14

Two-Substrate Kinetics

INTRODUCTION

Although some enzymes have only a single substrate, the vast majority have two or more. The equations developed in Chap. 14 describing the initial rate behavior of one-substrate enzymes and developing the ideas of reversible inhibitors must be modified to deal with the more common two-substrate enzyme. In this chapter the initial rate behavior of two-substrate enzymes is considered.

Before proceeding, the concept of "formal kinetic mechanism" for an enzyme-catalyzed reaction must be defined. This involves a description of the order in which the substrates bind to the enzyme as well as the sequence of product release after catalysis has taken place. Two basic classes of multisubstrate mechanisms exist. Those involving addition of *all* of the substrates prior to catalysis are (in the case of a two-substrate mechanism) referred to as *ternary complex mechanisms.* In the class where a formed product leaves the enzyme active site prior to the second substrate binding, the mechanism often involves a group transfer from one substrate to another, with that group forming a covalent intermediate with the enzyme prior to its transfer to the second substrate. This mechanism does not require formation of a ternary complex and is referred to as an *enzyme-substituted mechanism.*

In a two-substrate enzyme-substituted mechanism there is a required order of substrate addition to the enzyme: The substrate with the group to be transferred must bind first, react with the enzyme to form the substituted enzyme, and allow the first product to be released. However, in ternary complex mechanisms a number of options are possible. The enzyme may have to bind one substrate initially to induce a conformational change in the protein that allows the second substrate to bind. Such

an enzyme would be described as having a *compulsory order* of substrate addition. Alternatively, it may make no difference to the enzyme that substrate binds first; the other substrate will then bind to give the ternary complex. This enzyme kinetic mechanism is referred to as *random order.*

With all of these a final component of the description of the formal kinetic mechanism involves knowledge of whether it is "*steady-state*" or "*rapid equilibrium.*" Both concepts were dealt with in Chap. 13 and are not reiterated here. It is the goal of an enzyme kinetic study to determine both the order and nature of substrate addition and, where appropriate, product release.

As a result of these considerations, for a two-substrate enzyme we have five formal kinetic mechanisms to consider. They are: compulsory order, steady state; compulsory order, equilibrium; random order, rapid equilibrium; random order, steady state; and enzyme substituted. In the next section equations for each of these are developed and, as will be seen, these equations (with one exception) fit the general form proposed by Dalziel:

$$\frac{e}{V_0} = \theta_0 + \frac{\theta_1}{\mathrm{A}} + \frac{\theta_2}{\mathrm{B}} + \frac{\theta_{12}}{\mathrm{AB}} \tag{14-1}$$

This general rate equation is useful in two ways: It is easy to remember and it forms the basis for a way of analyzing initial rate data to give a series of parameters, whose properties give an indication of which formal kinetic mechanism is the most likely. The formal kinetic mechanisms examined in this chapter are not meant to form a comprehensive list, but a list of the most common. Each mechanism considered can be made more complex by the inclusion of other enzyme–substrate or enzyme–product complexes, and more complex equations describing such mechanisms derived. The more complex mechanisms usually still fit the generalized rate equation given in Eq. (14-1). Many enzyme kineticists adhere to the truism that one can never *prove* a formal kinetic mechanism for an enzyme; all one can do is to eliminate from consideration all reasonable alternatives, leaving the simplest formal mechanism that is consistent with the experimental data. It is the purpose of this chapter to show what types of initial rate data lead to this sort of description for the formal kinetic mechanism of a two-substrate enzyme-catalyzed reaction.

An understanding of the formal mechanism is important since it allows the use of the appropriate rate equation to give a variety of information on substrate (or product) affinities and/or various rate constants of binding or release. When in Chap. 21 we consider how enzyme reactions are regulated, information of this type will be essential to understanding the biological role of such regulation.

RATE EQUATIONS FOR TWO SUBSTRATE REACTIONS

Consider the reaction $\mathrm{A} + \mathrm{B} \rightleftharpoons \mathrm{P} + \mathrm{Q}$. There are a variety of kinetic mechanisms that can be considered for it, and each will be examined in turn to derive a rate equation. Once again, these are: (1) compulsory order, steady state; (2) compulsory order,

equilibrium; (3) random order, rapid equilibrium; (4) random order, steady state; and (5) enzyme substituted.

Compulsory Order, Steady State

This mechanism, illustrated in Fig. 14-1, involves the obligatory addition of one substrate prior to the addition of the second. Together with a compulsory order of substrate addition, the products are released in a compulsory order.

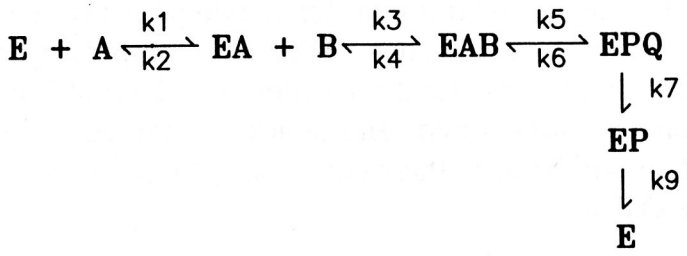

Figure 14-1 Reaction pathway for a compulsory-order, steady-state kinetic mechanism.

The enzyme conservation equation for this mechanism is given in

$$e = \mathrm{E} + \mathrm{EA} + \mathrm{EAB} + \mathrm{EPQ} + \mathrm{EP} \tag{14-2}$$

There are five enzyme-containing species, and hence four steady-state equations are required:

$$\frac{d\mathrm{EA}}{dt} = k_1 \cdot \mathrm{E} \cdot \mathrm{A} - k_2 \cdot \mathrm{EA} - k_3 \cdot \mathrm{EA} \cdot \mathrm{B} + k_4 \cdot \mathrm{EAB} = 0 \tag{14-3}$$

$$\frac{d\mathrm{EAB}}{dt} = k_3 \cdot \mathrm{EA} \cdot \mathrm{B} - \mathrm{EAB}(k_4 + k_5) + \mathrm{EPQ} \cdot k_6 = 0 \tag{14-4}$$

$$\frac{d\mathrm{EPQ}}{dt} = k_5 \cdot \mathrm{EAB} - \mathrm{EPQ}(k_6 + k_7) = 0 \tag{14-5}$$

$$\frac{d\mathrm{EP}}{dt} = k_7 \cdot \mathrm{EPQ} - k_9 \cdot \mathrm{EP} = 0 \tag{14-6}$$

For convenience we can start the derivation with the assumption that the initial rate (V_0) is given by

$$V_0 = k_9 \cdot \mathrm{EP} \tag{14-7}$$

However, other starting points can be used and give the same final equation. From Eq. (14-6)

$$\mathrm{EP} = \frac{k_7 \cdot \mathrm{EPQ}}{k_9}$$

Thus

$$\mathrm{EPQ} = \frac{\mathrm{EP} \cdot k_9}{k_7} \tag{14-8}$$

From Eq. (14-5),

$$\mathrm{EAB} = \frac{\mathrm{EPQ}(k_6 + k_7)}{k_5} \tag{14-9}$$

and hence

$$\mathrm{EAB} = \frac{\mathrm{EP} \cdot k_9(k_6 + k_7)}{k_5 \cdot k_7} \tag{14-10}$$

From Eq. (14-4)

$$\mathrm{EAB}(k_4 + k_5) = \mathrm{EPQ} \cdot k_6 + \mathrm{EA} \cdot k_3 \cdot \mathrm{B} \tag{14-11}$$

Thus

$$\mathrm{EA} \cdot k_3 \cdot \mathrm{B} = \mathrm{EAB}(k_4 + k_5) - \mathrm{EPQ} \cdot k_6 \tag{14-12}$$

