Initial Rate Kinetics of Multi-Substrate Enzymes

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Synonyms

Steady State Kinetics, Initial Velocity Kinetics, Enzyme Kinetics

Synopsis

The format of the initial rate equations for two and three substrate enzymes, derived using either the steady state assumption or the rapid equilibrium assumption provides insight into both the ways that the formal kinetic mechanism of an enzyme can be established and to the physical interpretation of various kinetic constants. The existence of various terms in the generalized rate equation for a three substrate enzyme is diagnostic of whether a quaternary complex or enzyme substituted mechanism is followed and provides insight into whether substrate addition is ordered or random and the overall mechanism steady state or equilibrium. For two substrate enzymes, enzyme substituted or compulsory order equilibrium mechanisms give unique formats of the generalized rate equation. Random order, rapid equilibrium and compulsory order steady state ternary complex mechanisms can be distinguished using either alternate substrate kinetics or by the inhibition patterns obtained using analog inhibitors.

Initial Rate Kinetics of Multi-Substrate Enzymes

Introduction

The principles of either the equilibrium assumption (Michaelis and Menten, 1913) or the less restrictive steady state assumption (Briggs and Haldane, 1925) can be applied to the derivation of rate equations for various multi-substrate formal kinetic mechanisms. Unlike the case with simple one substrate kinetics the format of the resultant equations is not always the same with multi-substrate enzymes. These equations can be used in conjunction with initial rate kinetic studies to give information about the physical meaning of the various kinetic parameters such as V_{max} and K_m that are obtained from experimental data. Unfortunately extracting the significance of these parameters requires knowledge of the formal kinetic mechanism of the enzyme. This

involves the collection and analysis of sufficient experimental data to eliminate reasonable alternatives. While there are different nomenclatures used in derivations of multi-substrate enzyme kinetic equations following Alberty (Alberty, 1956, 1962), Dalziel(Dalziel, 1957, Engel and Dalziel 1970) or Cleland (Cleland, 1963a,b,c), all lead to the same basic approaches to distinguish formal kinetic mechanisms and in the following discussion the approach developed by Dalziel for 2 and 3 substrate enzymes is used.

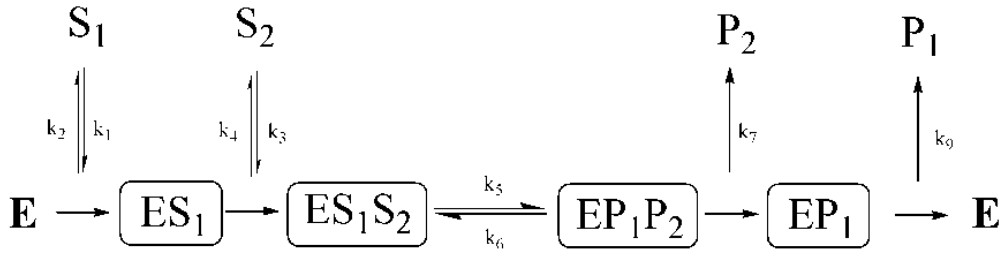
Two Substrate Kinetic Mechanisms

The appropriate rate equations derived for various 2 substrate mechanisms shown in scheme 1(Compulsory Order, Steady State, COSS, Compulsory Order, Rapid Equilibrium, CORE, Random Order, Rapid Equilibrium, RORE, and enzyme substituted)

Scheme 1: Schematic representation of various 2 substrate kinetic mechanisms,

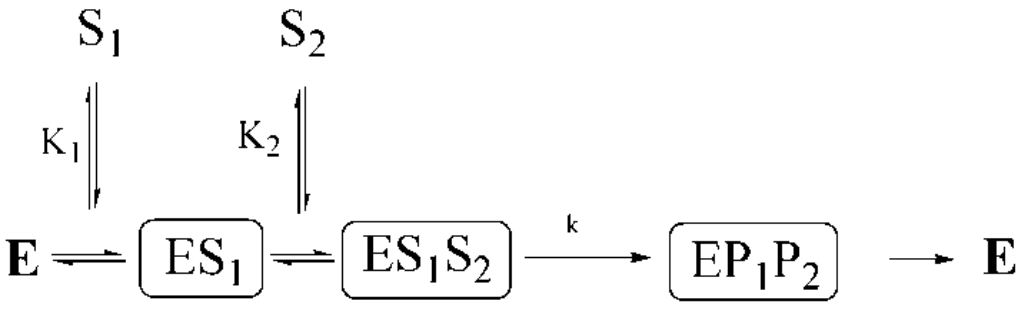
a)

