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A Kinetic Analysis of Enzyme Systems Involving Four Substrates

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A treatment of kinetic data for enzyme mechanisms involving four substrates is described. The initial-rate equations and product-inhibition patterns for such mechanisms are presented. The treatment is extended to include analysis of enzyme mechanisms involving three substrates in which two molecules of one substrate are used.

A systematic treatment of initial-rate equations for enzyme-catalysed reactions involving two substrates has been given by a number of authors (e.g. Alberty, 1953; Dalziel, 1957; Cleland, 1963*a*). Cleland (1963*b*) has also derived and classified inhibition patterns for both product and dead-end inhibitors. Dalziel (1969) has published a systematic treatment of initial-rate equations for three substrate reactions, and concluded that far from being more confusing, the extra variable permits greater discrimination between kinetic mechanisms. The use of competitive inhibitors to distinguish various mechanisms involving three substrates has been studied by Fromm (1967), but no detailed treatment of product inhibition has been carried out.

Some enzymes catalyse reactions involving four true substrates, e.g. NAD^+ synthase (EC 6.3.5.1) (Preiss & Handler, 1958):

$$ATP + \text{deamido-}NAD^+ + \text{glutamine} + H_2O \rightleftharpoons AMP + \text{pyrophosphate} + NAD^+ + \text{glutamate}$$

and GMP synthase (EC 6.3.5.2) (Lagerkvist, 1958):

$$ATP + \text{xanthosine 5'-phosphate} + \text{glutamine} + H_2O \rightleftharpoons AMP + \text{pyrophosphate} + GMP + \text{glutamate}$$

whereas some enzymes utilize three substrates, of which two molecules of one are required, e.g. carbamoyl phosphate synthetase (EC 2.7.2.5) (Metzenberg *et al.*, 1957)

$$2ATP + HCO_3^- + NH_4^+ \rightleftharpoons 2ADP + \text{carbamoyl phosphate} + \text{phosphate}$$

and β-hydroxymethylglutaryl-CoA reductase (EC 1.1.1.34) (Knappe *et al.*, 1959)

$$\text{Mevalonate} + \text{CoASH} + 2NADP^+ \rightleftharpoons \beta\text{-hydroxymethylglutaryl-CoA} + 2NADPH$$

Examples are also known of enzymes utilizing two substrates, with three molecules of one being used, e.g. nitrite reductase (EC 1.6.6.4) catalyses the overall reaction (Lazzarini & Atkinson, 1961):

$$3NADPH + NO_2^- \rightleftharpoons 3NADPH^+ + NH_2OH + H_2O$$

Other enzymes may be considered to use four 'substrates' if an essential activator is included as a 'substrate', e.g. pyruvate carboxylase (EC 6.4.1.1) (Utter & Keech, 1960):

$$ATP + HCO_3^- + \text{pyruvate} \xrightleftharpoons{\text{Acetyl-CoA}} ADP + \text{phosphate} + \text{oxaloacetate}$$

and many enzymes require an essential metal activator, especially magnesium with enzymes utilizing ATP, e.g. succinyl-CoA synthetase (EC 6.2.1.5) (Palmer & Wedding, 1966)

$$ATP + \text{succinate} + \text{CoASH} \xrightleftharpoons{Mg^{2+}} ADP + \text{phosphate} + \text{succinyl-CoA}$$

In this theoretical treatment of kinetic data for reactions involving four substrates, which has been motivated by a study of the enzyme carbamoyl phosphate synthetase (Elliott & Tipton, 1974*a*,*b*), an attempt has been made to give a systematic treatment of both initial-rate equations and product-inhibition patterns for plausible kinetic mechanisms involving either three or four products.

Initial-rate Equations

The general initial-rate equation for a four-substrate reaction may be derived by an extension of the equations for two- and three-substrate reactions. The general equation for the two-substrate enzyme-catalysed reaction:*

$$A + B \rightleftharpoons P(+Q) \qquad (1)$$

may be written as follows (see e.g. Alberty, 1953):

$$v = \frac{V}{1 + \frac{K_m^A}{A} + \frac{K_m^B}{B} + \frac{K_s^A K_m^B}{AB}} \qquad (2)$$

* Throughout this paper substrates are represented by A, B, C and D, and products by P, Q, R and T.

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where v is the initial velocity, V is the maximum velocity, A and B are the concentrations of substrates A and B, K_m^A and K_m^B are the concentrations of A and B respectively when $v = V/2$ and the second substrate has been extrapolated to an infinite concentration, and K_s^A is the apparent dissociation constant of the complex of the enzyme with A. In some mechanisms one of the constant terms in eqn. (2) may become zero. Similarly the theoretical equation describing the initial-rate behaviour of an enzyme-catalysed three-substrate reaction:

$$A+B+C \rightleftharpoons P+Q(+R) \qquad (3)$$

(assuming that linear reciprocal plots are obtained for each substrate at constant concentrations of the other two) is as follows (adapted from Dalziel, 1969)

$$v = \frac{V}{1+\frac{K_m^A}{A}+\frac{K_m^B}{B}+\frac{K_m^C}{C}+\frac{K_s^A K_m^B}{AB}+\frac{K_s^A K_m^C}{AC}+\frac{K_{s'}^B K_m^C}{BC}+\frac{K_s^A K_{s'}^B K_m^C}{ABC}} \qquad (4)$$

where $K_{s'}^B$ is the apparent dissociation constant of the complex of B with the enzyme species to which it binds. For a three-substrate reaction, in all types of mechanism, except a totally random-order equilibrium mechanism, at least one of the constants becomes zero.