Using the expression for EAB in terms of EPQ, we get

$$\mathrm{EA} \cdot k_3 \cdot \mathrm{B} = \mathrm{EPQ} \cdot \frac{(k_6 + k_7) \cdot (k_4 + k_5)}{k_5 - k_6} \tag{14-13}$$

$$= \frac{\mathrm{EPQ}}{k_5 \cdot (k_4k_6 + k_4k_7 + k_5k_7)} \tag{14-14}$$

$$= \frac{\mathrm{EP} \cdot k_9}{k_5k_7 \cdot (k_4k_6 + k_4k_7 + k_5k_7)} \tag{14-15}$$

Therefore,

$$\mathrm{EA} = \frac{(\mathrm{EP} \cdot k_9)(k_4k_6 + k_4k_7 + k_5k_7)}{k_3k_5k_7 \cdot \mathrm{B}} \tag{14-16}$$

An expression for E in terms of EP is now obtained from either Eq. (14-3) or the steady-state equation for E,

$$\frac{d\mathrm{E}}{dt} = \mathrm{EP} \cdot k_9 + \mathrm{EA} \cdot k_2 - \mathrm{E} \cdot k_1 \cdot \mathrm{A} = 0 \tag{14-17}$$

Hence

$$\mathrm{E} \cdot k_1 \cdot \mathrm{A} = \mathrm{EP} \cdot k_9 + \mathrm{EA} \cdot k_2 \tag{14-18}$$

Therefore,

$$\mathrm{E} = \mathrm{EP} \cdot \left(\frac{k_9}{ki\mathrm{A}} + \frac{k_2k_9(k_4k_6 + k_4k_7 + k_5k_7)}{k_1k_3k_5k_7 \cdot \mathrm{A} \cdot \mathrm{B}} \right) \tag{14-19}$$

Using the enzyme conservation equation [Eq. (14-2)], the expression for the total enzyme concentration, e, becomes

$$e = \mathrm{EP}\left(1 + \frac{k_9}{k_7} + \frac{k_9(k_6 + k_7)}{k_5k_7} + \frac{k_9(k_4k_6 + k_4k_7 + k_5k_7)}{k_3k_5k_7 \cdot \mathrm{B}} + \frac{k_9}{k_1 \cdot \mathrm{A}} + \frac{k_2k_9(k_4k_6 + k_4k_7 + k_5k_7)}{k_1k_3k_5k_7 \cdot \mathrm{A} \cdot \mathrm{B}}\right) \tag{14-20}$$

Since from Eq. (14-7), $V_0 = k_9 \cdot \mathrm{EP}$, we obtain an expression for e/V_0, given in

$$\frac{e}{V} = \frac{1/k_9 + 1/k_7 + (k_6 + k_7)}{k_5k_7} + \frac{k_4k_6 + k_4k_7 + k_5k_7}{k_3k_5k_7 \cdot \mathrm{B}} + \frac{1}{k_1 \cdot \mathrm{A}} + \frac{k_2(k_4k_6 + k_4k_7 + k_5k_7)}{k_1k_3k_5k_7 \cdot \mathrm{A} \cdot \mathrm{B}} \tag{14-21}$$

In terms of Dalziel's θ parameters, this equation may be written:

$$\frac{e}{V_0} = \theta_0 + \frac{\theta_1}{\mathrm{A}} + \frac{\theta_2}{\mathrm{B}} + \frac{\theta_{12}}{\mathrm{AB}} \tag{14-22}$$

where the θ parameters are the appropriate groups of constants from the Eq. (14-21).

If both substrates are considered to be saturating, Eq. (14-22) reduces to

$$\frac{e}{V_0} = \theta_0 \tag{14-23}$$

Under these conditions the enzyme is operating at its maximum rate, V_{max}, which is related to θ_0 by

$$\frac{V_{max}}{e} = \frac{1}{\theta_0} \tag{14-24}$$

If only one of the substrates is set at a saturating concentration, Eq. (14-22) becomes either Eq. (14-25) or (14-26), depending on the saturating substrate.

$$\frac{e}{V_0} = \theta_0 + \frac{\theta_1}{\mathrm{A}} \qquad \text{Sat. B} \tag{14-25}$$

$$\frac{e}{V_0} = \theta_0 + \frac{\theta_2}{\mathrm{B}} \qquad \text{Sat. A} \tag{14-26}$$

By rearrangement we obtain the Michaelis–Menten form of these equations:

$$V_0 = \frac{V_{max}\,\mathrm{A}}{\mathrm{A} + \theta_1/\theta_0} \tag{14-27}$$

$$V_0 = \frac{V_{max}\mathrm{B}}{\mathrm{B} + \theta_2/\theta_0} \tag{14-28}$$

Equilibrium Treatment of a Compulsory-Order Mechanism

As discussed for one substrate systems, when the more restrictive equilibrium assumption is applied the derivation of a rate equation is a simpler process. The rate-limiting step is determined by the equilibrium assumption as the catalytic step EAB → E + products. Product release steps need not be considered, and the enzyme–substrate complexes can be described by the appropriate equilibrium equations.

$$\mathbf{E} + \mathbf{A} \underset{}{\overset{K1}{\rightleftharpoons}} \mathbf{EA} + \mathbf{B} \overset{K2}{\rightleftharpoons} \mathbf{EAB} \overset{k}{\rightleftharpoons} \mathbf{EPQ}$$

Figure 14-2 Reaction pathway for an equilibrium compulsory-order kinetic mechanism.

For the reaction shown in Fig. 14-2 we can write two equilibrium expressions:

$$K_1 = \frac{\mathrm{E} \cdot \mathrm{A}}{\mathrm{EA}} \tag{14-29}$$

$$K_2 = \frac{\mathrm{EA} \cdot \mathrm{B}}{\mathrm{EAB}} \tag{14-30}$$

The initial velocity V_0 is obtained by substitution from Eqs. (14-29) and (14-30) into the expression $V_0 = k \cdot \mathrm{EAB}$, as in

$$V_0 = k \cdot \mathrm{EAB} = \frac{k \cdot \mathrm{EA} \cdot \mathrm{B}}{K_2} = \frac{k \cdot \mathrm{E} \cdot \mathrm{A} \cdot \mathrm{B}}{K_1 \cdot K_2} \tag{14-31}$$

The enzyme conservation equation for the reaction is given by

$$e = \mathrm{E} + \mathrm{EA} + \mathrm{EAB} \tag{14-32}$$

which, using Eqs. (14-29) and (14-30) to substitute into Eq. (14-32) gives, after rearrangement,

$$e = \mathrm{E}\left(1 + \frac{\mathrm{A}}{K_1} + \frac{\mathrm{A} \cdot \mathrm{B}}{K_1 \cdot K_2}\right) \tag{14-33}$$

Thus

$$\mathrm{E} = \frac{e}{1 + \mathrm{A}/K_1 + \mathrm{AB}/(K_1 \cdot K_2)} \tag{14-34}$$

and

$$V_0 = \frac{k \cdot e \cdot \mathrm{A} \cdot \mathrm{B}}{(K_1 \cdot K_2)[1 + \mathrm{A}/K_1 + \mathrm{A} \cdot \mathrm{B}/(K_1 \cdot K_2)]} \tag{14-35}$$

$$= \frac{k \cdot e \cdot \mathrm{A} \cdot \mathrm{B}}{K_1 \cdot K_2 + \mathrm{A} \cdot \mathrm{B} + K_2 \cdot \mathrm{A}} \tag{14-36}$$

and

$$\frac{e}{V_0} = \frac{K_1 \cdot K_2 + \mathrm{A} \cdot \mathrm{B} + K_2 \cdot \mathrm{A}}{k \cdot \mathrm{A} \cdot \mathrm{B}} \tag{14-37}$$

$$= \frac{1}{k} + \frac{K_2}{K \cdot \mathrm{B}} + \frac{K_1 \cdot K_2}{k \cdot \mathrm{A} \cdot \mathrm{B}} \tag{14-38}$$

which, in terms of Dalziel's θ parameters, becomes

$$\frac{e}{V_0} = \theta_0 + \frac{\theta_2}{\mathrm{B}} + \frac{\theta_{12}}{\mathrm{AB}} \tag{14-39}$$

It is apparent that for such a mechanism, θ, is equal to 0.