Compulsory Order, Steady State



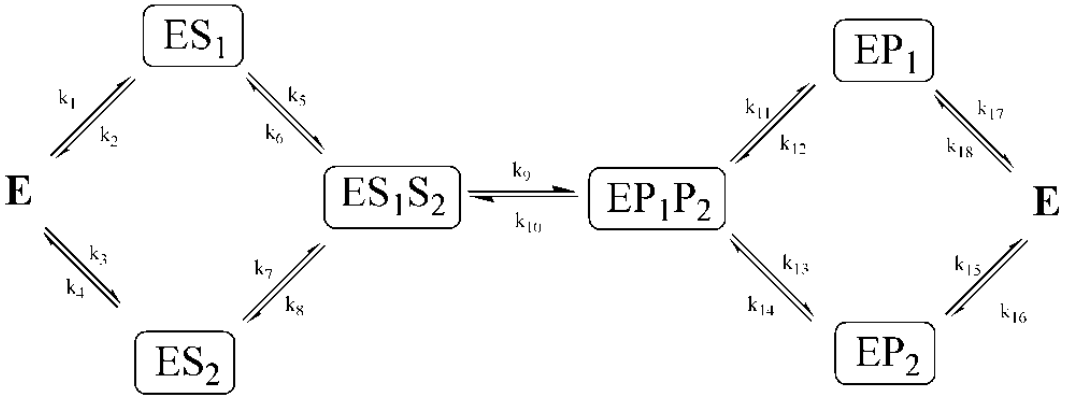
b)

Compulsory Order, Rapid Equilibrium



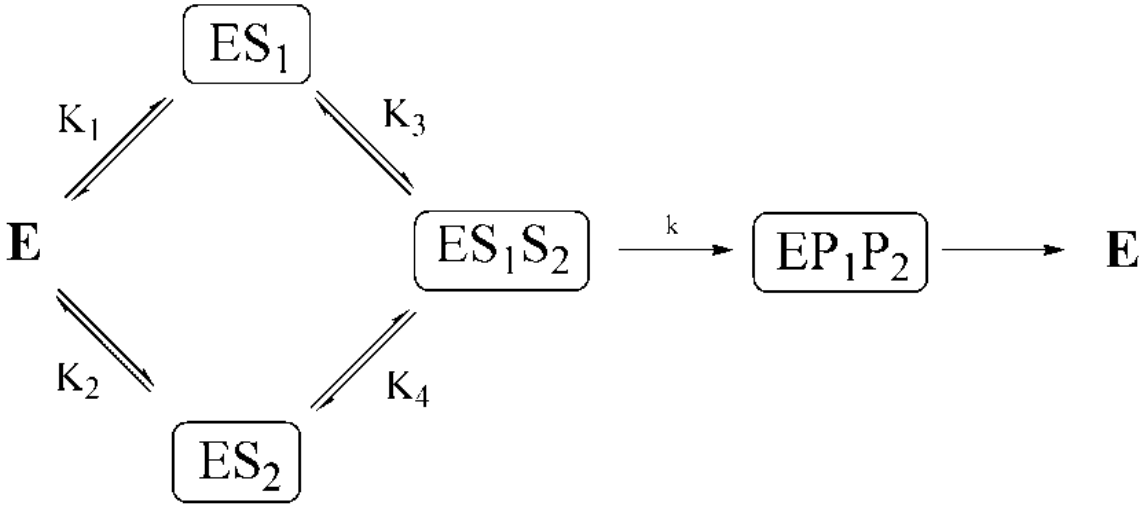
c)

Random Order, Steady State



d)

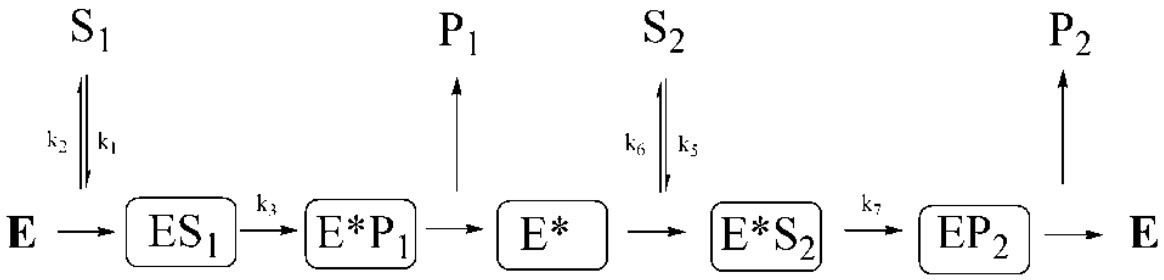
Random Order, Rapid Equilibrium



and

e)

Enzyme Substituted



show that four of the five mechanisms considered give rise to the general form of a rate equation for two substrates as proposed by Dalziel (Dalziel, 1957). The random order, steady-state mechanism gives an equation that cannot, without making simplifying assumptions, give rise to linear Lineweaver- Burk plots. Table1 summarizes the values of the four Φ parameters of the generalized rate equation for these four mechanisms, together with expressions for V_{max} and the Michaelis constants for the two substrates S_1 and S_2.

Table 1. Relationships involving initial rate parameters and kinetic mechanisms for 2 substrate systems. The various rate constants (k) and dissociation constants (K) are designated as in scheme 1.

