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Rapid-equilibrium rate equations for the enzymatic catalysis of $A+B=P+Q$ over a range of pH

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Abstract

This article shows how p*K*s for the enzymatic site and enzyme-substrate complexes can be obtained from kinetic experiments on the reaction $A+B=P+Q$, with and without the consumption of hydrogen ions. The rapid-equilibrium rate equation makes it possible to obtain the p*K*s and chemical equilibrium constants involved in the mechanism, the apparent equilibrium constant K' for the catalyzed reaction, and the number of hydrogen ions consumed in the rate-determining reaction. Experimentally-determined Michaelis constants can be adjusted for the p*K*s of the substrates *A*, *B*, *P*, and *Q* so that it is easier to obtain the p*K*s of E, EA, EB, EAB, EQ, and EPQ, and the chemical equilibrium constants. Reaction rates are discussed for the forward reaction ordered $A+B=$ordered $P+Q$ with zero, one, or two hydrogen ions consumed in the rate-determining reaction and for random $A+B=$ordered $P+Q$ with zero, one, or two hydrogen ions consumed in the rate-determining reaction. When hydrogen ions are consumed in the rate-determining reaction, there is a new factor $10^{n\mathrm{pH}}$ in the rate equation, where *n* is the number of hydrogen ions consumed in the rate-determining reaction for the forward reaction. The integer *n* can be obtained from rate measurements over a range of pH, but it cannot be determined from thermodynamic measurements.

Keywords: Enzyme kinetics; Rapid-equilibrium rate equations; pH effects in kinetics; Change in binding of hydrogen ions; Rate equations

1. Introduction

This article is about enzyme mechanisms for the reaction ordered $A+B=$ordered $P+Q$ and random $A+B=$ordered $P+Q$ that have a rate-determining reaction. When enzyme-catalyzed reactions of these types do not follow the rate equations in this article, they have mechanisms without rate-determining reactions and have more complicated rate equations. The pH dependencies of the initial rates of enzyme-catalyzed reactions are not due solely to the p*K*s of the enzymatic site, enzyme-substrate complexes, and substrates, but also to the consumption of hydrogen ions in the rate-determining reaction. These two different types of pH effects have been discussed for S=P and $S+H_2O=P$ [1], three mechanisms for a type of reductase reaction [2], and for $A+B=$products when the reactants do not have p*K*s in the pH range of interest [3]. The rapid-equilibrium assumption has been used in these three papers because: (1) The number of p*K*s and chemical equilibrium constants involved in these mechanisms is large. (2) The emphasis is on the consumption of one or more hydrogen ions in the rate-determining reaction because these effects extend over the whole range of pH. Consumption of hydrogen ions in reactions prior to the rate-determining reaction will not have effects that extend over the whole pH range because the reactions preceding the rate-determining reaction compete with each other.

It is important to distinguish between the consumption of *n* hydrogen ions in the rate-determining reaction in kinetics and the change in binding of hydrogen ions $\Delta_r N_H$ in the over-all reaction that is obtained by thermodynamic measurements or calculations. $\Delta_r N_H$ can be obtained by taking the negative derivative of the logarithm of the apparent equilibrium constant K' with respect to pH [4] or from the p*K*s of the substrates and information on the number of hydrogen atoms in the most basic

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form. The changes in binding of hydrogen ions have been calculated for over 200 enzyme-catalyzed reactions [4–6]. The number n of hydrogen ions consumed in the rate-determining reaction can be determined by measuring initial reaction velocities over a range of pH, as described in this article.

The symbol for the number of hydrogen ions consumed in the rate-determining reaction can be considered to have its origin in the following equation that has been used in biochemical thermodynamics for a long time [7,8,9].

$$K' = K_{\text{ref}} 10^{n\text{pH}} f(\text{pH}) \tag{1}$$

K_{ref} is the equilibrium constant for a chemical reference reaction, n is the stoichiometric number of hydrogen ions in the reference reaction (a positive integer when hydrogen ions are produced and a negative integer when hydrogen ions are consumed). The function f(pH) brings in the pKs of the substrates; its form depends on the choice of a reference reaction. This equation suggests that hydrogen ions play two different roles in enzyme-catalyzed reactions. However, in biochemical thermodynamics the choice of a reference reaction is arbitrary, and many different choices can be made. Therefore, biochemical thermodynamics cannot be used to determine the number of hydrogen ions consumed in the rate-determining reaction, but enzyme kinetics can. When hydrogen ions are consumed in the rate-determining reaction, n is a negative integer. There are reactions for which $n = -8$ [2].

The mechanisms for ordered $A+B$=ordered $P+Q$ and random $A+B$=ordered $P+Q$ are considered here for $n = 0, -1, -2, \ldots$ In the four sections discussing these calculations, it is assumed that substrates, the enzymatic site, and enzyme-substrate complexes each consists of three species, but this number of species can be readily changed. The first section is on the mechanism ordered $A+B$=ordered $P+Q$ when no hydrogen ions are consumed in the rate-determining reaction. The second section is on random $A+B$=ordered $P+Q$ when no hydrogen ions are consumed in the rate-determining reaction. The third section is on ordered $A+B$= ordered $P+Q$ when one or two hydrogen ions are consumed in the rate-determining reaction. The fourth section is on random $A+B$=ordered $P+Q$ when one or two hydrogen ions are consumed in the rate-determining reaction.

2. Rapid-equilibrium rate equation for the enzymatic catalysis of ordered $A+B$=ordered $P+Q$ when the rate-determining reaction does not consume hydrogen ions

The ordered mechanism is represented by

$$E + A = EA \quad K_{IA} = [E][A]/[EA] \tag{2}$$

$$EA + B = EAB \quad K_B = [EA][B]/[EAB] \tag{3}$$

$$EAB \Leftrightarrow EPQ \tag{4}$$

$$EPQ = EQ + P \quad K_P = [EQ][P]/[EPQ] \tag{5}$$

$$EQ = E + Q \quad K_{IQ} = [E][Q]/[EQ] \tag{6}$$

The equal signs in this mechanism indicate that equilibria in these reactions are adjusted rapidly, and ⇔ indicates the rate-determining reaction. When the rapid-equilibrium assumption is used, the Michaelis constants are equilibrium constants [10,11]. The extension of mechanism (2)–(6) to include pH effects provides for three species of each reactant:

$$\begin{array}{ccccc} E^- & & A^- & & HEA^- \\ \| & & \| & & \| \\ HE & + & HA & = & H_2EA \\ \| & & \| & & \| \\ H_2E^+ & & H_2A^+ & & H_3EA^+ \end{array} \tag{7}$$

$$\begin{array}{ccccccccccc} HEA^- & & B^- & & H_2EAB^- & & H_2EPQ^- & & HEQ^- & & P^- \\ \| & & \| & & \| & k_{\text{f}} & \| & & \| & & \| \\ H_2EA & + & HB & = & H_3EAB & \Leftrightarrow & H_3EPQ & = & H_2EQ & + & HP \\ \| & & \| & & \| & k_{\text{r}} & \| & & \| & & \| \\ H_3EA^+ & & H_2B^+ & & H_4EAB^+ & & H_4EPQ^+ & & H_3EQ^+ & & H_2P^+ \end{array} \tag{8}$$

$$\begin{array}{ccccc} HEQ^- & & E^- & & Q^- \\ \| & & \| & & \| \\ H_2EQ & = & HE & + & HQ \\ \| & & \| & & \| \\ H_3EQ^+ & & H_2E^+ & & H_2Q^+ \end{array} \tag{9}$$

The rate constants for the rate-determining reaction are k_f and k_r. The chemical equilibrium constant expressions for the "main line" reactions are

$$K_{H2EA} = [HE][HA]/[H_2EA] \tag{10}$$

$$K_{H3EAB} = [HB][H_2EA]/[H_3EAB] \tag{11}$$

$$K_{H3EPQ} = [HP][H_2EQ]/[H_3EPQ] \tag{12}$$

$$K_{H2EQ} = [HE][HQ]/[H_2EQ] \tag{13}$$

The rate of reaction v for the rapid-equilibrium ordered $A+B=$ordered $P+Q$ reaction [10,11,12] is

$$v = \frac{\frac{V_f[A][B]}{K_{IA}K_B} - \frac{V_r[P][Q]}{K_PK_{IQ}}}{1 + \frac{[A]}{K_{IA}} + \frac{[A][B]}{K_{IA}K_B} + \frac{[Q]}{K_{IQ}} + \frac{[P][Q]}{K_{IQ}K_P}} \tag{14}$$

The pH dependencies for limiting velocities in the forward and reverse directions are given by

$$V_f = \frac{k_f[E]_t}{\left(1 + 10^{\mathrm{pH}-\mathrm{p}K_{1EAB}} + 10^{-\mathrm{pH}+\mathrm{p}K_{2EAB}}\right)} \tag{15}$$

$$V_r = \frac{k_r[E]_t}{\left(1 + 10^{\mathrm{pH}-\mathrm{p}K_{1EPQ}} + 10^{-\mathrm{pH}+\mathrm{p}K_{2EPQ}}\right)} \tag{16}$$

where $[E]_t$ is the total concentration of enzymatic sites. Note that $\mathrm{p}K_1 > \mathrm{p}K_2$. Plots of V_f and V_r versus pH each yield three kinetic constants.