Random Order, Rapid Equilibrium

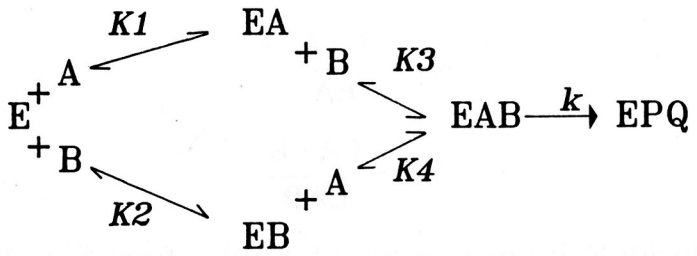

Figure 14-3 Reaction mechanism for a random-order, rapid-equilibrium kinetic mechanism.

As with the previous mechanism, derivation of the rate equation is quite simple. From the mechanism shown in Fig. 14-3, the four equilibrium constants K_1, K_2, K_3, and K_4 are defined by

$$K_1 = \frac{\mathrm{E} \cdot \mathrm{A}}{\mathrm{EA}} \tag{14-40}$$

$$K_2 = \frac{\mathrm{E} \cdot \mathrm{B}}{\mathrm{EB}} \tag{14-41}$$

$$K_3 = \frac{\mathrm{EA} \cdot \mathrm{B}}{\mathrm{EAB}} \tag{14-42}$$

$$K_4 = \frac{\mathrm{EB} \cdot \mathrm{A}}{\mathrm{EAB}} \tag{14-43}$$

The initial rate, V_0, is given by

$$V_0 = k\mathrm{EAB} = \frac{k \cdot \mathrm{EA} \cdot \mathrm{B}}{K_3} = \frac{k \cdot \mathrm{E} \cdot \mathrm{A} \cdot \mathrm{B}}{K_1 \cdot K_3} \tag{14-44}$$

The enzyme conservation equation for this mechanism is

$$e = \mathrm{E} + \mathrm{EA} + \mathrm{EB} + \mathrm{EAB} \tag{14-45}$$

which, using Eqs. 14-40 to 14-43, becomes

$$e = \mathrm{E}\left(1 + \frac{\mathrm{A}}{K_1} + \frac{\mathrm{B}}{K_2} + \frac{\mathrm{A}\cdot\mathrm{B}}{K_1\cdot K_3}\right) \tag{14-46}$$

Thus

$$V_0 = \frac{k\cdot e\cdot \mathrm{A}\cdot \mathrm{B}}{K_1\cdot K_3[1 + \mathrm{A}/K_1 + \mathrm{B}/K_2 + \mathrm{A}\cdot\mathrm{B}/(K_1\cdot K_3)]} \tag{14-47}$$

$$= \frac{k\cdot e\cdot \mathrm{A}\cdot \mathrm{B}}{K_1\cdot K_3 + K_3\cdot \mathrm{A} + K_1\cdot K_3\cdot \mathrm{B}/K_2 + \mathrm{A}\cdot\mathrm{B}} \tag{14-48}$$

and

$$\frac{e}{V_0} = \frac{K_1\cdot K_3}{k\cdot \mathrm{A}\cdot \mathrm{B}} + \frac{K_3}{k\cdot \mathrm{B}} + \frac{K_1\cdot K_3}{K_2\cdot k\cdot \mathrm{A}} + \frac{1}{k} \tag{14-49}$$

However, $K_1\cdot K_3 = K_2\cdot K_4$ and therefore $K_1\cdot K_3/K_2 = K_4$, and Eq. (15-49) becomes

$$\frac{e}{V_0} = \frac{K_1\cdot K_3}{k\cdot \mathrm{A}\cdot \mathrm{B}} + \frac{K_3}{k\cdot \mathrm{B}} + \frac{K_4}{k\cdot \mathrm{A}} + \frac{1}{k} \tag{14-50}$$

which of course fits the generalized form of Dalziel's θ parameter equation,

$$\frac{e}{V_0} = \theta_0 + \frac{\theta_1}{\mathrm{A}} + \frac{\theta_2}{\mathrm{B}} + \frac{\theta_{12}}{\mathrm{AB}} \tag{14-51}$$

Random Order, Steady State

If one does not apply the restrictive equilibrium assumption to the previous mechanism, in place of the equilibrium constants the individual rate constants for substrate or product binding and release must be used. A scheme for these rate constants is shown in Fig. 14-4.

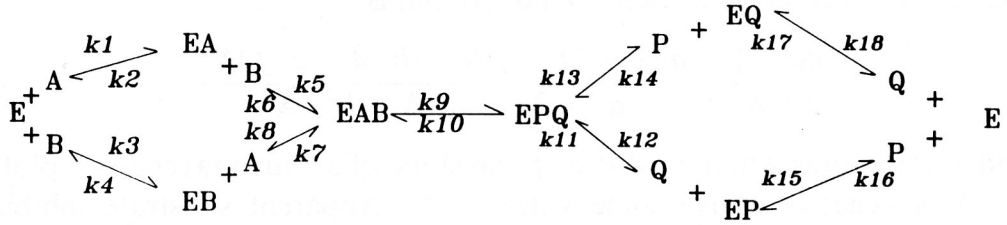

Figure 14-4 Reaction mechanism for a random-order, steady-state kinetic mechanism, showing rate constants for various ligand binding or release steps.

The enzyme conservation equation here includes two additional terms when compared to that for the compulsory order steady-state mechanism, and is given by

$$e = \mathrm{E} + \mathrm{EA} + \mathrm{EB} + \mathrm{EAB} + \mathrm{EPQ} + \mathrm{EP} + \mathrm{EQ} \tag{14-52}$$

with EB and EQ being the additional terms.

In the absence of product (which eliminates the consideration of k_{12}, k_{14} and k_{16}), the steady-state expression, derived in an analogous manner to that for the compulsory order steady-state mechanism, is given by

$$\frac{e}{V} = \frac{\text{numerator}}{\text{denominator}} \tag{14-53}$$

where

$$\begin{aligned}\text{numerator} &= k_2k_4[Y(k_6 + k_8) + k_9] \\ &\quad + \mathrm{A} \cdot \{k_1 \cdot k_4[Y \cdot (k_6 + k_8) + k_9] + k_2k_7(Yk_6 + k_9)\} \\ &\quad + \mathrm{B} \cdot \{k_2 \cdot k_3[Y \cdot (k_6 + k_8) + k_9] + k_4k_7(Yk_8 + k_9)\} \\ &\quad + \mathrm{A}^2 \cdot k_1k_7(Y \cdot k_6k_9) + \mathrm{B}^2 \cdot k_3 \cdot k_5(Y \cdot k_8 + k_9) \\ &\quad + \mathrm{A} \cdot \mathrm{B} \cdot \{k_1 \cdot k_5[(Y + Z) \cdot (k_4 + k_7 \cdot \mathrm{A}) + Yk_7]\} \\ &\quad + k_3k_7 \cdot [(\mathrm{Y} + Z) \cdot (k_2 + k_5\mathrm{B}) + Yk_6] + k_5k_7k_9\end{aligned}$$

$$\text{denominator} = \mathrm{A} \cdot \mathrm{B} \cdot k_9[k_1 \cdot k_5(k_4 + k_7 \cdot \mathrm{A}) + k_3k_7(k_2 + k_5 \cdot \mathrm{B})]$$

and where

$$Y = 1 + \frac{k_{10}}{k_{13} + k_{11}}$$

$$Z = \frac{k_9(k_{17} \cdot k_{15} + k_{17} \cdot k_{11} + k_{13} \cdot k_{15})}{k_{17} \cdot k_{15}(k_{13} + k_{11})}$$

This equation clearly does not fit the linear format of those describing the other mechanisms thus far considered, but rather is of the form

$$\frac{e}{V} = \frac{e + d \cdot \mathrm{A} + f \cdot \mathrm{A}^2}{a \cdot \mathrm{A} + b \cdot \mathrm{A}^2} \tag{14-54}$$

where one substrate, B, is held at fixed concentrations, and where a, b, c, d, and f, are functions of the fixed substrate, B, and various rate constants.