Table 1

Relationships Involving Initial Rate Parameters and Kinetic Mechanisms

Mechanism	Φ_0	Φ_1	Φ_2	Φ_{12}	V_{max} ($1/\Phi 0$)	$K_m^{S_1}$ (Φ_1/Φ_0)	$K_m^{S_2}$ (Φ_2/Φ_0)
COSS	$1/k_9+1/k_7+(k_6+k_7)/k_5k_7$	$1/k_1$	$\frac{k_4k_6+k_4k_7+k_5k_7}{k_3k_5k_7}$	$\frac{k_2(k_4k_6+k_4k_7+k_5k_7}{k_1k_3k_5k_7}$	complex	complex	complex
CORE	$1/k$	0	k_2/k	K_1K_2/k	k		k_2
RORE	$1/k$	K_4/k	K_3/k	K_1K_3/k	k	K_4	K_3
Enzyme Substituted	$1/k_3 + 1/k_7$	$(k_2+k_3)/k_1k_3$	$(k_6+k_7)/k_5k_7$	0	complex	complex	complex

COSS: Compulsory Order, Steady State

CORE: Compulsory Order, Rapid Equilibrium

RORE: Random Order, Rapid Equilibrium

EXPERIMENTAL DETERMINATION OF Φ PARAMETERS

Experimentally, Φ parameters are determined from a series of initial rate measurements with varied concentrations of the first substrate at several fixed concentrations of the second. The general rate equation may be written in two forms, depending on whether substrate S_1 or substrate S_2 is varied,

$$e/v_o = \Phi_0 + \Phi_1/[S_1] + \Phi_2/[S_2] + \Phi_{12}/[S_1 \cdot S_2]$$

$$= (\Phi_0 + \Phi_2/[S_2]) + (\Phi_1 + \Phi_{12}/[S_2]) \cdot (1/[S_1]) \quad \text{for plots with } S_1 \text{ as the varied substrate}$$

$$= (\Phi_0 + \Phi_1/[S_1]) + (\Phi_2 + \Phi_{12}/[S_1]) \cdot (1/[S_2]) \quad \text{for plots with } S_2 \text{ as the varied substrate}$$

$$y = \text{intercept} + \text{slope} \cdot x$$

And these two equations form the basis of the experimental determination of Φ parameters from kinetic data. Primary Lineweaver-Burk plots of data (Fig 1a) give slopes and intercepts that are replotted versus the reciprocal of the concentration of the second substrate (Fig 1b),

Figure 1a: Primary Kinetic Data obtained with varied concentrations of S_1 collected at a series of fixed concentrations of S_2.

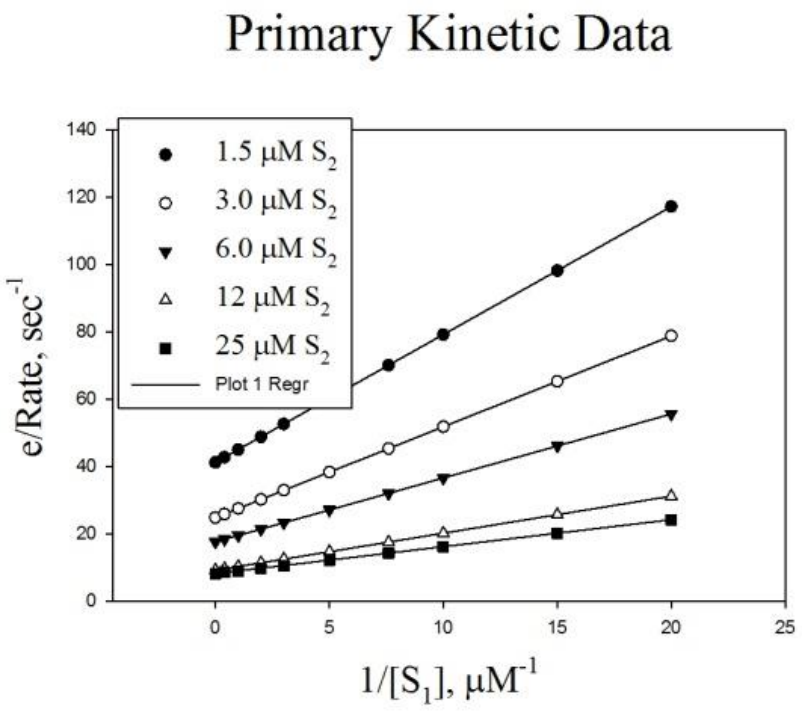
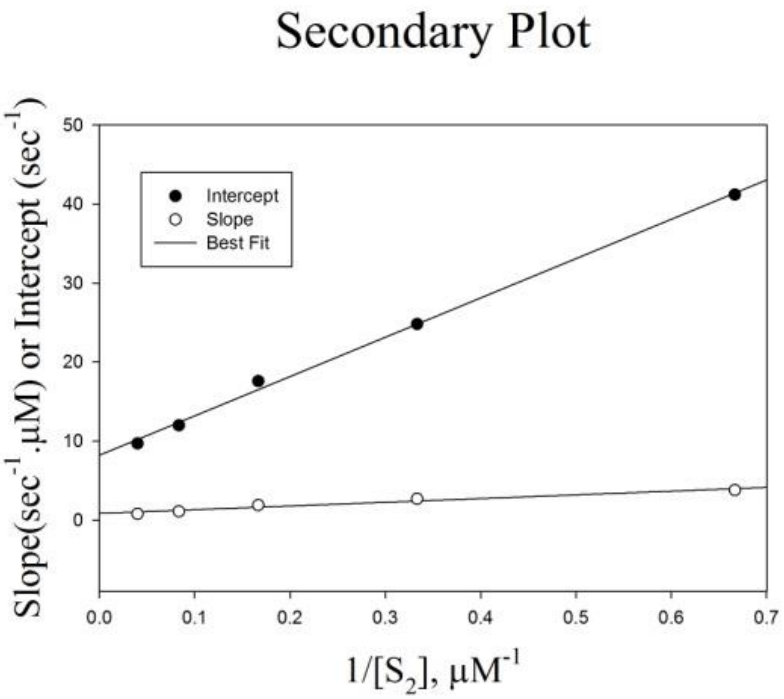