The pH dependencies of the Michaelis constants K_{IA}, K_B, K_P, and K_{IQ} are given by

$$K_{IA} = \frac{K_{H2EA}\left(1 + 10^{\mathrm{pH}-\mathrm{p}K_{1E}} + 10^{-\mathrm{pH}+\mathrm{p}K_{2E}}\right)\left(1 + 10^{\mathrm{pH}-\mathrm{p}K_{1A}} + 10^{-\mathrm{pH}+\mathrm{p}K_{2A}}\right)}{\left(1 + 10^{\mathrm{pH}-\mathrm{p}K_{1EA}} + 10^{-\mathrm{pH}+\mathrm{p}K_{2EA}}\right)} \tag{17}$$

$$K_B = \frac{K_{H3EAB}\left(1 + 10^{\mathrm{pH}-\mathrm{p}K_{1EA}} + 10^{-\mathrm{pH}+\mathrm{p}K_{2EA}}\right)\left(1 + 10^{\mathrm{pH}-\mathrm{p}K_{1B}} + 10^{-\mathrm{pH}+\mathrm{p}K_{2B}}\right)}{\left(1 + 10^{\mathrm{pH}-\mathrm{p}K_{1EAB}} + 10^{-\mathrm{pH}+\mathrm{p}K_{2EAB}}\right)} \tag{18}$$

$$K_P = \frac{K_{H3EPQ}\left(1 + 10^{\mathrm{pH}-\mathrm{p}K_{1EQ}} + 10^{-\mathrm{pH}+\mathrm{p}K_{2EQ}}\right)\left(1 + 10^{\mathrm{pH}-\mathrm{p}K_{1P}} + 10^{-\mathrm{pH}+\mathrm{p}K_{2P}}\right)}{\left(1 + 10^{\mathrm{pH}-\mathrm{p}K_{1EPQ}} + 10^{-\mathrm{pH}+\mathrm{p}K_{2EPQ}}\right)} \tag{19}$$

$$K_{IQ} = \frac{K_{H2EQ}\left(1 + 10^{\mathrm{pH}-\mathrm{p}K_{1E}} + 10^{-\mathrm{pH}+\mathrm{p}K_{2E}}\right)\left(1 + 10^{\mathrm{pH}-\mathrm{p}K_{1Q}} + 10^{-\mathrm{pH}+\mathrm{p}K_{2Q}}\right)}{\left(1 + 10^{\mathrm{pH}-\mathrm{p}K_{1EQ}} + 10^{-\mathrm{pH}+\mathrm{p}K_{2EQ}}\right)} \tag{20}$$

The Haldane equation obtained from Eq. (14) is

$$K'(A+B=P+Q) = \frac{V_fK_PK_{IQ}}{V_rK_{IA}K_B} = K_{\mathrm{ref}}\frac{\left(1 + 10^{\mathrm{pH}-\mathrm{p}K_{1P}} + 10^{\mathrm{p}K_{2P}-\mathrm{pH}}\right)\left(1 + 10^{\mathrm{pH}-\mathrm{p}K_{1Q}} + 10^{\mathrm{p}K_{2Q}-\mathrm{pH}}\right)}{\left(1 + 10^{\mathrm{pH}-\mathrm{p}K_{1A}} + 10^{\mathrm{p}K_{2A}-\mathrm{pH}}\right)\left(1 + 10^{\mathrm{pH}-\mathrm{p}K_{1B}} + 10^{\mathrm{p}K_{2B}-\mathrm{pH}}\right)} \tag{21}$$

where

$$K_{\mathrm{ref}} = \frac{[HP][HQ]}{[HA][HB]} = \frac{k_fK_{H3EPQ}K_{H2EQ}}{k_rK_{H3EAB}K_{H2EA}} \tag{22}$$

is the expression for the chemical equilibrium constant for the reference reaction $HA+HB=HP+HQ$ in terms of the parameters in mechanism (7)–(9).

According to mechanism (7)–(9), the pH dependencies of V_f and V_r each involve three parameters, as shown in Eqs. (15) and (16). Thus $k_f[E]_t$, $\mathrm{p}K_{1\mathrm{EAB}}$, $\mathrm{p}K_{2\mathrm{EAB}}$, $k_r[E]_t$, $\mathrm{p}K_{1\mathrm{EPQ}}$, and $\mathrm{p}K_{2\mathrm{EPQ}}$ can readily be obtained from experimental data. However, each of the

Table 1
Input of kinetic constants for derABPQkinHB in addition to that in the text

Input	Section 1	Section 2	Section 3	Section 4
pK_{1EB}	12	7.5	12	7.5
pK_{2EB}	2	5.5	2	5.5
K_{H2EB}	10,000	0.5	10,000	0.5
N	0	0	−1,−2	−1,−2
X	0	0	7.5	7.5

Michaelis constants involves six p*K*s and a chemical equilibrium constant. The eight p*K*s of A, B, P, and Q can be determined by acid titrations, and so the effects of these p*K*s on the Michaelis constants can be moved to the left-hand sides of Eqs. (17)–(20) to obtain the following adjusted Michaelis constants:

$$K_{IA\mathrm{adj}} = K_{IA}/\left(1+10^{\mathrm{pH}-\mathrm{p}K_{1A}}+10^{\mathrm{p}K_{2A}-\mathrm{pH}}\right) = \frac{K_{H2EA}\left(1+10^{\mathrm{pH}-\mathrm{p}K_{1E}}+10^{-\mathrm{pH}+\mathrm{p}K_{2E}}\right)}{\left(1+10^{\mathrm{pH}-\mathrm{p}K_{1EA}}+10^{-\mathrm{pH}+\mathrm{p}K_{2EA}}\right)} \tag{23}$$

$$K_{B\mathrm{adj}} = K_{B}/\left(1+10^{\mathrm{pH}-\mathrm{p}K_{1B}}+10^{\mathrm{p}K_{2B}-\mathrm{pH}}\right) = \frac{K_{H3EAB}\left(1+10^{\mathrm{pH}-\mathrm{p}K_{1EA}}+10^{-\mathrm{pH}+\mathrm{p}K_{2EA}}\right)}{\left(1+10^{\mathrm{pH}-\mathrm{p}K_{1EAB}}+10^{-\mathrm{pH}+\mathrm{p}K_{2EAB}}\right)} \tag{24}$$

$$K_{P\mathrm{adj}} = K_{P}/\left(1+10^{\mathrm{pH}-\mathrm{p}K_{1P}}+10^{\mathrm{p}K_{2P}-\mathrm{pH}}\right) = \frac{K_{H3EPQ}\left(1+10^{\mathrm{pH}-\mathrm{p}K_{1EQ}}+10^{-\mathrm{pH}+\mathrm{p}K_{2EQ}}\right)}{\left(1+10^{\mathrm{pH}-\mathrm{p}K_{1EPQ}}+10^{-\mathrm{pH}+\mathrm{p}K_{2EPQ}}\right)} \tag{25}$$

$$K_{IQ\mathrm{adj}} = K_{IQ}/\left(1+10^{\mathrm{pH}-\mathrm{p}K_{1Q}}+10^{\mathrm{p}K_{2Q}-\mathrm{pH}}\right) = \frac{K_{H2EQ}\left(1+10^{\mathrm{pH}-\mathrm{p}K_{1E}}+10^{-\mathrm{pH}+\mathrm{p}K_{2E}}\right)}{\left(1+10^{\mathrm{pH}-\mathrm{p}K_{1EQ}}+10^{-\mathrm{pH}+\mathrm{p}K_{2EQ}}\right)} \tag{26}$$

These adjustments can be applied to individual values of Michaelis constants obtained experimentally. Each of the adjusted Michaelis constants involves five kinetic constants, which makes it difficult to obtain these kinetic constants by curve fitting. The two rate constants, four chemical equilibrium constants, and twelve p*K*s can be determined as follows from the experimental pH dependencies of the two limiting velocities and four adjusted Michaelis constants: The plot of V_f/K_{Badj} yields K_{H3EAB}, $\mathrm{p}K_{\mathrm{1EA}}$, and $\mathrm{p}K_{\mathrm{2EA}}$ as shown by

$$\frac{V_f}{K_{B\mathrm{adj}}} = \frac{k_f[E]_t}{K_{H3EAB}\left(1+10^{\mathrm{pH}-\mathrm{p}K_{1EA}}+10^{\mathrm{p}K_{2EA}-\mathrm{pH}}\right)} \tag{27}$$