The first differential coefficient of Eq. (14-54) is

$$\frac{d(e/V)}{d(1/\mathrm{A})} = \frac{a \cdot c + 2b \cdot c \cdot \mathrm{A} + (b \cdot d - a \cdot f)\mathrm{A}^2}{a^2 + 2 \cdot a \cdot b \cdot \mathrm{A} + b^2 \cdot \mathrm{A}^2} \tag{14-55}$$

Under conditions where $b \cdot d < a \cdot f$, the slope of a Lineweaver-Burk plot of e/V versus 1/A is negative above some value of A. Apparent substrate inhibition is observed.

The second differential coefficient of Eq. (14-54) is

$$\frac{d^2(e/V)}{d(1/\mathrm{A})^2} = \frac{2F \cdot \mathrm{A}^3}{(a + b\mathrm{A})^3} \tag{14-56}$$

where $F = a^2 \cdot f + b^2 \cdot c - a \cdot b \cdot d$ and may be positive or negative depending on the fixed substrate concentration and the relative values of the rate constants that make up a, b, c, d, and f.

If conditions are such that F is positive, the slope of the Lineweaver-Burk plot increases with increasing value of 1/A, and the plot is concave upward. An equivalent plot of V versus [A] is sigmoidal. If, on the other hand, F is negative, the Lineweaver–Burk plot is concave downward and resembles substrate activation.

Enzyme-Substituted Mechanism

In this mechanism (sometimes also referred to as double displacement or ping-pong) both substrates are not required to be bound to the enzyme together, in distinction to the previous four mechanisms where a ternary EAB complex must be formed. The formal scheme for this mechanism is shown in Fig. 14-5.

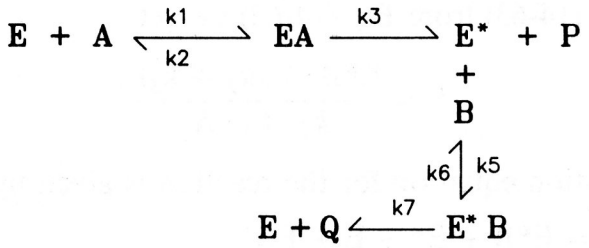

Figure 14-5 Reaction mechanism for an enzyme-substituted mechanism. E* is a covalent intermediate formed from E and A, with release of P.

As with other steady-state mechanisms, the product addition steps need not be considered, and the following steady-state equations are required:

$$\frac{d\mathrm{E}}{dt} = \mathrm{EA} \cdot k_2 + \mathrm{E^*B} \cdot k_7 - \mathrm{E} \cdot k_1 \cdot \mathrm{A} = 0 \tag{14-57}$$

$$\frac{d\mathrm{E^*B}}{dt} = \mathrm{E^*} \cdot k_5 \cdot \mathrm{B} - \mathrm{E^*B}(k_7 + k_6) = 0 \tag{14-58}$$

$$\frac{d\mathrm{EA}}{dt} = \mathrm{E} \cdot k_1 \cdot \mathrm{A} - \mathrm{EA}(k_2 + k_3) = 0 \tag{14-59}$$

$$\frac{d\mathrm{E^*}}{dt} = \mathrm{EA} \cdot k_3 + \mathrm{E^*B} \cdot k_6 - \mathrm{E^*} \cdot k_5 \cdot \mathrm{B} = 0 \tag{14-60}$$

From Eq. (14-58) we get an expression for E*:

$$\mathrm{E^*} \cdot k_5 \cdot \mathrm{B} = \mathrm{E^*B}(k_7 + k_6)$$

Therefore,

$$\mathrm{E^*} = \frac{\mathrm{E^*B}(k_7 + k_6)}{k_5 \cdot \mathrm{B}} \tag{14-61}$$

From Eq. (14-59) we get an expression for EA:

$$\mathrm{E} \cdot k_1 \cdot \mathrm{A} = \mathrm{EA}(k_2 + k_3)$$

Therefore,

$$\mathrm{EA} = \frac{\mathrm{E} \cdot k_1 \cdot \mathrm{A}}{k_2 + k_3} \tag{14-62}$$

For this particular mechanism, the net flow from E to EA, from EA to E*, and from E* to E*B must be the same. Therefore,

$$\mathrm{EA} \cdot k_3 = \mathrm{E^*B} \cdot k_7$$

Therefore,

$$\mathrm{EA} = \frac{\mathrm{E^*B} \cdot k_7}{k_3} \tag{14-63}$$

Substituting into Eq. (14-63) from Eq. (14-62) we get

$$\mathrm{E} = \frac{\mathrm{E^*B} \cdot k_7(k_2 + k_3)}{k_1 \cdot k_3 \cdot \mathrm{A}} \tag{14-64}$$

The enzyme conservation equation for the reaction is given by

$$\begin{aligned} e &= \mathrm{E^*B} + \mathrm{E^*} + \mathrm{E} + \mathrm{EA} \\ &= \mathrm{E^*B}\left(1 + \frac{k_7 + k_6}{k_5 \cdot \mathrm{B}} + \frac{k_7}{k_3} + k_7 \frac{(k_2 + k_3)}{k_1 \cdot k_3 \cdot \mathrm{A}}\right) \end{aligned} \tag{14-65}$$

Since $V_0 = k_7 \cdot \mathrm{E^*B}$, then

$$\frac{e}{V_0} = \frac{1}{k_7} + \frac{1}{k_3} + \frac{k_2 + k_3}{k_1 k_3 \cdot \mathrm{A}} + \frac{k_6 + k_7}{k_5 \cdot k_7 \cdot \mathrm{B}} \tag{14-66}$$

As with most of the other cases considered, this equation is in the general form of Dalziel's θ parameter equation, where the θ_{12} parameter is equal to 0.

$$\frac{e}{V_0} = \theta_0 + \frac{\theta_1}{\mathrm{A}} + \frac{\theta_2}{\mathrm{B}} \tag{14-67}$$

TABLE 14-1 Form of kinetic constants for two-substrate mechanisms

	Constant		
Mechanism[a]	V_{max} $1/\theta_0$	K_m(A) θ_1/θ_0	K_m(B) θ_2/θ_0
1	$\dfrac{1}{1/k_9 + 1/k_7 + (k_6 + k_7)/k_5k_7)}$	$\dfrac{1}{k_1[1/k_9 + 1/k_7 + (k_6 + k_7)/k_5k_7]}$	$\dfrac{k_4k_6 + k_4k_7 + k_5k_7}{k_3[(k_5k_7/k_9) + k_5 + k_6 + k_7]}$
2	k	0	K_2
3	k	K_4	K_3
4	$\dfrac{1}{1/k_7 + 1/k_3}$	$\dfrac{k_2 + k_3}{k_1k_3/k_7 + k_1}$	$\dfrac{k_6 + k_7}{k_5 + k_5k_7/k_3}$

[a] 1, Compulsory order, steady state; 2, compulsory order, equilibrium; 3, random order, rapid equilibrium; 4, enzyme substituted.