Figure 1b: Secondary Plots of slopes and intercepts- y axis- from the primary plots plotted against $1/[S_2]$



The slopes and intercepts of the primary plot with S_1 as the varied substrate are given by the expressions:

Slope $= \Phi_1 + \Phi_{12} \cdot 1/[S_2]$, and Intercept $= \Phi_0 + \Phi_2 \cdot 1/[S_2]$

And a secondary plot of slope vs $1/[S_2]$ is linear with a slope of Φ_{12} and an intercept of Φ_1 .

Likewise a secondary plot of intercept vs $1/[S_2]$ is linear with a slope of Φ_2 and an intercept of Φ_0 .(fig 1b)

Since the general rate equation is symmetrical it does not matter whether the primary plots are constructed with S_1 or S_2 as the initial varied substrate, equivalent, and independent, values for the Φ parameters are obtained. The principal advantage of analyzing kinetic data with the Dalziel equation is that the initial rate parameters are obtained without any prior assumptions concerning the formal kinetic mechanism of the enzyme. It is also possible to assess the experimental accuracy of the parameters by suitable statistical analysis of the primary kinetic data rather than by using overall fit to one particular kinetic mechanism.

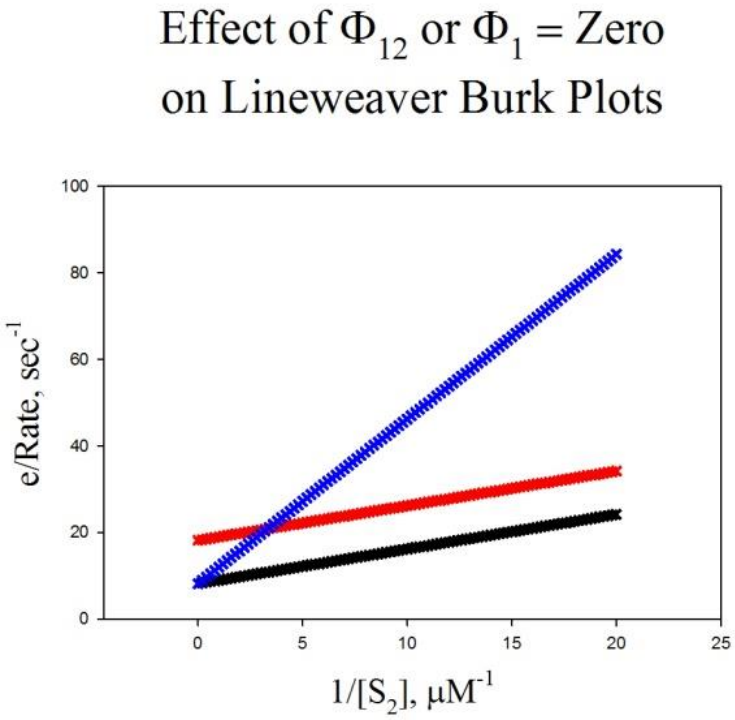
EXPERIMENTAL DISTINCTION BETWEEN VARIOUS TWO-SUBSTRATE KINETIC MECHANISMS

There are several methods for distinguishing among these kinetic mechanisms using initial rate kinetics. Examination of the derived rate equations (table 1) reveals two basic approaches, one involving the overall form of the rate equation and the other the physical significance of individualΦ parameters in the generalized rate equations for various mechanisms.

Primary Plots of Initial Rate Kinetic Data

With the exception of the random-order, steady-state mechanism, the most likely two substrate mechanisms conform to the generalized rate equation but two of the four are lacking one term in the rate equation. With the enzyme-substituted mechanism, the Φ_{12} term is equal to 0. The pattern of Lineweaver- Burk plots obtained when one substrate concentration is varied at a series of fixed concentrations of the other reflects this (figure 2), with one of either S_1 or S_2 varied only the intercept changes as a function of the non-varied substrate concentration. The resultant series of parallel lines obtained are a quite distinctive feature of enzyme-substituted mechanisms.

Figure 2: Effects of zero values for certain Φ Parameters on Lineweaver Burk plots with S_2 varied at different fixed concentrations of S_1.Black: Data with highest concentration of S_1, Red: Data with lower concentration of S_1 if Φ_{12} = zero, Blue: Data with lower concentration of S_1 if Φ_1 = zero



Ternary complex mechanisms are all characterized by a positive value for Φ_{12}, and the slopes of Lineweaver-Burk plots change as a function of the concentration of the non-varied substrate. With one of these ternary complex mechanisms however, there is a distinctive feature. In the compulsory-order, equilibrium mechanism the Φ_1 parameter is equal to 0, and in Lineweaver-Burk plots with S_1 as the varied substrate, the intercept is equal to 1/k and is independent of the concentration of S_2.(figure 2) With this mechanism, Lineweaver-Burk plots with S_2 as the varied substrate intersect on the e/v_o axis for various fixed concentrations of S_1. With S_1 as the varied substrate, of course, a normal pattern of plots is obtained: Both slope (= $K_1 \cdot K_2/S_2$) and intercept (= 1/k +

$K_2/k \cdot S_2$) vary as the concentration of $S_2$is changed.