The plot of V_r/K_{Padj} yields K_{H3EPQ}, $\mathrm{p}K_{\mathrm{1EQ}}$, and $\mathrm{p}K_{\mathrm{2EQ}}$ as shown by

$$\frac{V_r}{K_{P\mathrm{adj}}} = \frac{k_r[E]_t}{K_{H3EPQ}\left(1+10^{\mathrm{pH}-\mathrm{p}K_{1EQ}}+10^{\mathrm{p}K_{2EQ}-\mathrm{pH}}\right)} \tag{28}$$

The plot of $V_f/K_{\mathrm{Badj}}K_{\mathrm{IAadj}}$ yields K_{H2EA}, $\mathrm{p}K_{\mathrm{1E}}$, and $\mathrm{p}K_{\mathrm{2E}}$ as shown by

$$\frac{V_f}{K_{B\mathrm{adj}}K_{IA\mathrm{adj}}} = \frac{k_f[E]_t}{K_{H2EA}\left(1+10^{\mathrm{pH}-\mathrm{p}K_{1E}}+10^{\mathrm{p}K_{2E}-\mathrm{pH}}\right)} \tag{29}$$

The plot of $V_r/K_{\mathrm{Padj}}K_{\mathrm{IQadj}}$ yields K_{H2EQ} and verifies $\mathrm{p}K_{\mathrm{1E}}$, and $\mathrm{p}K_{\mathrm{2E}}$ as shown by

$$\frac{V_r}{K_{P\mathrm{adj}}K_{IQ\mathrm{adj}}} = \frac{k_r[E]_t}{K_{H2EQ}\left(1+10^{\mathrm{pH}-\mathrm{p}K_{1E}}+10^{\mathrm{p}K_{2E}-\mathrm{pH}}\right)} \tag{30}$$

This is not all new information, but the emphasis here is on the use of adjusted Michaelis constants. When *A*, *B*, *P*, and *Q* do not have any p*K*s in the pH range of interest, the experimental Michaelis constants are equal to adjusted Michaelis constants.

The calculation of initial reaction velocities *v* for ordered $A+B=$ordered $P+Q$ requires 24 kinetic constants (eighteen p*K*s, two rate constants, and four chemical equilibrium constants). Fortunately, Mathematica$^{\mathrm{R}}$ is well suited to making calculations of this type because of its symbolic capabilities. Fourteen kinetic parameters need to be calculated (two limiting velocities, four Michaelis constants, four adjusted Michaelis constants, and four ratios that are useful). The function for the initial reaction velocity v also needs to be derived. A Mathematica program can be written to do this, but this program can be extended to calculate kinetic properties for random $A+B=$ordered $P+Q$ and the consumption of hydrogen ions in the rate-determining reaction. In order to provide for these possibilities, it is necessary to include five more inputs (pK_{1EB}, pK_{2EB}, K_{H2EB}, n, and x). The program derABPQkinHB requires input of 29 constants, and it produces 19 kinetic parameters, including v. This program, which is given in the Appendix, has been used to make all the calculations in this paper. Twentyfour of the kinetic constants involved in ordered $A+B=$ordered $P+Q$ are required in all the calculations presented here. The following constants have been set arbitrarily to make it possible to show what this program can do: $pK_{1E}=7$, $pK_{2E}=6$, $pK_{1EA}=8$, $pK_{2EA}=6$, $pK_{1EQ}=8$, $pK_{2EQ}=7$, $pK_{1EAB}=8$, $pK_{2EAB}=7$, $pK_{1EPQ}=6$, $pK_{2EPQ}=5$, $pK_{1A}=8$, $pK_{2A}=7$, $pK_{1B}=7.7$, $pK_{2B}=6.5$, $pK_{1P}=8$, $pK_{2P}=7$, $pK_{1Q}=8$, $pK_{2Q}=6$, $k_f[E]_t=2$, $k_r[E]_t=1$, $K_{H2EA}=1$, $K_{H3EAB}=2$, $K_{H2EQ}=3$, and $K_{H3EQ}=4$. When some of the pKs are not needed, pK_1 can be set outside the range of interest, for example 12, and pK_2 can be set at 2. A chemical equilibrium constant that is not needed can be set very high, for example 10,000. This program shows how it is possible to make calculations on several types of enzyme mechanisms with a single program. This program can be used to calculate all the kinetic parameters and the velocity for ordered $A+B=$ordered $P+Q$, random $A+B=$ordered $A+B$, and ordered $A+B=$random $P+Q$ at $n=0,-1,-2,\ldots$ Table 1 shows the additional input required to treat the random mechanism and the consumption of hydrogen ions in the rate-determining reaction.

Fig. 1 shows the pH dependencies of the two limiting velocities and four Michaelis constants. In treating experimental data, the limiting velocities each involve three kinetic constants that can be readily calculated from the first two plots, but the Michaelis constants are each determined by seven kinetic constants. Since the pKs of the substrates can be determined by acid titrations, it is

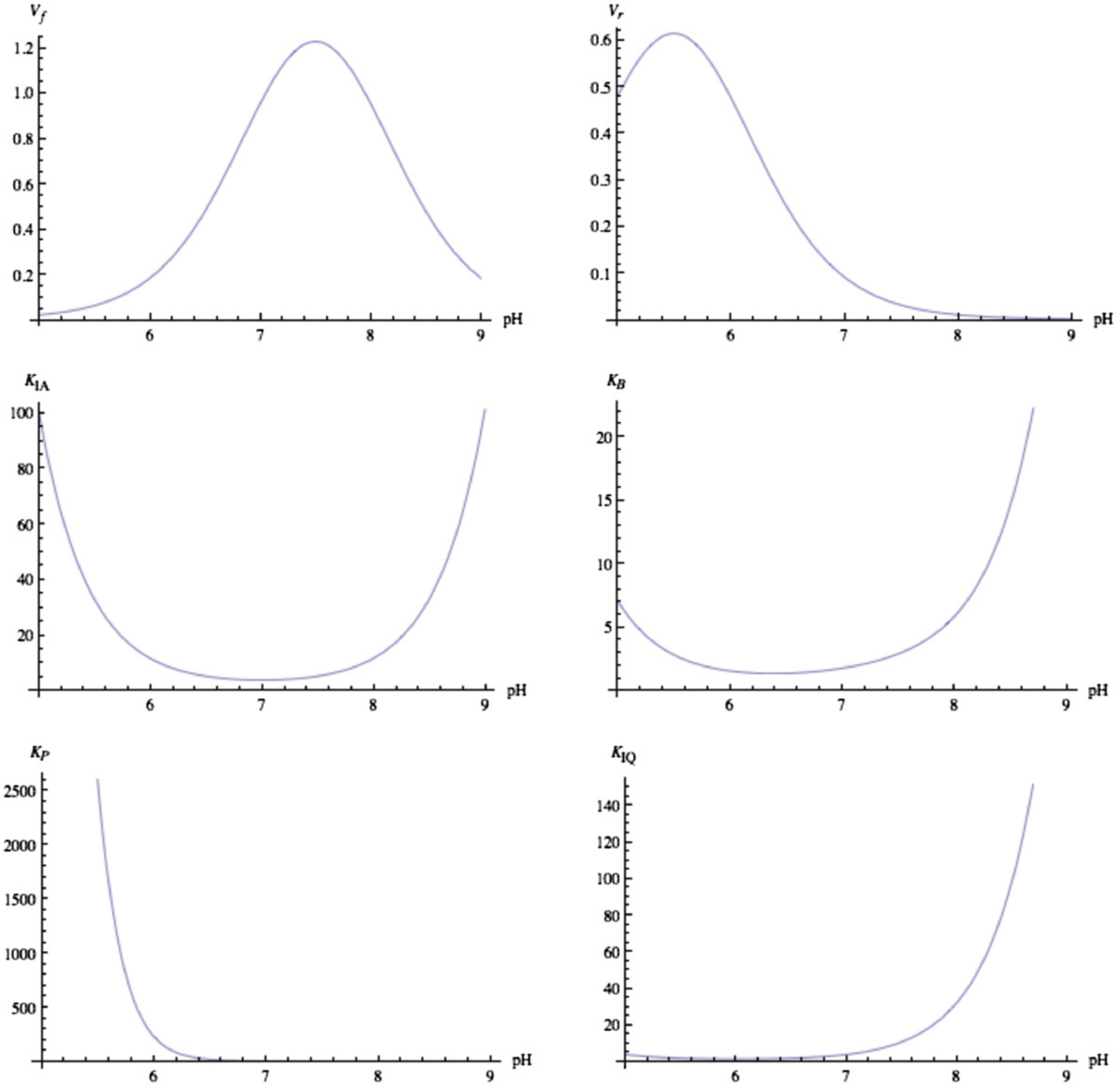


Fig. 1. Kinetic parameters for ordered $A+B=$ordered $P+Q$ as functions of pH when no hydrogen ions are consumed in the rate-determining reaction. The kinetic constants are given in the text.