TABLE 14-2 Summary of θ parameters for two-substrate mechanisms

Mechanism[a]	Parameter			
	θ_0	θ_1	θ_2	θ_{12}
1	$\frac{1}{k_9}+\frac{1}{k_7}+\frac{k_6+k_7}{k_5k_7}$	$\frac{1}{k_1}$	$\frac{k_4k_6+k_4k_7+k_5k_7}{k_3k_5k_7}$	$\frac{k_2(k_4k_6+k_4k_7+k_5k_7)}{k_1k_3k_5k_7}$
2	$\frac{1}{k}$	0	$\frac{k_2}{k}$	$K_1\frac{K_2}{k}$
3	$\frac{1}{k}$	$\frac{K_4}{k}$	$\frac{K_3}{k}$	$K_1\frac{K_3}{k}$
4	$\frac{1}{k_7}+\frac{1}{k_3}$	$\frac{k_2+k_3}{k_1k_3}$	$\frac{k_6+k_7}{k_5k_7}$	0

[a] 1, Compulsory order, steady state; 2, compulsory order, equilibrium: 3, random order, rapid equilibrium; 4, enzyme substituted.

From these derivations it is clear that four of the five mechanisms considered give rise to the general form of a rate equation for two substrates as proposed by Dalziel. Only the random order, steady-state mechanism yields an equation that cannot, without further simplifying assumptions, give rise to linear Lineweaver–Burk plots. Over limited ranges of substrate concentrations even this mechanism may give an apparently linear Lineweaver–Burk plot, and "fit" to the Dalziel equation. Thus it is important to establish "linearity" over as wide a substrate concentration range as practical. Table 14–1 summarizes the values of the four θ parameters of the generalized rate equation for these four mechanisms. In Table 14–2 expressions for V_{max} and the Michaelis constants for the two substrates A and B are given.

EXPERIMENTAL DETERMINATION OF θ PARAMETERS

Experimentally, θ 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 A or substrate B is varied.

$$\frac{e}{V_0}=\left(\theta_0+\frac{\theta_2}{\mathrm{B}}\right)+\left(\theta_1+\frac{\theta_{12}}{\mathrm{B}}\right)\frac{1}{\mathrm{A}} \tag{14-68}$$

$$\frac{e}{V_0}=\left(\theta_0+\frac{\theta_1}{\mathrm{A}}\right)+\left(\theta_2+\frac{\theta_{12}}{\mathrm{A}}\right)\frac{1}{\mathrm{B}} \tag{14-69}$$

These two equations form the basis of the experimental determination of θ parameters from kinetic data. The slope and intercept of the appropriate Lineweaver–Burk plot is related to various constants and the concentration of the nonvaried substrate. Primary Lineweaver–Burk plots of data (Fig. 14-6) give slopes and intercepts

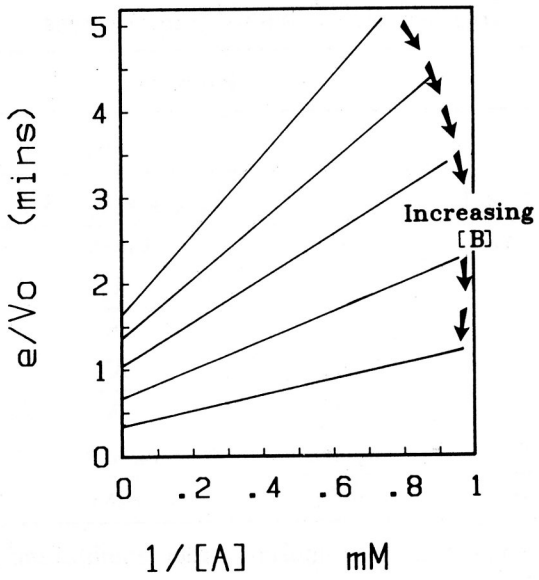

Figure 14-6 Primary Lineweaver–Burk plot of initial rate data according to Eq. (14-68). From this primary plot, slopes and intercepts of plots of e/V_0 versus 1/A are obtained at a series of fixed concentrations of B.

that are replotted versus the reciprocal of the concentration of the second substrate; for example, from Eq. (14-68), the slope of the primary plot with A as the varied substrate is given by the expression

$$\text{slope} = \theta_1 + \frac{\theta_{12}}{\text{B}} \tag{14-70}$$

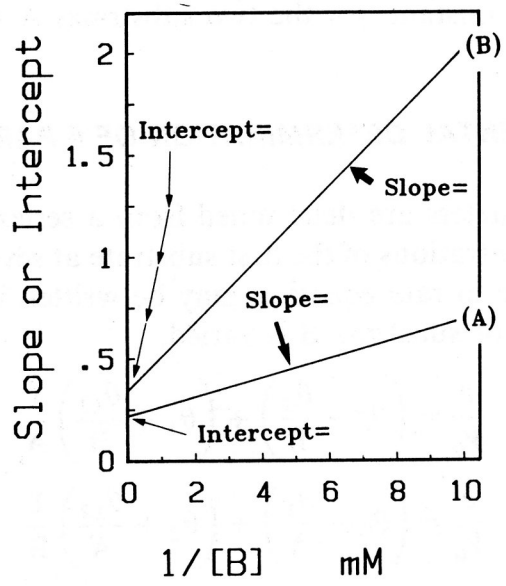

Figure 14-7 Secondary plots of (A) slopes, or (B) intercepts obtained from primary plots versus 1/B. Individual θ parameters are obtained from the slopes and intercepts of the secondary plots as indicated.

and the intercept by the expression

$$\text{intercept} = \theta_0 + \frac{\theta_2}{B} \tag{14-71}$$

Clearly, as illustrated in Fig. 14-7, individual θ parameters are then obtained from the slopes and intercepts of such secondary plots, which are constructed by plotting either slopes or intercepts obtained from the primary plots versus 1/B, according to Eq. (14-70) or Eq. (14-71), respectively.

Since the general rate equation is symmetrical [compare Eqs. (14-68) and (14-69)], it does not matter whether the primary plots are constructed with A or B as the varied substrate—equivalent values for the θ 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

Based on the discussion to date and the rate equations derived for the various two-substrate kinetic mechanisms we are concerned with here, several methods for distinguishing among these mechanisms can be considered. Examination of the derived rate equations reveals two basic approaches, one involving the overall form of the rate equation and the other the physical significance of individual θ parameters in the generalized rate equations for various mechanisms.

Primary Plots of Kinetic Data

While all the mechanisms except the random-order, steady-state mechanism conform to the generalized rate equation proposed by Dalziel, two of the four are lacking one term in the rate equation. With the enzyme-substituted mechanism, the θ_{12} term is equal to 0. This has immediate implications for the pattern of Lineweaver–Burk plots obtained when one substrate concentration is varied at a series of fixed concentrations of the other. From the rate equation for the mechanism [Eq. (14-66)], it is evident that in Lineweaver–Burk plots with either A or B as the varied substrate, only the intercept changes as a function of the nonvaried substrate concentration. The slopes of the Lineweaver–Burk plots are either $(k_2 + k_3)/(k_1k_3)$, when A is the varied substrate, or $(k_6 + k_7)/(k_5k_7)$, when B is the varied substrate, and are of course independent of substrate concentration. The resultant series of parallel lines obtained are a quite distinctive feature of enzyme-substituted mechanisms.