As seen in the previous discussion, several kinetic mechanisms have characteristic forms of the generalized rate equation which lead to distinctive patterns of Lineweaver-Burk plots, however care must be taken assigning a parameter value equal to 0 and the experimental determination of Φ parameters has to cover a sufficient range of substrate concentrations to allow the unequivocal demonstration of the presence or absence of a particular parameter. For example, a random-order, rapid-equilibrium mechanism where one of the substrates binds extremely tightly to the enzyme in the presence of the other, (consider K_4 in scheme 1 tending towards zero indicating extremely tight binding of S_1 in the ES_1S_2 complex) the mechanism may, if sufficiently low concentrations of S_1 are not used in Φ parameter determinations, resemble a compulsory-order, equilibrium mechanism.

The major problem facing the enzymologist considering two substrate mechanisms is the distinction between a compulsory-order, steady-state and a random-order, rapid-equilibrium mechanism, both of which contain all 4 Φ parameters in their generalized rate equation. A number of simple approaches are frequently used to assist in the distinction between these mechanisms. They can be grouped into three areas: (1) the use of alternate substrates, (2) comparison of kinetically derived parameters with independently derived parameters, and (3) the use of substrate analogs as reversible inhibitors.

Use of Alternative Substrates

Where several alternative substrates for a particular enzyme-catalyzed reaction are available it is often possible, from the results of simple initial rate measurements, to distinguish between a compulsory-order, steady-state mechanism and a random order, rapid equilibrium mechanism. The basis for such a distinction is the fact that for the compulsory-order, steady-state mechanism, $\Phi_1 = 1/k_1$, where k_1 is the rate constant for the first substrate (S_1) binding to the enzyme. If the □□parameters in the generalized rate equation are determined with a series of alternative substrates, S_2, S_2' and S_2'' etc, the value of Φ_1 for the different alternate substrates is constant if the mechanism is compulsory order, steady state where-as if the mechanism is really random order, rapid equilibrium or compulsory order, steady state, with S_2 as the first substrate, Φ_1 varies as the nature of S_2 varies. The use of alternative substrates to S_1 as well as to S_2 allows these two alternates to be distinguished (Dalziel & Dickinson, 1966). If Φ_1 varies as a function the nature of S_2, the determinations are repeated with a single S_2 but a variety of alternative substrates for S_1. Constant values for Φ_2 as the nature of S_1 is varied indicate a compulsory order, steady-state mechanism with S_2 as the first substrate.

Comparison of Kinetically Derived Constants with Independently Determined Values

As summarized in table 1,the Φ parameters of the generalized rate equation have specific physical significance depending on the kinetic mechanism (Dalziel, 1962). This allows a comparison to be made between various Φ parameters (or ratios of Φ parameters) and directly determined values for specific constants in the appropriate mechanism.

For example, in a random-order, rapid-equilibrium mechanism the Michaelis constants for the

reaction, Φ_1/Φ_0 and Φ_2/Φ_0 give values for K_3 and K_4, the dissociation constants for S_1 and S_2 from the ternary ES_1S_2 complex, respectively. Since for all of the ternary complex mechanisms Φ_{12}/Φ_2 is the dissociation constant for S_1 binding to the free enzyme, one can use this in conjunction with the equality $K_1 \cdot K_3 = K_2 \cdot K_4$ for the rapid equilibrium random order mechanism to obtain values for all of the dissociation constants for substrate binding which is the only one that gives a value for K_2. Direct determination of K_2 by such approaches as equilibrium dialysis or spectroscopic titrations and comparison with Φ_{12}/Φ_1 can give supportive evidence for a random-order, rapid-equilibrium mechanism. Likewise, in a compulsory-order, steady-state mechanism,$\Phi_1 = 1/k_1$, where k_1 is the rate constant for S_1binding to enzyme. Direct determination of k_1 by rapid reaction techniques and comparison with Φ_1 determined via enzyme kinetics gives information about whether or not a compulsory-order, steady-state mechanism is applicable. In both cases, agreement between such constants is merely consistent with the mechanism: disagreement disproves the mechanism.

Use of Analog Inhibitors

A substrate analog of one of the substrates can bind in place of that substrate in either one or two sites, depending on whether a compulsory-order or a random-order mechanism exists. Consider an analog of S_2 (X_2) in these mechanisms. In compulsory order, steady state, X_2 can bind only to an ES_1 complex to give ES_1X_2, with a dissociation constant of K_{X2}(this is in reality a K_i - inhibition constant- but to indicate that it is an analog of the substrate we use the designation X) and the interaction can be described in terms of a dissociation constant K_{X2}; Derivation of the rate equation for such a mechanism in the presence of X_2 leads to the generalized format:

$$e/vo = \Phi_0 + \Phi_1/S_1 + \Phi_2/S_2 \cdot (1 + X_2/K_{X2}) + \Phi_{12}/S_1 \cdot S_2$$

With the random-order, rapid-equilibrium mechanism however, X_2 can combine to free enzyme as well as to the ES_1 complex, and two additional steps must be included leading to two additional terms in the generalized rate equation:

$$e/vo = \Phi_0 + \Phi_1/S_1 + \Phi_2/S_2 \cdot (1 + X_2/K_{X2}) + \Phi_{12}/S_1 \cdot S_2(1 + X_2/K'_{X2})$$

where K'_{X2} is the dissociation constant for binding to the free enzyme and K_{X2} is the dissociation constant for binding to ES_1.