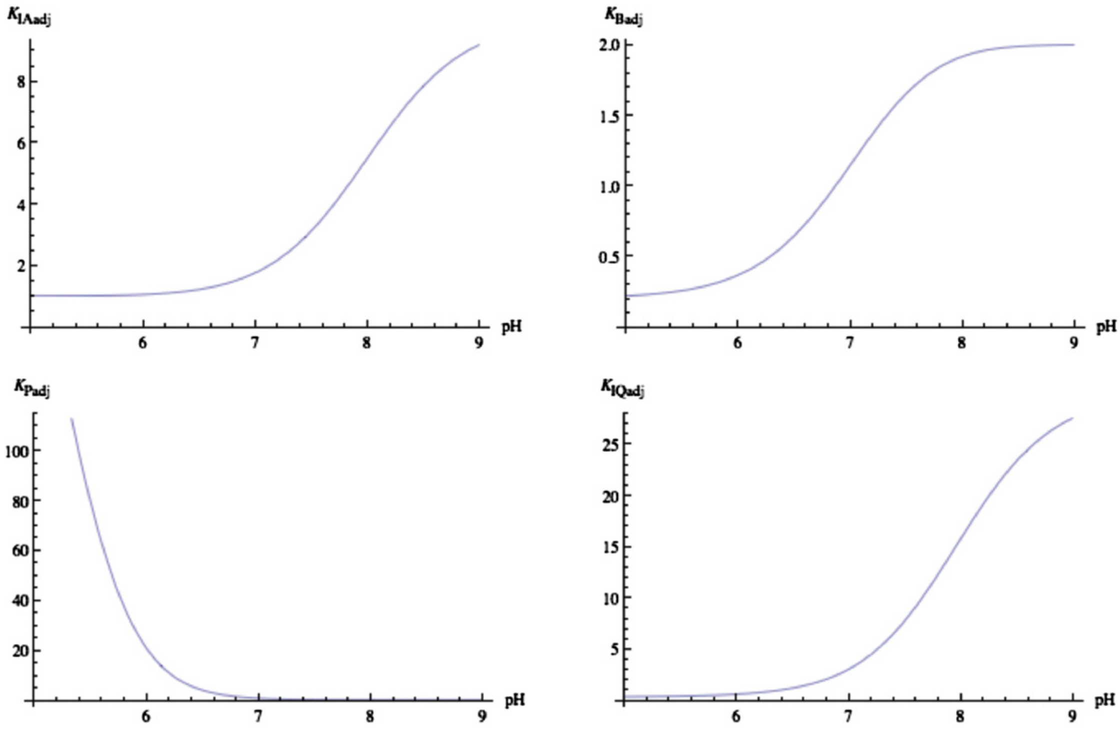


Fig. 2. Adjusted Michaelis constants for ordered $A+B=$ordered $P+Q$ obtained by removing the substrate p*K*s. These kinetic parameters are each determined by five kinetic constants.

useful to take them out and make further calculations with adjusted Michaelis constants (Eqs. (23)–(26)). Fig. 2 shows the pH dependencies of the adjusted Michaelis constants. Since it is difficult to obtain five kinetic constants from each of the plots of K_{IAadj}, K_{Badj}, K_{Padj}, and K_{IQadj}, it is useful to plot the ratios given in Eqs. (27)–(30). The plots shown in Fig. 3 make it convenient to calculate the three kinetic parameters involved in each of the plots. Figs. 1 and 3 yield all 24 kinetic constants in the following way: 6 kinetic constants are obtained from V_{f} and V_{r}, 8 p*K*s are determined by acid titrations of the substrates, and 10 kinetic constants are obtained from the bell-shaped plots in Fig. 3. The plot $V_{\mathrm{r}}/K_{\mathrm{IQadj}}K_{\mathrm{Padj}}$ confirms $\mathrm{p}K_{1\mathrm{E}}$ and $\mathrm{p}K_{2\mathrm{E}}$, but it also yields a chemical equilibrium constant K_{H2EQ}.

The expression for the initial velocity of the catalyzed reaction as a function of pH is too big to write out here, but Mathematica does write it out. Since the initial reaction velocity of the forward reaction is a function of [*A*], [*B*], and pH, it can be shown in a three-dimensional plot at a specified pH. These plots are given in Fig. 4 for pHs 5, 6, 7, and 8.

At high concentrations of *A*, the plots show the Michaelis constant for *B*.

3. Rapid-equilibrium rate equation for the enzymatic catalysis of random *A* + *B* = ordered *P* + *Q* when the rate-determining reaction does not consume hydrogen ions

When A and B are bound by the enzyme at random, the following reaction has to be added to mechanism (2)–(6).

$$E + B = EB \quad K_{IB} = \frac{[E][B]}{[EB]} \tag{31}$$

The acid dissociations and additional chemical equilibrium constant are shown by

$$\begin{array}{lll} E^{-} & B^{-} & HEB^{-} \\ || & || & || \\ HE & + HB = & H_2EB \\ || & || & || \\ H_2E^{+} & H_2B^{+} & H_3EB^{+} \end{array} \qquad K_{H2EB} = \frac{[HE][HB]}{[H_2EB]} \tag{32}$$

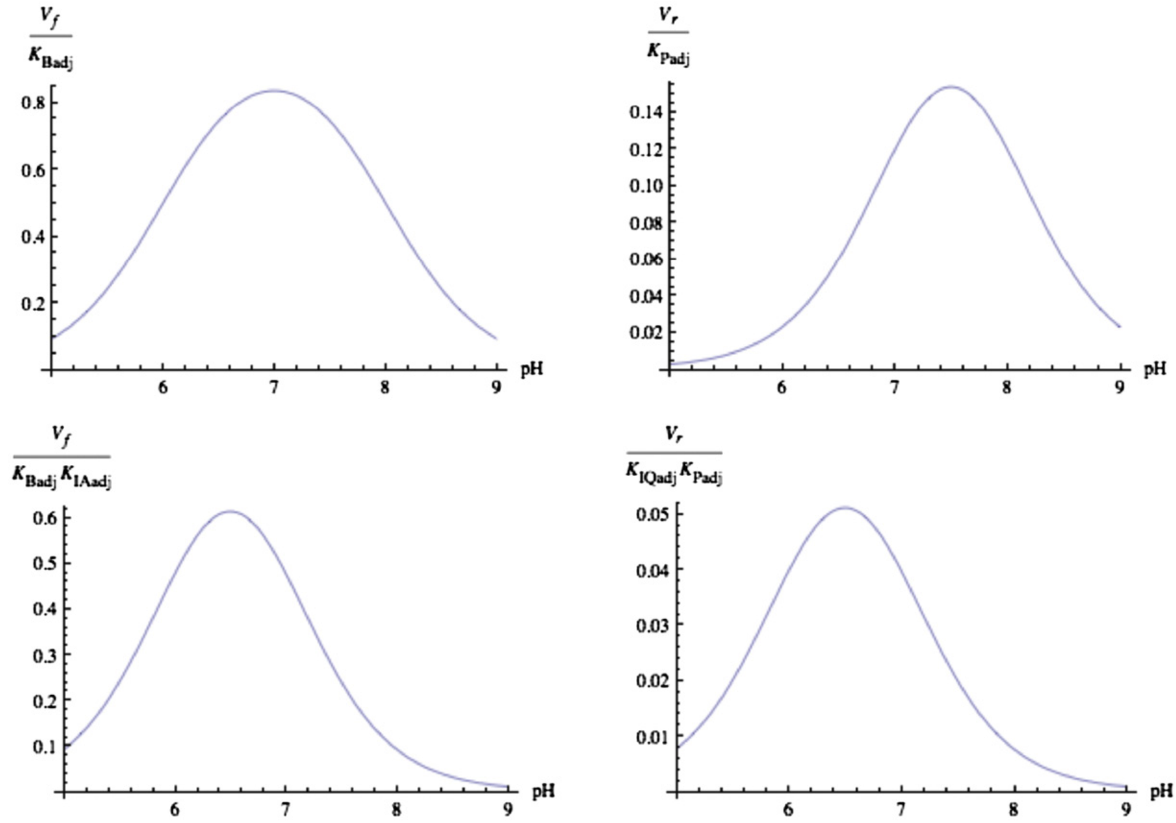


Fig. 3. Ratios of kinetic parameters for ordered $A+B=$ordered $P+Q$ versus pH when no hydrogen ions are consumed in the rate-determining reaction. These plots of experimental data each yield three kinetic constants. The last two plots both yield p$K_{1\mathrm{E}}$ and p$K_{2\mathrm{E}}$.