The ternary complex mechanisms are all characterized by a positive value for θ_{12}, requiring that in such mechanisms slopes of Lineweaver–Burk plots change as a function of the concentration of the nonvaried substrate. With one of the three ternary

complex mechanisms, whose rate equations do fit into the generalized Dalziel rate equations however, there is a distinctive feature. In the compulsory-order, equilibrium mechanism the θ_1 parameter is equal to 0. From the rate equation for the mechanism [Eq. (14-38)], it is apparent that in Lineweaver–Burk plots with B as the varied substrate, the intercept is equal to $1/k$ and is independent of the concentration of substrate A. With such a mechanism Lineweaver–Burk plots with B as the varied substrate intersect on the e/V axis for various fixed concentrations of the substrate A. With A as the varied substrate, of course, a normal pattern of plots is obtained: Both slope ($= K_1 \cdot K_2/\mathrm{B}$) and intercept ($= 1/k + K_2/k \cdot \mathrm{B}$) vary as the concentration of B is changed.

As we have 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. On the surface, compulsory-order, equilibrium, and enzyme-substituted mechanisms are easily distinguishable from the others considered. Care must be taken, however, in assigning a θ-parameter value equal to 0 and the experimental determination of θ parameters has to cover a sufficient range of substrate concentrations to allow the unequivocal demonstration of the presence or absence of a particular θ parameter. Consider the case of a random-order, rapid-equilibrium mechanism where one of the substrates binds extremely tightly to the enzyme in the presence of the other. The effect on the overall rate equation [Eq. (14-50)] of extremely tight binding of substrate A to EAB (i.e. $K_4 \rightarrow 0$) is that the term $K_4/(K\mathrm{A})$ tends to approach 0 and the mechanism may, if sufficiently low concentrations of A are not used in θ-parameter determinations, resemble a compulsory-order, equilibrium mechanism.

Such considerations aside, however, 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. A number of simple approaches are examined here (others are considered in Chap. 19) that 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 from the rate equation for the compulsory-order, steady-state mechanism it is evident that $\theta_1 = 1/k_1$, where k_1 is the rate constant for the first substrate (A) binding to the enzyme. In the random-order, rapid equilibrium mechanism, this θ parameter does not have this simple physical significance. Consider then the effect of determining the four θ parameters in the generalized rate equation with a series of alternative substrates, B, B_1, B_2, and so on. If the mechanism is in fact compulsory order, steady

state with B as the second substrate, the value of θ_1 determined for the different alternate substrates is constant. If the mechanism is really random order, rapid equilibrium or compulsory order, steady state, with B as the *first* substrate, θ_1 varies as the nature of B varies. To govern against this possibility it is necessary to use alternative substrates to A as well as to B. If θ_1 varies as a function of B, the determinations are repeated with a single B but a variety of alternative substrates for A. Constant values for the θ_2 parameter as the nature of A is varied indicate a compulsory order, steady-state mechanism with B as the first subtrate.

As is discussed in more detail in Chap. 19 alternative substrates can be used in a variety of other ways to assist in the establishment of kinetic mechanisms. The approach described here is a simple and readily interpretable one that has been used with a variety of enzymes to help distinguish between a compulsory-order, steady-state and a random-order, rapid-equilibrium mechanism.

Comparison of Kinetically Derived Constants with Independently Determined Values

As was outlined earlier and summarized in Table 14-1, the θ parameters of the generalized rate equation have specific physical significance depending on the kinetic mechanism. This allows a comparison to be made between various θ parameters (or ratios of θ parameters) and directly determined values for specific constants in the appropriate mechanism.

Consider the rate equation derived earlier for a random-order, rapid-equilibrium mechanism [Eq. (14-50)]. The Michaelis constants for the reaction (θ_1 and θ_2 divided by θ_0) give values for K_3 and K_4, the dissociation constants for A and B from the ternary EAB complex, respectively. Alternatively, if we take the θ_{12} parameter and divide by the θ_2 parameter, we get K_1, the dissociation constant for A from the EA complex. Since as discussed earlier, $K_1K_3 = K_2K_4$, the θ_{12} parameter divided by θ_1 gives K_2, the dissociation constant for B from the EB complex. Table 14-3 summarizes ratios between θ parameters for particular mechanisms.

While both the compulsory-order, steady-state and the random-order, rapid-equilibrium mechanism give values for K_1, the latter mechanism, as you would expect, is the only one that gives a value for K_2. Direct determination of K_2 by such

TABLE 14-3 Relationships between θ parameters for two-substrate systems

	Relationship			
Mechanism[a]	θ_1/θ_0	θ_2/θ_0	θ_{12}/θ_1	θ_{12}/θ_2
1	Complex	Complex	Complex	$= K_1$
2	$= 0$	Complex	$= 0$	$= K_1$
3	$= K_4$	$= K_3$	$= K_2$	$= K_1$
4	Complex	Complex	$= 0$	$= 0$

[a] 1, Compulsory order, steady state: 2, compulsory order, equilibrium: 3, random order, rapid equilibrium; 4, enzyme substituted.

approaches as equilibrium dialysis or spectroscopic titrations (both of which are considered in Chap. 17) and comparison with the ratio θ_{12}/θ_1 can give supportive evidence for a random-order, rapid-equilibrium mechanism.

The second facet of direct comparison with independently determined parameters involves the determination of individual rate constants for particular steps in the reaction. As we have discussed, in a compulsory-order, steady-state mechanism the θ_1 parameter $= 1/k_1$, where k_1 is the rate constant for substrate A binding to enzyme. Direct determination of k_1 by rapid reaction techniques such as stopped or continuous flow, or temperature jump (all of which are discussed in Chap. 18), and comparison with the θ_1 parameter determined via enzyme kinetics gives information about whether or not a compulsory-order, steady-state mechanism is applicable.

The comparison of directly and independently determined constants with those derived from initial rate kinetic studies by assuming particular formal kinetic mechanisms reiterates the truism stated in the introduction to this chapter. Agreement between such constants is merely consistent with the mechanism: Disagreement disproves the mechanism.

Use of Analog Inhibitors

The basic concepts and equations associated with the idea of a competitive inhibitor being an analog of one of the substrates of an enzyme were discussed in Chap. 13; with two-substrate systems, however, a more complex situation holds. 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 mechanism or a random-order mechanism exists. Consider an analog (BX) of substrate B in these mechanisms. In compulsory order, steady state, BX can bind only to an EA complex ($E \cdot A + BX \rightleftharpoons E \cdot A \cdot BX$) and the interaction can be described in terms of a dissociation constant K_i. Derivation of the rate equation for such a mechanism in the presence of BX leads to the generalized format

$$\frac{e}{V_0} = \theta_0 + \frac{\theta_1}{A} + \frac{\theta_2}{B}\left(1 + \frac{BX}{K_i}\right) + \frac{\theta_{12}}{AB} \tag{14-72}$$

With the random-order, rapid-equilibrium mechanism however, BX can combine to free enzyme as well as to the EA complex, and two additional steps must be considered in the derivation of the rate equation, $E + BX \rightleftharpoons E \cdot BX$ and $E \cdot A + BX \rightleftharpoons E \cdot A \cdot BX$, with dissociation constants of K_i' and K_i, respectively.

Derivation of the rate equation with the addition of these two steps yields

$$\frac{e}{V_0} = \theta_0 + \frac{\theta_1}{A} + \frac{\theta_2}{B}\left(1 + \frac{BX}{K_i}\right) + \frac{\theta_{12}}{AB}\left(1 + \frac{BX}{K_i'}\right) \tag{14-73}$$

Consider first Lineweaver–Burk plots with B as the varied substrate. With either mechanism only the slope is affected and the inhibition, as expected, is competitive, with the slope in the presence of the inhibitor being

$$(a) = \theta_2\left(1 + \frac{BX}{K_i}\right) + \frac{\theta_{12}}{A} \tag{14-74}$$

or

$$(b) = \theta_2\left(1 + \frac{BX}{K_i}\right) + \theta_{12}\left(1 + \frac{BX}{K_i'}\right) \tag{14-75}$$

depending on whether a compulsory-order mechanism (a) or a random-order mechanism (b) is operating.

