB.

$$E + A = EA \qquad K_A = [E][A]/[EA] \quad (64)$$

$$EA + B = EAB \qquad K_B = [EA][B]/[EAB] \quad (65)$$

$$EAB + H^+ = EABH^+ \qquad K_{ABH} = [EAB][H^+]/[EABH^+] \quad (66)$$

$$EABH^+ \rightarrow \text{products} \qquad v = k_f[EABH^+] \quad (67)$$

This mechanism has been kept as simple as possible to illustrate the consumption of a hydrogen ion prior to the rate-determining reaction. The rapid-equilibrium rate equation can be derived, and it shows that the limiting velocity is not proportional to $[H^+]$.

TABLE 7: Values of Kinetic Parameters for the Completely Ordered Mechanism for A + B + C → Products Obtained by Using {[A],[B],[C]} = {200,200,200}, {0.5,200,200}, {200,0.5,200}, and {200,200,0.5} and Testing the Effects of 5% Errors in Velocities, One at a Time

	V_{fexp}	K_A	K_{AB}	K_{ABC}
no errors	1.00	5.00	20.00	35.00
$1.05 \times v_1$	1.06	6.63	20.00	37.12
$1.05 \times v_2$	1.00	3.11	20.17	35.01
$1.05 \times v_3$	1.00	5.30	18.74	35.21
$1.05 \times v_4$	0.99	5.00	21.11	32.84

The mechanism for a third possibility involves the consumption of a hydrogen ion by EA.

$$E + A = EA \qquad K_A = [E][A]/[EA] \quad (68)$$

$$EA + H^+ = EAH^+ \qquad K_{EAH} = [EA][H^+]/[EAH^+] \quad (69)$$

$$EAH^+ + B = EABH^+ \qquad K_{EABH} = [EAH^+][B]/[EABH^+] \quad (70)$$

$$EABH^+ \rightarrow \text{products} \qquad v = k_f[EABH^+] \quad (71)$$

The rate equation for this mechanism shows that the limiting velocity is not proportional to $[H^+]$.

Thermodynamics of Rapid-Equilibrium Enzyme Kinetics. In the discussion of the rate equation for A → products, it was pointed out that $\Delta_rG'^\circ$ for E + A = EA can be calculated from the Michaelis constant for A at a particular temperature, pH, and ionic strength. It will be of interest to determine the effects of ionic strength on $\Delta_rG'^\circ$ because the extended Debye−Hückel equation does not apply to a reaction that involves the enzymatic site. The extended Debye−Hückel only applies when the electric field around an ion is spherically symmetrical.

The estimation of equilibrium constants from rapid-equilibrium rate measurements is different from the estimation of equilibrium constants for the binding of small molecules by proteins by direct measurements, as determined by Klotz[38] for example, because direct studies involve the binding at various sites and not just at the enzymatic site. Kinetic measurements over a range of ionic strengths present the opportunity to determine the effects of ionic strength on reactions involving the enzymatic site.

A Mechanism That Can Be Represented by a Cuboid in *Mathematica*. When the enzymatic site E^- can bind A, B, and H^+ in any order and both EAB^- and HEAB can produce products, the mechanism can be represented by a cuboid in *Mathematica*, as shown in Figure 2. The cube has eight corners with eight different species, and A, B, and H^+ also count as species. Thus, the mechanism involves 11 different chemical species. The number of components is four (E, H^+, A, and B). Linear algebra can be used to show that the number of species is equal to the number of components plus the number of independent reactions, $N = C + R$. Thus, the number of independent reactions is $R = N - C = 11 - 4 = 7$. The number of reactions in the cuboid mechanism is 12, and therefore, 5 of the reactions in the cuboid are not independent. Each face represents the equation for a thermodynamic cycle (see eq 57), and therefore, there are six thermodynamic cycles in this mechanism.

A mechanism like this can be tested by (1) deriving the rate equation, (2) substituting arbitrary values of the kinetic parameters, (3) calculating velocities at arbitrary sets of substrate concentrations, and (4) using Solve to calculate the kinetic parameters from the velocities.

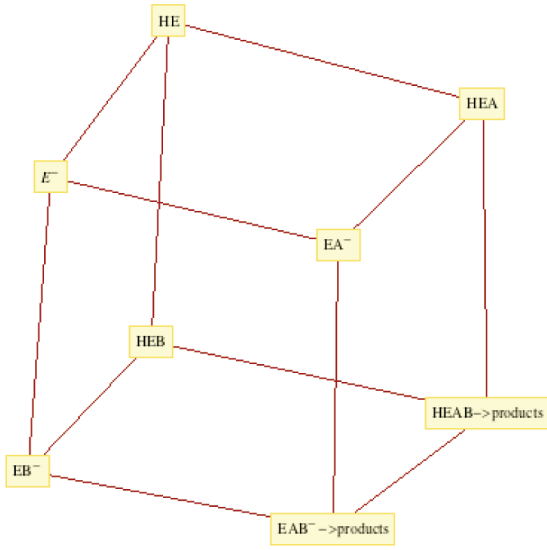

Figure 2. Cuboid representing the mechanism when the enzymatic site E^- can bind A, B, and H^+ in any order and EAB^- and HEAB can produce products.

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Appendix

The Solve operation in *Mathematica* can be used to derive the rapid-equilibrium rate equation for ordered A + B → products. Variables in *Mathematica* have to start with lowercase letters because operations, like Solve, start with capital letters. The first step is to derive the expression for the equilibrium concentration of EAB.

```
In: Solve
 [{kIA==e*a/ea,kB==ea*b/eab,et==e+ea+eab},{eab},
                                          {e,ea}]

Out: {{eab=a*b*et/(a*b+a*kB+kB*kIA)
```

In *Mathematica*, the multiply sign is * or a space. The rapid-equilibrium velocity of the reaction is given by kf*eab. When the expression for eab is multiplied by kf, the rapid-equilibrium velocity is obtained, v = kf*eab. This leads to the rate equation

```
vord = a*b*kfet/(a*b+a*kB+kB*kIA)
```

The symbol kfet is used, rather than kf*et, because the separate effects of kf and et are not discussed here; kfet is referred to as the limiting velocity.

There are three kinetic parameters for ordered A + B → products, and therefore, three velocities have to be measured to estimate the three kinetic parameters. When the kinetic parameters are kfet = 1, kIA = 5, and kB = 20, Figure 1 suggests that the three velocities should be measured at {a,b} = {100,100}, {1,100}, and {100,1}. The rate equation can be used to calculate the following three velocities $v(100,100) = 0.8265$, $v(1,100) = 0.4545$, and $v(100,1) = 0.04545$. This leads to three simultaneous equations that can be solved using Solve in *Mathematica*. This yields the correct values of the three kinetic parameters, but the effects of experimental errors in the velocity measurements have to be considered. This can be done by multiplying the three velocities by 1.05, one at a time.

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