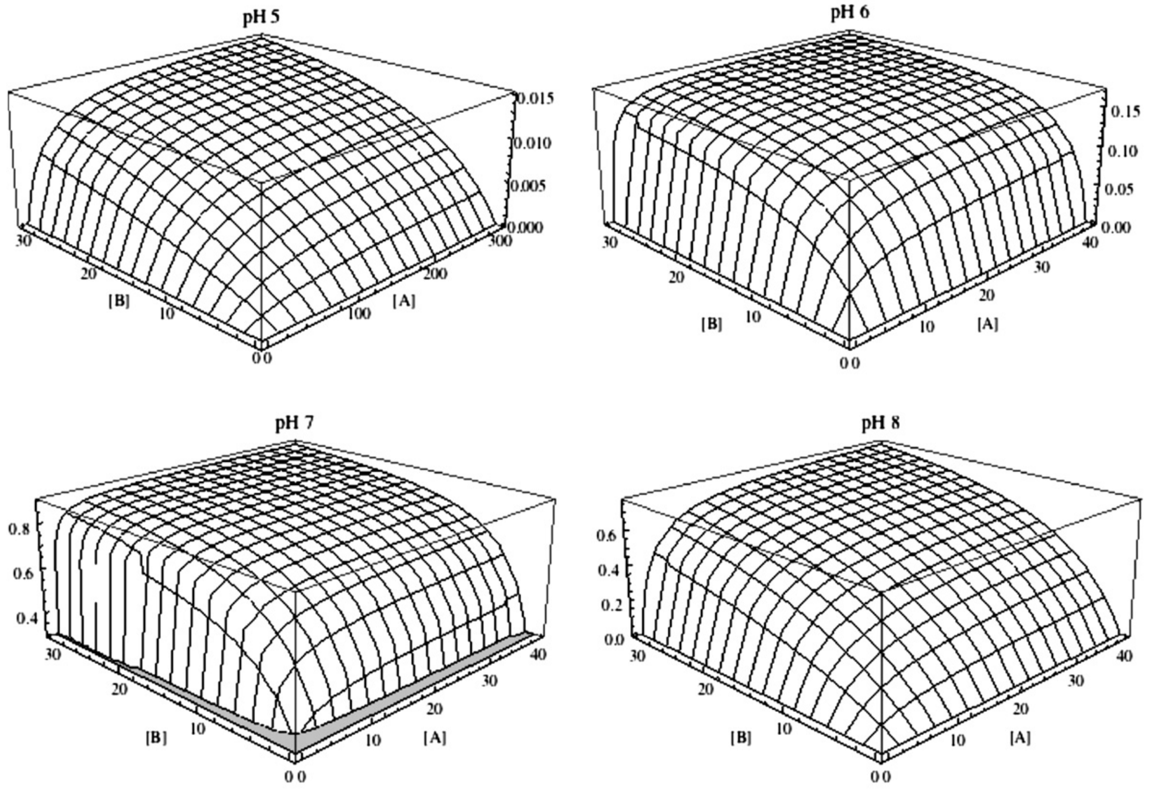


Fig. 4. Three-dimensional plots of initial velocities of the forward reaction at four pHs when no hydrogen ions are consumed in the rate-determining reaction. The ordinate is the initial reaction velocity *v*.

The pH dependence of K_{IB} is shown by

$$K_{IB} = \frac{K_{H2EB}\left(1 + 10^{\mathrm{pH}-\mathrm{p}K_{1E}} + 10^{-\mathrm{pH}+\mathrm{p}K_{2E}}\right)\left(1 + 10^{\mathrm{pH}-\mathrm{p}K_{1B}} + 10^{-\mathrm{pH}+\mathrm{p}K_{2B}}\right)}{\left(1 + 10^{\mathrm{pH}-\mathrm{p}K_{1EB}} + 10^{-\mathrm{pH}+\mathrm{p}K_{2EB}}\right)} \tag{33}$$

The effects of pK_{1EB} and pK_{2EB} can be taken out of the Michaelis constant K_{IB} as shown by

$$K_{IB\mathrm{adj}} = \frac{K_{H2EB}\left(1 + 10^{\mathrm{pH}-pK_{1E}} + 10^{-\mathrm{pH}+\mathrm{p}K_{2E}}\right)}{\left(1 + 10^{\mathrm{pH}-\mathrm{p}K_{1EB}} + 10^{-\mathrm{pH}+\mathrm{p}K_{2EB}}\right)} \tag{34}$$

The following ratio makes it possible to obtain pK_{1EB}, pK_{2EB}, and K_{H2EB} from experimental data.

$$\frac{K_{IB\mathrm{adj}}V_f}{K_{IA\mathrm{adj}}K_{B\mathrm{adj}}} = \frac{K_{H2EB}k_f[E]_t}{K_{H2EA}K_{H3EAB}\left(1 + 10^{\mathrm{pH}-\mathrm{p}K_{1EB}} + 10^{\mathrm{p}K_{2EB}-\mathrm{pH}}\right)} \tag{35}$$

The rapid-equilibrium rate of reaction v for the random $A+B$=ordered $P+Q$ mechanism [10,11] is

$$v = \frac{\frac{V_f[A][B]}{K_{IA}K_B} - \frac{V_r[P][Q]}{K_P K_{IQ}}}{1 + \frac{[A]}{K_{IA}} + \frac{[B]}{K_{IB}} + \frac{[A][B]}{K_{IA}K_B} + \frac{[Q]}{K_{IQ}} + \frac{[P][Q]}{K_{IQ}K_P}} \tag{36}$$

There is an additional term in the denominator for B. The Haldane equation in Eq. (21) is not changed because the numerator of the rate equation is not affected the binding of B at the enzymatic site. The equations for the limiting velocities and Michaelis constants in the preceding section are not affected by the inclusion of $E+B=EB$. Fig. 5 shows the comparison of the pH dependencies of K_{B} and K_{IB}. The ratio of kinetic parameters $K_{\mathrm{IBadj}}V_{\mathrm{f}}/K_{\mathrm{IAadj}}K_{\mathrm{Badj}}$ is also shown in Fig. 5. The random mechanism reduces the initial velocity of the forward reaction, as shown by Fig. 6, but the limiting velocity is not affected.

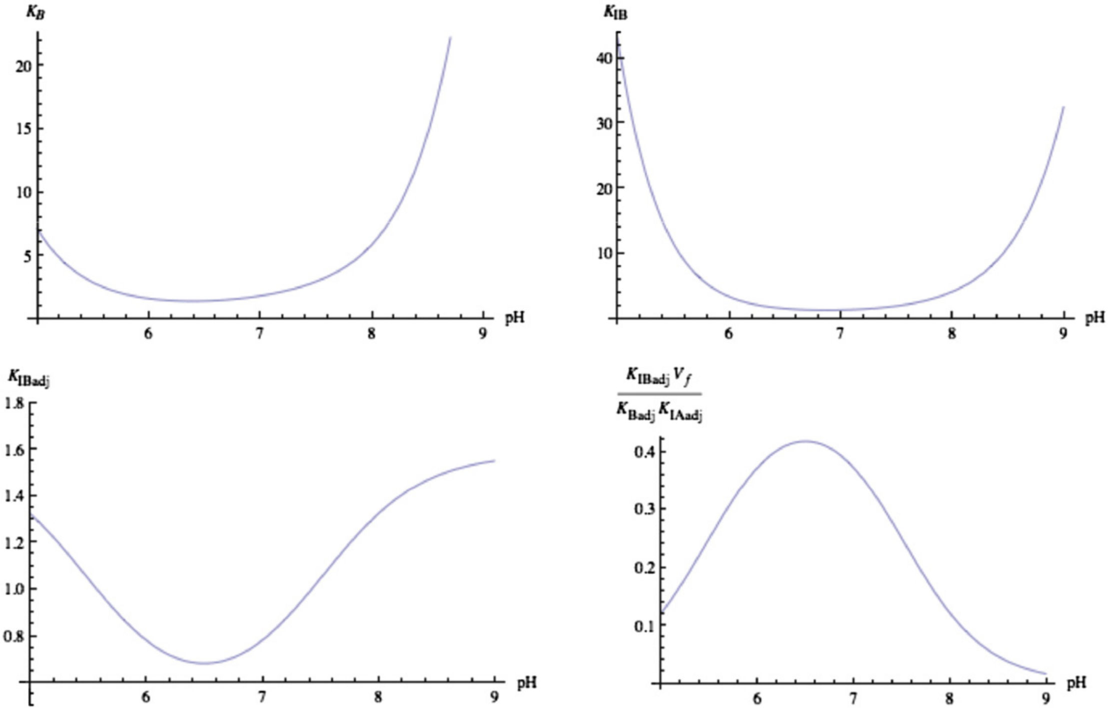
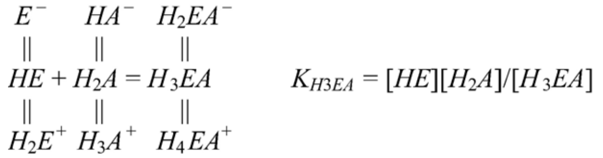


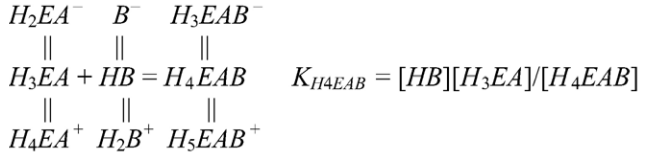
Fig. 5. Michaelis constants for B and a ratio of kinetic parameters for the random $A+B$=ordered $P+Q$ reaction. The plot for K_{B} is duplicated from Fig. 1. The ratio plot yields pK_{1EB}, K_{2EB}, and K_{H2EB}.