When Lineweaver–Burk plots with A 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. With a compulsory-order, steady-state mechanism an analog of B is an uncompetitive inhibitor with respect to A. With a random-order, rapid-equilibrium mechanism, an analog of B is a noncompetitive inhibitor with respect to A.

For the compulsory-order mechanism, the intercept becomes

$$\text{intercept} = \theta_0 + \frac{\theta_2}{B}\left(1 + \frac{BX}{K_i}\right) \tag{14-76}$$

while in the random-order mechanism the intercept and slope are given by

$$\text{intercept} = \theta_0 + \theta_2\left(1 + \frac{BX}{K_i}\right) \tag{14-77}$$

$$\text{slope} = \theta_1 + \frac{\theta_{12}}{B}\left(1 + \frac{BX}{K_i'}\right) \tag{14-78}$$

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 θ parameters in the presence and absence of the inhibitor.

EXAMPLES OF THE DETERMINATION OF KINETIC MECHANISMS

Histone Acetyltransferase

Reference: L.-J. Wong, and S. Wong, *Biochemistry*, *22*, 4637–4642 (1983).

This enzyme catalyzes the acetylation of nuclear histones using acetyl-CoA as substrate. The second substrate is the side-chain amino groups of lysine residues close to the N termini of various histones. Initial rate studies with the enzyme purified from calf thymus can be conducted by following the transfer of tritiated acetyl groups from tritiated acetyl-CoA into histone, using ethanol precipitation of the protein substrate and product to separate the labeled product from remaining tritiated acetyl-CoA. Lineweaver–Burk plots with either histone or acetyl-CoA as the varied substrate are given in Fig. 14-8. Both primary plots obtained show a series of parallel lines indicating the absence of the θ_{12} parameter from the rate equation. These data clearly prove that histone acetyltransferase follows an enzyme-substituted mechanism.

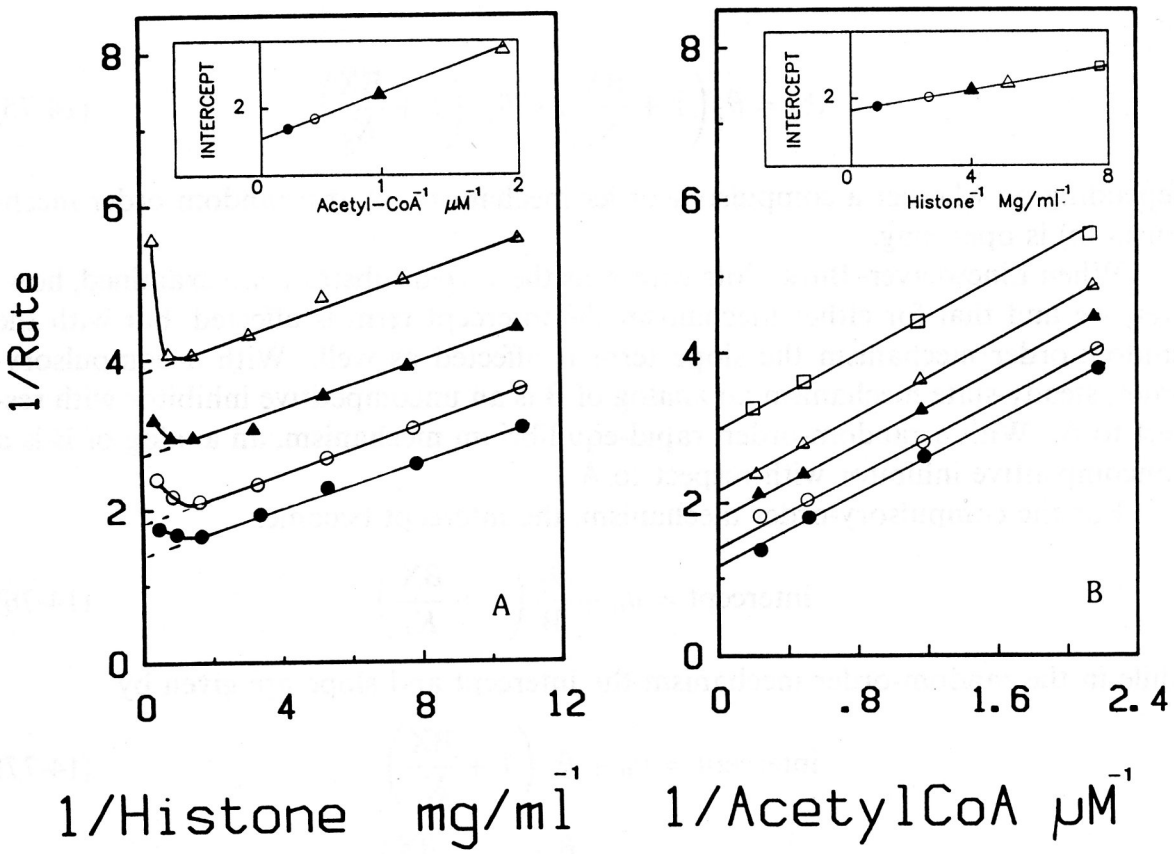

Figure 14-8 Primary Lineweaver–Burk plots for histone acetyltransferase with (A) histone concentrations varied at several acetyl-CoA concentrations, and (B) acetyl-CoA concentrations varied at several histone concentrations. The inserts are the secondary plots of intercepts from A or B versus the reciprocal of the concentration of the nonvaried substrate in the primary plots. (Reprinted with permission from: L.-J. C. Wong and S. S. Wong, *Biochemistry*, *22*, 4637–4641. Copyright 1983 American Chemical Society, Washington, D. C.)

Liver Alcohol Dehydrogenase

Reference: K. Dalziel, and F. Dickinson, *Biochem. J. 100*, 34–46 (1966).

This enzyme catalyzes the reversible oxidation–reduction of a variety of alcohols and aldehydes using NAD or NADH. The reaction is readily monitored either by following absorbance changes at 340 nm or by following fluorescence changes with emission at 450 nm and excitation at 340 nm (since the reduced coenzyme absorbs at 340 nm whereas the oxidized coenzyme does not). Using a wide range of substrate concentrations, the oxidation reaction has been studied with a variety of primary alcohols, and the reduction reaction has been studied with the corresponding aldehydes. From these it was found that all four parameters of the generalized equation were present, eliminating various mechanisms from further consideration. Table 14-4 shows the θ parameters obtained for both oxidation and reduction reactions with various alcohols and aldehydes.

TABLE 14-4 Parameters for reactions

Substrate	θ_0 (sec)	θ_1 (μM-sec)	θ_2 (μM-sec)	θ_{12} (mM^2-sec)
Ethanol	0.37	1.1	66	0.0072
Propan-1-ol	0.31	1.2	19	0.0012
Butan-1-ol	0.35	1.1	4	0.0004
2-Methylpropan-1-ol	0.34	1.7	40	0.0032
Acetaldehyde	0.0075	0.1	3.3	[a]
Propionaldehyde	0.0095	0.14	0.43	[a]
Butyraldehyde	0.0075	0.10	0.17	[a]
2-Methylpropionaldehyde	0.009	0.12	0.25	[a]

[a] No accurate value obtainable.

With both the oxidative and the reductive reactions, the θ_1 parameter is invariant with the nature of the alcohol or the aldehyde substrate, even though the θ_2 and the θ_{12} parameters change. These results are consistent with a compulsory order of substrate addition for both reactions with coenzyme as the required first substrate. Interestingly, when secondary alcohols or aldehydes are used as substrates, these relationships do not hold, suggesting that while the various primary alcohols all follow a steady-state compulsory order mechanism, the secondary alcohols may follow a different one.