With either mechanism when S_2 is varied in the absence and presence of the analog inhibitor X_2, in a Lineweaver-Burk plot only the slope term is affected and the inhibition is competitive (figure 3a).

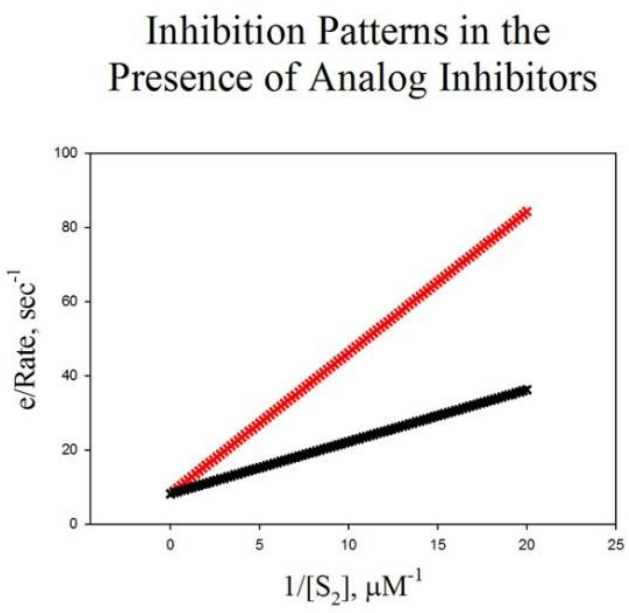
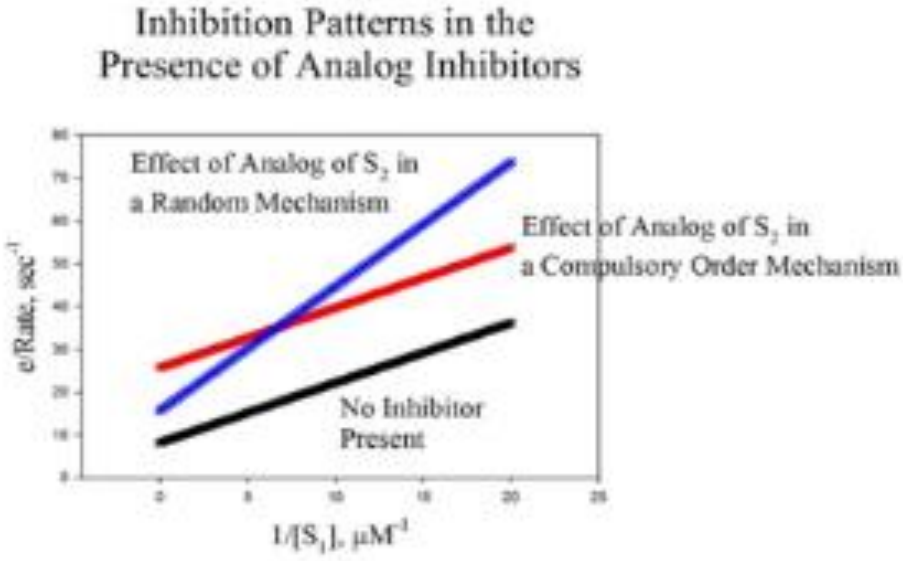


Figure 3: a) Competitive Inhibition obtained with an analog of S_2. Black line- no inhibitor present, red line plus inhibitor,



b) Inhibition Patterns obtained with an Analog of S2 when S1 is varied in a Lineweaver Burk plot. Black- no inhibitor present, Red- Uncompetitive Inhibition obtained if the mechanism is compulsory ordered steady state, Blue- Non Competitive (Mixed) Inhibition obtained if the mechanism is random ordered rapid equilibrium.

The slope terms for either “competitive” inhibition are: slope = $(\Phi_2/S_2 \cdot (1 + X_2/K_{X2}) + \Phi_{12}/S_1 \cdot S_2)$ for the compulsory order mechanism, and slope = $(\Phi_2/S_2 \cdot (1 + X_2/K_{X2}) + \Phi_{12}/S_1 \cdot S_2(1 + X_2/K'_{X2}))$ for the random order mechanism, compared to $(\Phi_2/S_2 + \Phi_{12}/S_1 \cdot S_2)$ for either mechanism. With values for the various Φ parameters, the inhibition constants, K_{X2} and K'_{X2} can be calculated

When Lineweaver-Burk plots with S_1 as the varied substrate are examined, however, we find that for either mechanism the intercept term is affected, but with the random-order mechanism the slope term is affected as well (figure 3b). With a compulsory order, steady-state mechanism an analog of S_2, X_2 is an uncompetitive inhibitor with respect to S_1 while in a random-order, rapid-equilibrium mechanism, X_2 is a noncompetitive inhibitor with respect to S_1.