4. Rapid-equilibrium rate equation for the enzymatic catalysis of ordered $A+B=$ordered $P+Q$ when one or two hydrogen ions are consumed in the rate-determining reaction

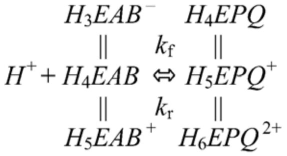
In the following mechanism a single hydrogen ion is consumed in the rate-determining reaction.



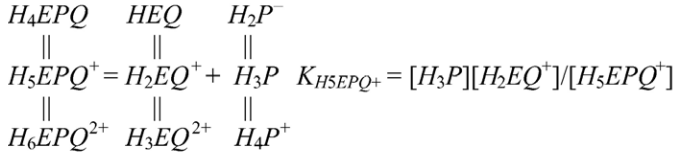
$$\begin{array}{ccc} E^- & HA^- & H_2EA^- \\ \| & \| & \| \\ HE\ + & H_2A\ = & H_3EA \\ \| & \| & \| \\ H_2E^+ & H_3A^+ & H_4EA^+ \end{array} \qquad K_{H3EA} = [HE][H_2A]/[H_3EA] \tag{37}$$



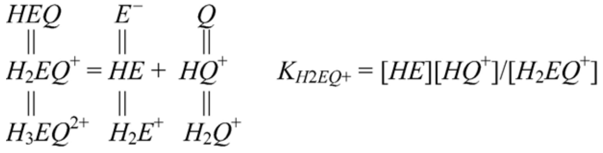
$$\begin{array}{ccc} H_2EA^- & B^- & H_3EAB^- \\ \| & \| & \| \\ H_3EA\ + & HB\ = & H_4EAB \\ \| & \| & \| \\ H_4EA^+ & H_2B^+ & H_5EAB^+ \end{array} \qquad K_{H4EAB} = [HB][H_3EA]/[H_4EAB] \tag{38}$$



$$\begin{array}{cccc} & H_3EAB^- & & H_4EPQ \\ & \| & k_f & \| \\ H^+\ + & H_4EAB & \Leftrightarrow & H_5EPQ^+ \\ & \| & k_r & \| \\ & H_5EAB^+ & & H_6EPQ^{2+} \end{array} \tag{39}$$



$$\begin{array}{ccc} H_4EPQ & HEQ & H_2P^- \\ \| & \| & \| \\ H_5EPQ^+ = & H_2EQ^+\ + & H_3P \\ \| & \| & \| \\ H_6EPQ^{2+} & H_3EQ^{2+} & H_4P^+ \end{array} \quad K_{H5EPQ+} = [H_3P][H_2EQ^+]/[H_5EPQ^+] \tag{40}$$



$$\begin{array}{ccc} HEQ & E^- & Q \\ \| & \| & \| \\ H_2EQ^+ = & HE\ + & HQ^+ \\ \| & \| & \| \\ H_3EQ^{2+} & H_2E^+ & H_2Q^+ \end{array} \qquad K_{H2EQ+} = [HE][HQ^+]/[H_2EQ^+] \tag{41}$$

The chemical reference reaction is given by

$$H^+ + H_2A + HB = H_3P + HQ^+ \quad K_{\mathrm{ref}} = \frac{[H_3P][HQ^+]}{[H^+][H_2A][HB]} = \frac{10^{\mathrm{pH}}[H_3P][HQ^+]}{[H_2A][HB]} \tag{42}$$

This reference reaction can be compared with the chemical reaction

$$\mathrm{H}^+ + \mathrm{NAD}_{\mathrm{red}}(\mathrm{H}_{27})^{2-} + \mathrm{acetaldehyde}(\mathrm{H}_4) = \mathrm{ethanol}(\mathrm{H}_6) + \mathrm{NAD}_{\mathrm{ox}}(\mathrm{H}_{26})^- \tag{43}$$

$\mathrm{NAD}_{\mathrm{red}}^{2-}$ is like $\mathrm{H_2A}$ in that it loses a hydrogen atom to form $\mathrm{NAD}_{\mathrm{ox}}^-$, which is like HQ^+. Acetaldehyde is like HB in that it gains two hydrogen atoms to form ethanol, which is like $\mathrm{H_3P}$.

The effect of the consumption of H^+ in the rate-determining reaction is that k_f in mechanism (7)–(9) is replaced by $k_f[\mathrm{H}^+]=k_f10^{-\mathrm{pH}}$ in the rate equation for mechanism (37)–(41). There are two ways this can be done in the rapid-equilibrium rate equation: replace $V_f=k_f[E]_t$ in rate Eq. (14) with $V_f=k_f10^{-\mathrm{pH}}[E]_t$ or replace V_f in rate Eq. (14) with $10^{-\mathrm{pH}}V_f$. The first choice is not useful because then V_f does not have the bell-shaped plot versus pH that is expected from Eq. (15) and is often obtained experimentally. The second choice preserves the bell-shaped plot of V_f and introduces the experimental limiting velocity $V_{\mathrm{fexp}}=10^{-\mathrm{pH}}V_f$, where V_f is given by Eq. (15). The expressions for the pH dependencies of V_f, V_r, and the four Michaelis constants are not affected by the consumption of a hydrogen ion in the rate-determining reaction.

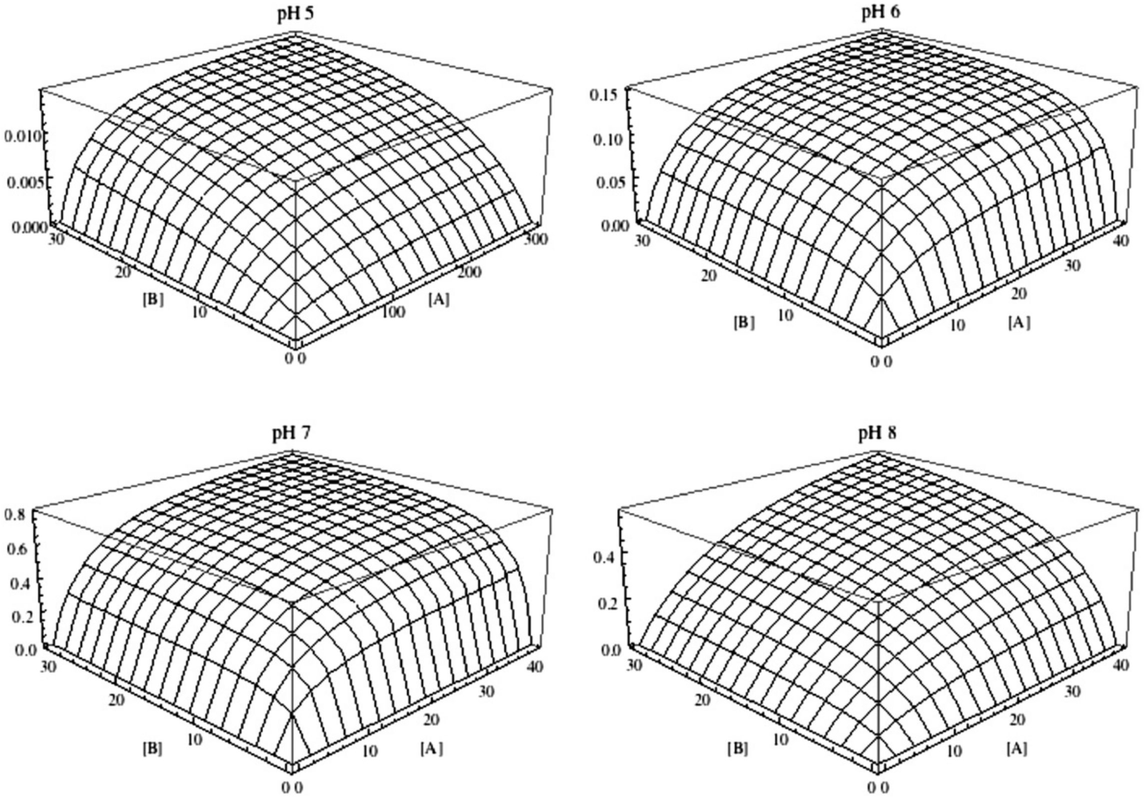


Fig. 6. Three-dimensional plots of initial velocities of the forward reaction at four pHs when no hydrogen ions are consumed in the rate-determining reaction and the mechanism of the forward reaction is random. The ordinate is the initial reaction velocity *v*. Introducing the K_{IB} term in the denominator reduces the reaction rate, as can be seen from Fig. 4 in Section 1.