Galactosyltransferase

Reference: J. E. Bell, T. Beyer, and R. Hill, *J. Biol. Chem.*, *251*, 3008–3013 (1976).

This enzyme catalyzes the transfer of galactose from UDP-galactose to an acceptor, which may be a glycoprotein or a small saccharide. The reaction is most conveniently followed using radioactive galactose in UDP-galactose and separating product from unused substrate chromatographically. Although the enzyme requires manganese, in the presence of saturating concentrations of $MnCl_2$, it can be regarded as a two-substrate enzyme. Initial rate studies with three different acceptor substrates showed that although the θ_1 parameter varied from 0.207 to 0.00068 depending on the nature of the acceptor substrate; all four parameters had positive values. These observations eliminated the enzyme-substituted mechanism and were inconsistent with a simple compulsory-order steady-state mechanism. Inhibition studies, using alternative substrates as analog inhibitors, are possible with this enzyme since the products obtained using, for example, *N*-acetylglucosamine and ovalbumin as acceptors, are readily separable. Figure 14-9 illustrates the results of experiments using *N*-acetylglucosamine as an inhibitor of the galactosylation of ovalbumin.

As expected, *N*-acetylglucosamine is a competitive inhibitor with respect to ovalbumin. When UDP-galactose concentrations are varied, *N*-acetylglucosamine is a noncompetitive inhibitor. Similar studies were performed with UDP-glucose,

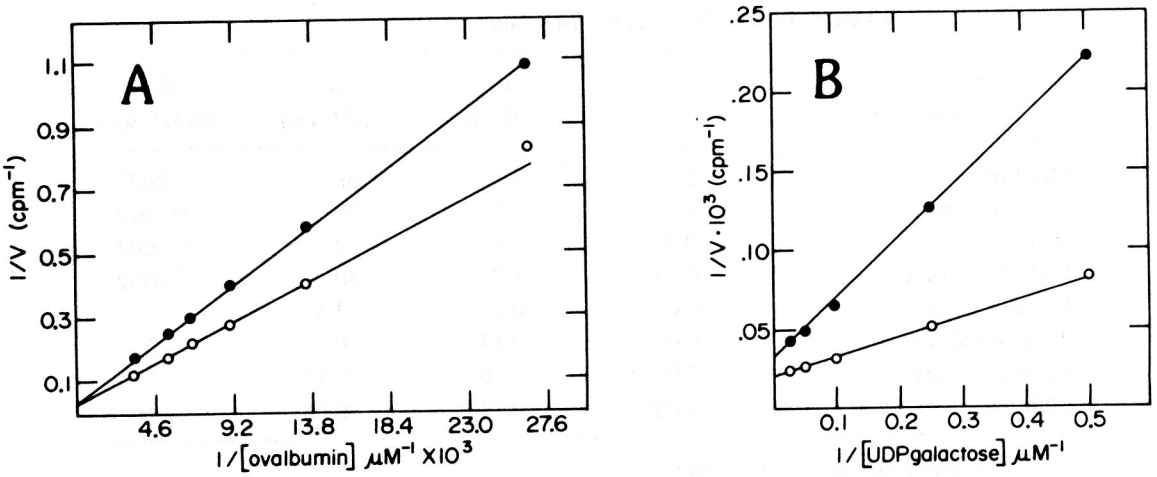

Figure 14-9 Inhibition of galactosyltransferase by *N*-acetylglucosamine with either ovalbumin (A) or UDP-galactose (B) as the varied substrate. ○, No *N*-acetylglucosamine; ●, +10 m*M* *N*-acetylglucosamine. [From J. E. Bell, T. A. Beyer and R. L. Hill, *J. of Biol. Chem.*, *251*, 3003–3013 (1976). Reprinted with the permission of the copyright owner, The American Society of Biological Chemists, Inc., Bethesda, Md.]

which was a competitive inhibitor versus UDP-galactose and a noncompetitive inhibitor versus ovalbumin. Table 14-5 summarizes the expected inhibition patterns for various possible two-substrate mechanisms for this enzyme.

The experimentally observed inhibition patterns are consistent only with the equilibrium random-order mechanism.

"*Norvaline*" *Dehydrogenase*

Reference: C. LiMuti and J. E. Bell, *Biochem. J.*, *211*, 99–107 (1983).

The enzyme glutamate dehydrogenase utilizes norvaline as a substrate for oxidative deamination, although the preferred substrate is glutamate. In detailed kinetic studies with norvaline as the varied substrate, Lineweaver–Burk plots could not be described

TABLE 14-5 Expected inhibition patterns[a]

Inhibitor	Compulsory order		Equilibrium random order
	UDP-galactose first	Acceptor first	
UDP-glucose			
versus UDP-Gal	C	C	C
versus ovalbumin	NC	UC	NC
N-Acetylglucosamine			
versus UDP-Gal	UC	NC	NC
versus ovalbumin	C	C	C

[a] C, Competitive inhibition; UC, uncompetitive inhibition; NC, noncompetitive inhibition.

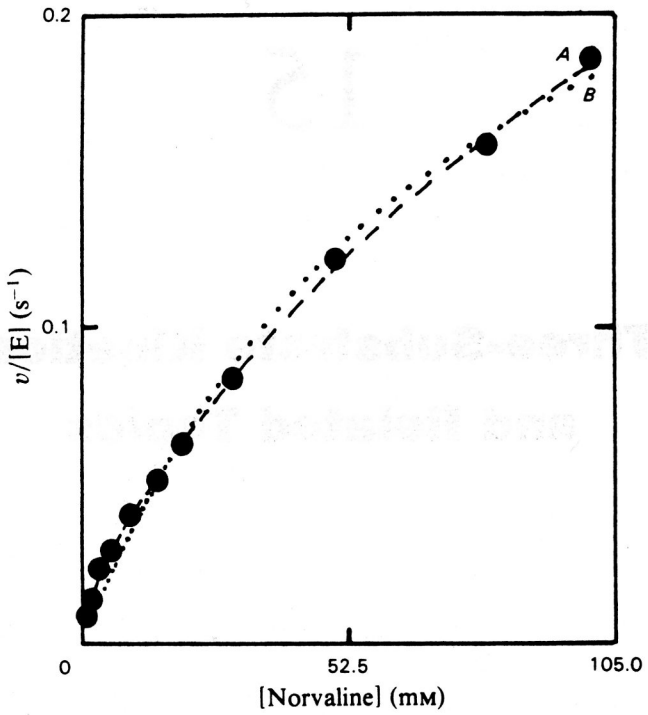

Figure 14-10 V_0 versus [norvaline] plot of data obtained with "norvaline" dehydrogenase showing theoretical curves for an equilibrium random-order mechanism (· · ·) and a steady-state random mechanism (——). (Reprinted with permission from: C. LiMuti and J. E. Bell, *Biochem. J.*, *211*, 99–107. Copyright 1983 The Biochemical Society, London.)

as linear when a wide range of substrate concentrations were used, suggesting the possibility of a steady-state random-order mechanism. Inhibition patterns with a variety of substrate analogs obtained using a limited range of substrate concentrations were consistent with a random order of substrate addition. As shown in Fig. 14-10, velocity versus [norvaline] plots are best described with an equation [in the form of Eq. (14-54)] containing second-order terms in the norvaline concentration.

From the theoretical fits to the data it is apparent that the steady-state mechanism is more appropriate than the equilibrium mechanism.

In this chapter a variety of enzyme initial rate kinetic approaches have been described that can yield information concerning the formal kinetic mechanism of an enzyme. In many cases initial rate kinetic studies must be used in conjunction with independently determined values for particular constants in the mechanism. This involves substrate equilibrium binding studies or studies of the rates of substrate binding. There are described in detail in Chap. 17 and 18, respectively.