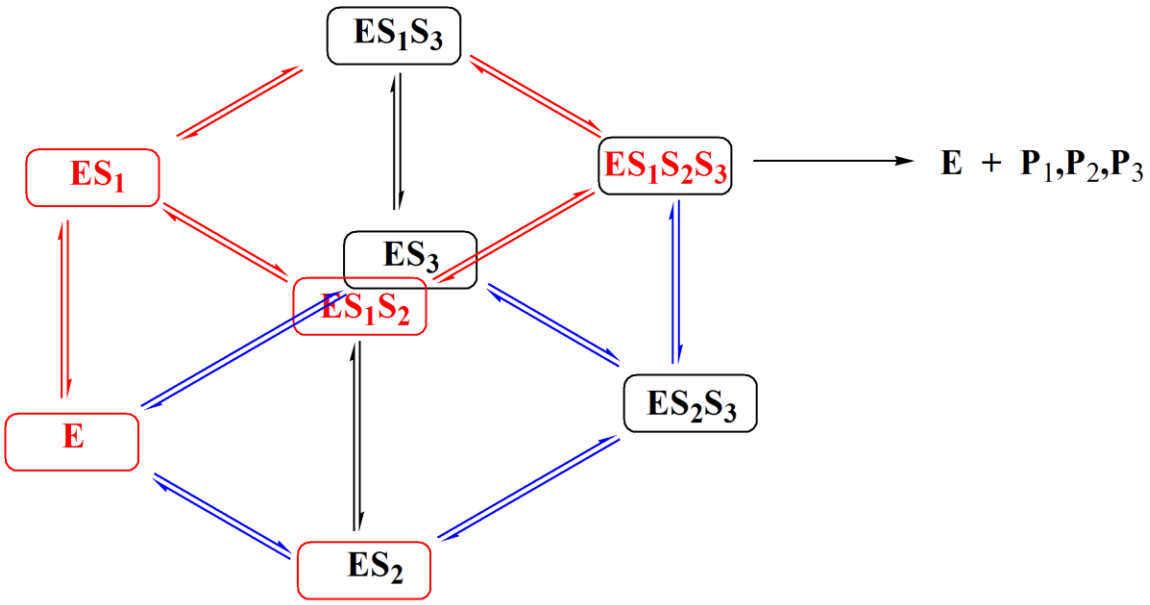
For either mechanism, the intercept becomes $(\Phi_0 + \Phi_2/S_2 \cdot (1 + X_2/K_{X2}))$ while in the random order mechanism the slope becomes $(\Phi_1 + \Phi_{12}/S_1 \cdot S_2(1 + X_2/K'_{X2}))$

A further important conclusion concerning reversible inhibitors in two-substrate systems is also apparent from this discussion. That is, the determination of K_i values requires knowledge of the values of individual Φ parameters in the presence and absence of the inhibitor.

3 Substrate Enzymes

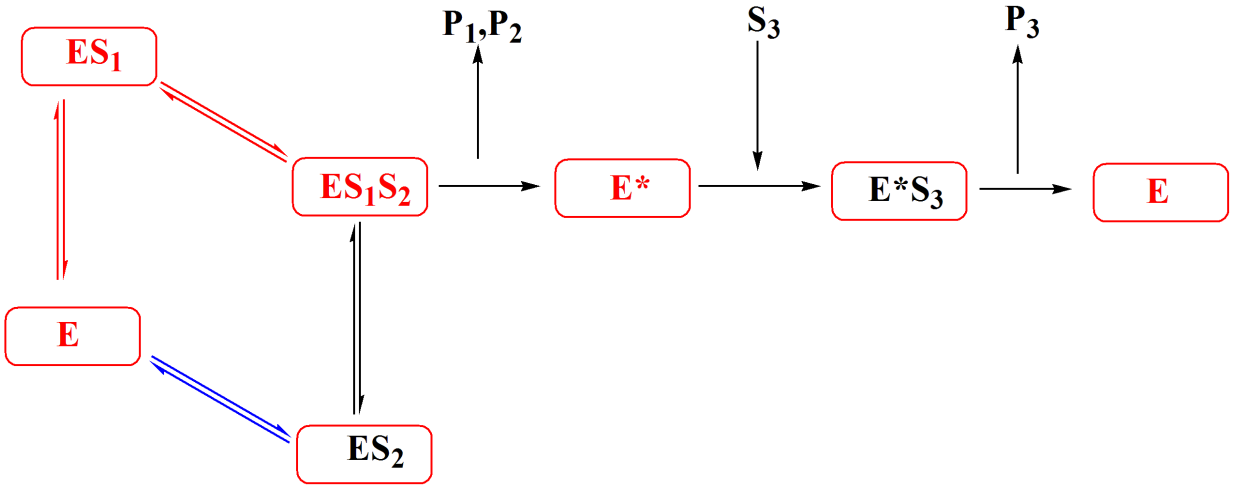
As with two-substrate enzyme kinetics we can separate possible formal kinetic mechanisms into two classes. In the first, all the substrates must be bound prior to the appearance of product and the mechanism is known as "quaternary complex". In the second class, one or more products appear before all the substrates have bound and, as with the two-substrate systems, the mechanism is said to be "enzyme substituted." With each of these classes there are considerations regarding the order of substrate addition and whether or not various steps are in rapid equilibrium or must be treated using the less restrictive steady-state assumption.