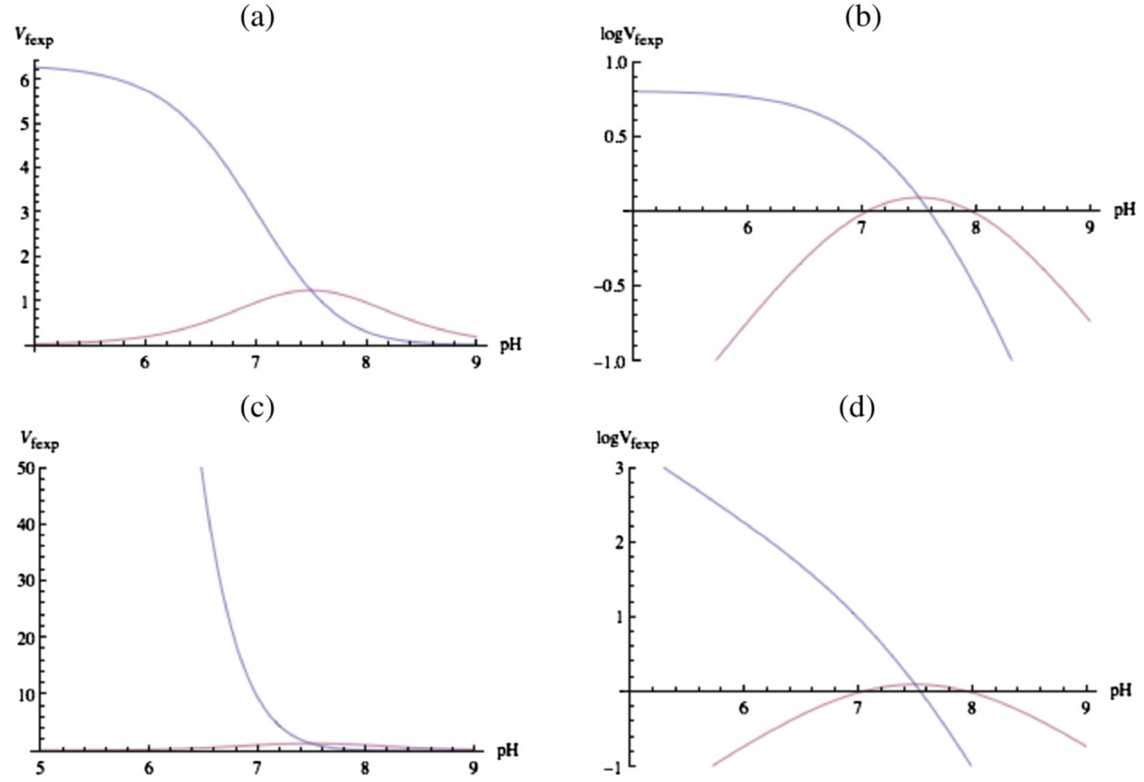


Fig. 7. (a) $V_{\mathrm{fexp}} = 10^{-(\mathrm{pH}-7.5)} V_{\mathrm{f}}$ versus pH when one hydrogen ion is consumed in the rate-determining reaction. The bell-shaped plot is for V_{f}. (b) LogV_{fexp} and LogV_{f} as functions of pH when one hydrogen ions are consumed. (c) $V_{\mathrm{fexp}} = 10^{-2(\mathrm{pH}-7.5)} V_{\mathrm{f}}$ versus pH when two hydrogen ions are consumed in the rate-determining reaction. (d) LogV_{fexp} and LogV_{f} as functions of pH when two hydrogen ions are consumed.

The new rapid-equilibrium rate equation for ordered $A+B$=ordered $P+Q$ when one hydrogen ion is consumed in the rate-determining reaction is

$$v=\frac{\frac{10^{-\mathrm{pH}}V_f[A][B]}{K_{IA}K_B}-\frac{V_r[P][Q]}{K_PK_{IQ}}}{1+\frac{[A]}{K_{IA}}+\frac{[A][B]}{K_{IA}K_B}+\frac{[Q]}{K_{IQ}}+\frac{[P][Q]}{K_{IQ}K_P}}=\frac{\frac{V_{f\mathrm{exp}}[A][B]}{K_{IA}K_B}-\frac{V_r[P][Q]}{K_PK_{IQ}}}{1+\frac{[A]}{K_{IA}}+\frac{[A][B]}{K_{IA}K_B}+\frac{[Q]}{K_{IQ}}+\frac{[P][Q]}{K_{IQ}K_P}} \tag{44}$$

This equation shows that the consumption of one hydrogen ion in the rate-determining reaction introduces $10^{-\mathrm{pH}}$ in the first term in the numerator of the rate equation. The Haldane equation obtained from Eq. (44) is

$$K'=\frac{[P][Q]}{[A][B]}=\frac{10^{-\mathrm{pH}}V_fK_PK_{IQ}}{V_rK_{IA}K_B}=\frac{V_{f\mathrm{exp}}K_PK_{IQ}}{V_rK_{IA}K_B}=\frac{K_{\mathrm{ref}}10^{-\mathrm{pH}}\left(1+10^{\mathrm{pH}-\mathrm{p}K_{1P}}+10^{\mathrm{p}K_{2P}-\mathrm{pH}}\right)\left(1+10^{\mathrm{pH}-\mathrm{p}K_{1Q}}+10^{\mathrm{p}K_{2Q}-\mathrm{pH}}\right)}{\left(1+10^{\mathrm{pH}-\mathrm{p}K_{1A}}+10^{\mathrm{p}K_{2A}-\mathrm{pH}}\right)\left(1+10^{\mathrm{pH}-\mathrm{p}K_{1B}}+10^{\mathrm{p}K_{2B}-\mathrm{pH}}\right)} \tag{45}$$

This Haldane equation yields an expression for K' that is of the form of Eq. (1), as used in biochemical thermodynamics.

The experimental limiting velocity of the forward reaction V_{fexp} is plotted in Fig. 7(a) in comparison with V_{f}. To make the two properties comparable, $V_{\mathrm{fexp}}=10^{-(\mathrm{pH}-7.5)}V_{\mathrm{f}}$ is plotted so that the two plots intersect at pH 7.5. The inclusion of $10^{7.5}$ in V_{fexp} is equivalent to changing $k_{\mathrm{f}}[E]_t$ in the input to the computer program. The pH at the intersection is referred to as x in the computer program. V_{fexp} levels off at low pH and appears to indicate a pK_1 of about 7, but this is an incorrect conclusion. LogV_{fexp} and logV_{f} are also plotted versus pH in Fig. 7(b).

The factors $10^{-\mathrm{pH}}$ in Eqs. (44) and (45) can be generalized to $10^{n\mathrm{pH}}$ where n is − 1, − 2, − 3,… when 1, 2, 3,… hydrogen ions are consumed in the rate-determining reaction. The generalized rate equation is given by

$$v=\frac{\frac{10^{n\mathrm{pH}}V_f[A][B]}{K_{IA}K_B}-\frac{V_r[P][Q]}{K_PK_{IQ}}}{1+\frac{[A]}{K_{IA}}+\frac{[A][B]}{K_{IA}K_B}+\frac{[Q]}{K_{IQ}}+\frac{[P][Q]}{K_{IQ}K_P}}=\frac{\frac{V_{f\mathrm{exp}}[A][B]}{K_{IA}K_B}-\frac{V_r[P][Q]}{K_PK_{IQ}}}{1+\frac{[A]}{K_{IA}}+\frac{[A][B]}{K_{IA}K_B}+\frac{[Q]}{K_{IQ}}+\frac{[P][Q]}{K_{IQ}K_P}} \tag{46}$$

Fig. 7(c) and (d) show V_{exp} and V_{f} as functions of pH when two hydrogen ions are conserved in the rate-determining reaction. The value of n can be determined by making kinetic measurements, but n cannot be determined from equilibrium measurements because thermodynamic properties are independent of mechanism. For some enzyme-catalyzed reactions, n can be − 8 [2].