Scheme 2: *Schematic representation of various 3 substrate kinetic mechanisms.*
a) Completely random addition of substrates to form the quaternary complex - shown in red arrows is an ordered first substrate followed by random addition of S2 and S3 – shown in blue is a random addition of S1 and S3 with a compulsory ordered addition of S3.



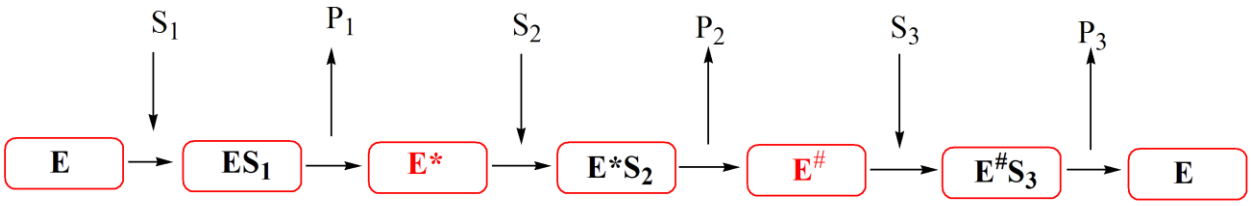
3 Substrate Quaternary Complex Mechanisms

b) Enzyme substituted mechanism with a single enzyme substituted species formed with either a random or ordered (red arrows) addition of S1 and S2.



3 Substrate Enzyme Substituted Mechanism 1

c) Enzyme substituted mechanism with two distinct enzyme substituted species and ordered addition of substrates.



3 Substrate Enzyme Substituted Mechanism 2

Five formal three-substrate mechanisms shown in Scheme 2 are considered. The first is the completely random-order addition of substrates in a quaternary complex mechanism. For the purposes of deriving a rate equation for this mechanism, the rapid-equilibrium assumption is made. The second general mechanism considered is one involving a compulsory order of addition of each substrate and the final quaternary complex mechanism involves an obligatory first substrate followed by a random-order addition of the second and third. The addition of the first substrate is usually treated using the steady-state approach, while the random order of the subsequent substrate additions is handled by the rapid-equilibrium assumption. As with two-substrate random-order mechanisms such treatment assumes that various steps prior to the quaternary complex, particularly the off rates, are much faster than the subsequent rate of catalytic interconversion of the quaternary complexes.

Two enzyme-substituted mechanisms are included, the first involving a triple transfer with two different enzyme-substituted species. In this mechanism there is an obligatory order of substrate addition and product release, and it is most simply treated by the steady-state approach.

In the other enzyme-substituted mechanism there is formation of a ternary ES_1S_2 complex prior to formation of the enzyme-substituted intermediate (and release of the first product) and subsequent addition of the final substrate. The addition of the first two substrates can be ordered or random. If the ternary complex formation is random ordered, the equilibrium approach can be employed, provided that the "off"-velocity constants are sufficiently rapid to allow the equilibrium condition to be reached. The ordered and random equilibrium versions of this mechanism give the same generalized rate equation.

Each of these mechanisms fit the overall generalized rate equation for a three substrate enzyme:

$$e/v_o = \Phi_0 + \Phi_1/S_1 + \Phi_2/S_2 + \Phi_3/S_3 + \Phi_{12}/S_1S_2 + \Phi_{13}/S_1S_3 + \Phi_{23}/S_2S_3 + \Phi_{123}/S_1S_2S_3$$

where the initial rate parameters, Φ , are determined from experimental data in a manner similar to that described earlier for 2 substrate systems.

The only one of the five considered mechanisms to contain all eight terms of this equation is the rapid-equilibrium random-order mechanism . The completely ordered mechanism lacks the Φ_{13}

term while the obligatory first substrate mechanism lacks both the Φ_{12} term and the Φ_{13} terms. Each of the quaternary complex mechanisms contains the Φ_{123} term. In contrast, the various enzyme-substituted mechanisms each lack this term. The triple transfer mechanism lacks four of the eight parameters; Φ_{12}, Φ_{13}, and $\Phi_{23}$are absent in addition to the Φ_{123} parameter. The other enzyme-substituted mechanism lacks the Φ_{23} and Φ_{13} parameters in addition to the Φ_{123} parameter. The generalized form of the rate equation is the same irrespective of whether the ternary complex is formed by a compulsory or random addition of the two substrates.

Overall, the formal kinetic mechanism of these three substrate types can be established solely by initial rate kinetics experiments although the same approaches as described for two substrate enzymes are also frequently used in conjunction with saturation by one of the three substrates (where any term in the generalized rate equation with that substrate in the denominator goes to zero). As with two substrate mechanisms, one the mechanism is established by eliminating reasonable alternatives, the physical interpretation of the initial rate parameters yields a wealth of information about the binding of substrates and the steps involved in the overall catalytic cycle of the enzyme.

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