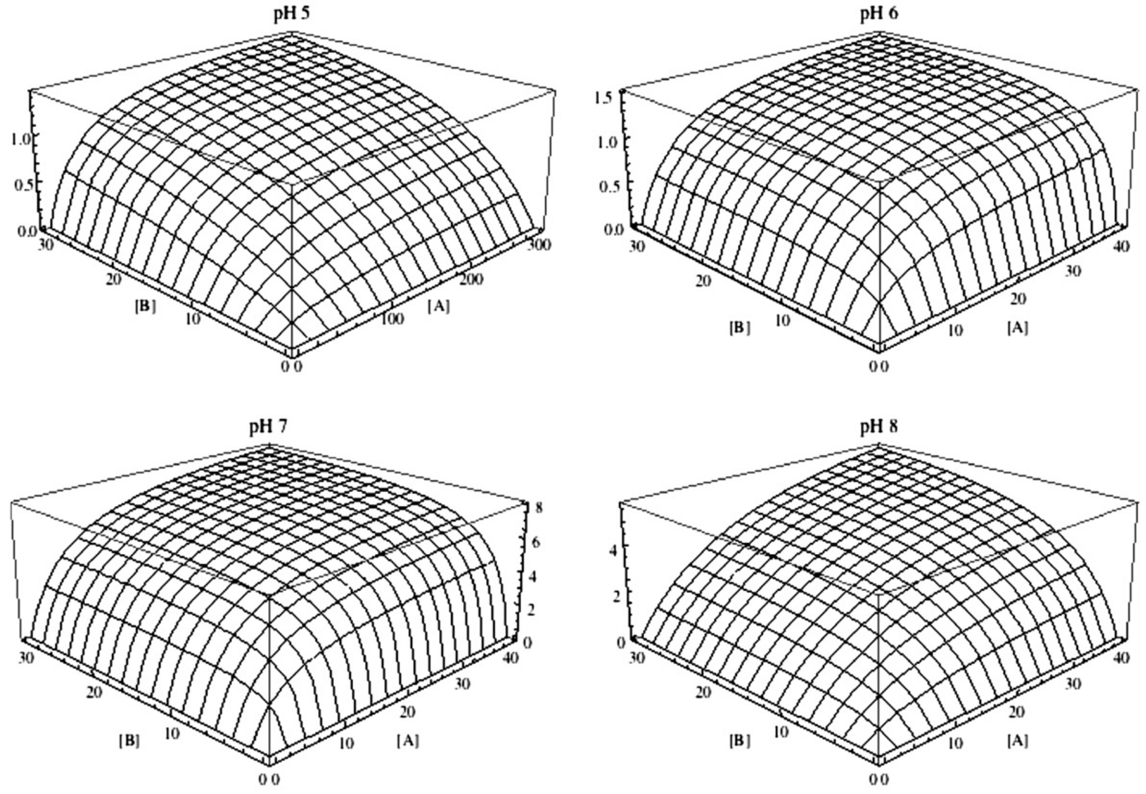


Fig. 8. Three-dimensional plots of initial velocities for the forward reaction of the random mechanism at four pHs when two hydrogen ions are consumed in the rate-determining reaction. The ordinate is the initial reaction velocity v. The ordinate in the first plot is v/1000, in the second v/100, in the third v, and in the fourth 100v. The scale changes by 10^5. Note that these plots have the same shapes as in Fig. 6 where no hydrogen ions are consumed in the rate-determining reaction.

5. Rapid-equilibrium rate equation for the enzymatic catalysis of random $A+B$ = ordered $P+Q$ when one or two hydrogen ions are consumed in the rate-determining reaction

The pH dependencies of the V_f, V_r, K_{IA}, K_B, K_{IB}, K_P, and K_{IQ} are the same as in Section 3. Fig. 8 shows three-dimensional plots of initial velocities of the forward reaction of the random mechanism when two hydrogen ions are consumed in the rate-determining reaction. Note that these plots have the same shapes as in Fig. 6 because these shapes are determined by the Michaelis constants. If the reaction rate increases with pH according to $10^{-n\mathrm{pH}}$, an error of 0.1 in pH will change the rate by a factor of 1.26 if $n=-1$ and 1.58 if $n=-2$. This makes it more difficult to determine kinetic parameters when $n=-2$.

6. Discussion

There are two types of pH effects in rapid-equilibrium enzyme kinetics. The effects of p*K*s extend over about two units of pH and the effects of the consumption of hydrogen ions in the rate-determining reaction extend over the whole pH range of interest. The existence of this second type of pH effect supports the hypothesis of a rate-determining reaction when the rate equations given here apply. When rate Eqs. (14), (36), (44), and (46) are not obeyed, other mechanisms and assumptions are required. When hydrogen ions are consumed in reactions prior to the rate-determining reaction, the rate equations given here will not be obeyed because there will be competition between the reactions prior to the rate-determining reaction. There are many ways that mechanisms can be more complicated than those discussed here, but they will not follow the rate equations given here. Bocklehurst [12] has written a useful review on pH dependence studies up to 1994. He discusses a number of complications including overlapping kinetically influential acid dissociations, parallel pathways, "mirage" pH effects, etc. Some of these can be incorporated into a computer program, but they will all yield more complicated rate equations than the four discussed here.

When hydrogen ions are consumed in the rate-determining reaction, the effects on initial velocities are large because *n*pH is in the exponent. This makes it easy to determine *n* using experimental kinetic data over a range of pH. This exponential term also appears in the expression for the apparent equilibrium constant because of the Haldane equation. But equilibrium measurements cannot yield the value of *n* because thermodynamics is independent of mechanism.

Since the p*K*s of the substrates *A*, *B*, *P*, and *Q* can be determined by acid titrations, their effects can be taken out of the expressions for experimentally-determined Michaelis constants to obtain adjusted Michaelis constants. It is easier to determine the p*K*s of the enzymatic site and enzyme-substrate complexes from the kinetic data by using adjusted Michaelis constants. Bell-shaped curves can be used to determine all the kinetic constants in the rate equations discussed here.

A computer program like calcABPQBH can be used to explore the behavior of a mechanism, and it can be used to test kinetic parameters that have been determined experimentally because the pH dependencies of the kinetic parameters can be calculated, and the initial rate for any set of substrate concentrations can be calculated. This computer program can also be used to calculate Lineweaver-Burk plots for a given set of parameters at any desired pH. The kinetic constants for a mechanism can be verified by using them to calculate the values of the kinetic parameters at any pH.

Acknowledgements

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Appendix A

derABPQkinHB[pK1e_,pK2e_,pK1ea_,pK2ea_,pK1eq_,pK2eq_, pK1eab_,pK2eab_,pK1epq_,pK2epq_,pK1a_,pK2a_,pK1b_,pK2b_,pK1p_,pK2p_,pK1q_,pK2q_,kfEt_,krEt_,kH2EA_,kH3EAB_,kH2EQ_,kH3EPQ_,pK1eb_,pK2eb_,kH2EB_,n_,x_]:=Module[{eafactor,ebfactor,eqfactor,efactor,eabfactor,epqfactor,afactor,bfactor,pfactor,qfactor,vf,vr,kia,kb,kiq,kp,kiaadj,kbadj,kibadj,kpadj,kiqadj,vfexp}, (*Calculates 7 kinetic parameters of the enzyme-catalyzed reaction ordered $A+B$=ordered $P+Q$ and random $A+B=P+Q$ at specified pH when $V_{\mathrm{fexp}}=10^{n(\mathrm{pH}-x)}V_{\mathrm{f}}$. It also calculates 5 adjusted Michaelis constants, 6 ratios of kinetic parameters, and the expression for the initial reaction velocity *v*. *n* is a negative integer that gives the number of hydrogen ions consumed in the rate-determining reaction. Specifying *x* is equivalent to setting kfEt. The calculations require 29 inputs. The output is a list of 19 functions. The last output is the rapid-equilibrium rate equation.*)

efactor$=1+10^{\mathrm{pK2e-pH}}+10^{\mathrm{pH-pK1e}}$;
eafactor$=1+10^{\mathrm{pK2ea-pH}}+10^{\mathrm{pH-pK1ea}}$;
ebfactor$=1+10^{\mathrm{pK2eb-pH}}+10^{\mathrm{pH-pK1eb}}$;
eabfactor$=1+10^{\mathrm{pK2eab-pH}}+10^{\mathrm{pH-pK1eab}}$;
eqfactor$=1+10^{\mathrm{pK2eq-pH}}+10^{\mathrm{pH-pK1eq}}$;
epqfactor$=1+10^{\mathrm{pK2epq-pH}}+10^{\mathrm{pH-pK1epq}}$;
afactor$=1+10^{\mathrm{pK2a-pH}}+10^{\mathrm{pH-pK1a}}$;
bfactor$=1+10^{\mathrm{pK2b-pH}}+10^{\mathrm{pH-pK1b}}$;
pfactor$=1+10^{\mathrm{pK2p-pH}}+10^{\mathrm{pH-pK1p}}$;
qfactor$=1+10^{\mathrm{pK2q-pH}}+10^{\mathrm{pH-pK1q}}$;
vf=kfEt/eabfactor;
vvr=krEt/epqfactor;
kia=kH2EA * efactor * afactor/eafactor;
kib=kH2EA * efactor * bfactor/ebfactor;
kb=kH3EAB * eafactor * bfactor/eabfactor;
kiq=kH2EQ * efactor * qfactor/eqfactor;
kp=kH3EPQ * eqfactor * pfactor/epqfactor;
kiaadj=kH2EA * efactor/eafactor;
kbadj=kH3EAB * eafactor/eabfactor;
kibadj=kH2EB * efactor/ebfactor;
kiqadj=kH2EQ * efactor/eqfactor;
kpadj=kH3EPQ * eqfactor/epqfactor;
vfexp=vf * 10^(*n* * (pH−*x*));

```
{vf,vr,kia,kib,kb,kp,kiq,kiaadj,kbadj,kibadj,kpadj,kiqadj,vf/
kbadj,(vf*kibadj)/,(kiaadj*kbadj),vr/kpadj,vf/(kiaadj*kbadj),
vr/(kiqadj*kpadj),vfexp,(((vfexp*a*b/(kia*kb))−vr*p*q/
(kiq*kp))))/(1+a/kia+bkib+a*b/(kia*kb)+q*p/(kiq*kp))}]
```

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