The equations may be extended to cover four-substrate reactions:

$$A+B+C+D \rightleftharpoons P+Q+R(+T) \qquad (5)$$

(assuming linear reciprocal plots) giving the theoretical equation:

$$v = \frac{V}{1+\frac{K_m^A}{A}+\frac{K_m^B}{B}+\frac{K_m^C}{C}+\frac{K_m^D}{D}+\frac{K_s^A K_m^B}{AB}+\frac{K_s^A K_m^C}{AC}+\frac{K_s^A K_m^D}{AD}+\frac{K_{s'}^B K_m^C}{BC}+\frac{K_{s'}^B K_m^D}{BD}+\frac{K_{s'}^C K_m^D}{CD}+\frac{K_s^A K_{s'}^B K_m^C}{ABC}+\frac{K_s^A K_{s'}^B K_m^D}{ABD}+\frac{K_s^A K_{s'}^C K_m^D}{ACD}+\frac{K_{s'}^B K_{s'}^C K_m^D}{BCD}+\frac{K_s^A K_{s'}^B K_{s'}^C K_m^D}{ABCD}} \qquad (6)$$

As for a three-substrate reaction only a completely random-order equilibrium mechanism will contain all the constants.

For a four-substrate reaction there are a very large number of plausible mechanisms that must be considered. However, the situation is simplified by considering that all the mechanisms must fall into one of the subdivisions in each of the following categories.

(1) (*a*) *Steady-state mechanisms.* The mechanism is best described by using the steady-state assumption.

(*b*) *Equilibrium mechanisms.* The mechanism is best described by using the equilibrium assumption.

(*c*) *Hybrid equilibrium and steady-state mechanisms.* Part of the mechanism may be considered to be in equilibrium while the rest is in a steady-state.

(2) (*a*) *Mechanisms in which a quinternary complex is formed.* A quinternary complex of the enzyme and all four substrates must be formed before products are released.

(*b*) *Mechanisms in which no quinternary complex is formed.* If no quinternary complex is formed a further subdivision applies.

(i) A situation may exist analogous to a double-displacement (Ping-Pong) mechanism where one or more products is released from the enzyme before all substrates are bound resulting in two or more stable forms of the enzyme during the reaction.

(ii) Products may be released from the enzyme before all substrates are bound, but without formation of a free modified form of the enzyme.

(3) (*a*) *Random-order binding of substrates.* The substrate may bind to the enzyme in a totally random order.

(*b*) *Compulsory-order binding of substrates.* The substrates must bind to the enzyme in a compulsory order.

(*c*) *Hybrid random–compulsory order binding of substrates.* The binding of substrates to the enzyme may be partially random, e.g. substrates A and B may bind first to the enzyme in a random order, followed by C and D in a compulsory order.

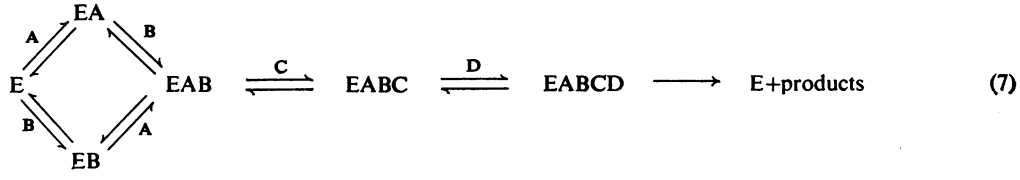
The initial-rate equations for the majority of the mechanisms considered below are derived wholly by use of the steady-state assumption by using the method of King & Altman (1956) as modified by Vol'kenshtein & Gol'dshtein (1966). Where equilibrium steps are involved the method of Cha (1968) was used to derive the equations.

Steady-State Mechanisms

Quinternary complex mechanisms

Random-order binding of substrates. Any random-order steady-state enzyme mechanism will tend to

lead to an initial-rate equation more complex than eqns. (2), (4) and (6) (cf. Wong & Hanes, 1962). With a four-substrate reaction the denominator will contain concentration terms raised to the power of up to eight giving very complex initial-rate equations and non-linear reciprocal plots. Even a partially random-order mechanism will lead to complex equations, e.g. the mechanism

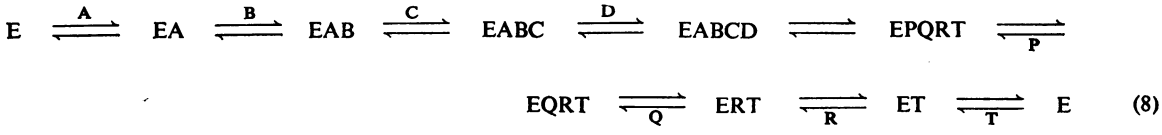


(7)

will be described by an initial-rate equation that is linear with respect to C and D but contains A^2 and B^2 terms.

Although the initial-rate equations may be non-linear it may not be practically possible to see the non-linearity on a reciprocal plot (see e.g. Pettersson, 1972). It is therefore sometimes not possible to distinguish between a steady-state and an equilibrium (see below) random-order mechanism on initial-rate data alone (cf. Cleland, 1970).

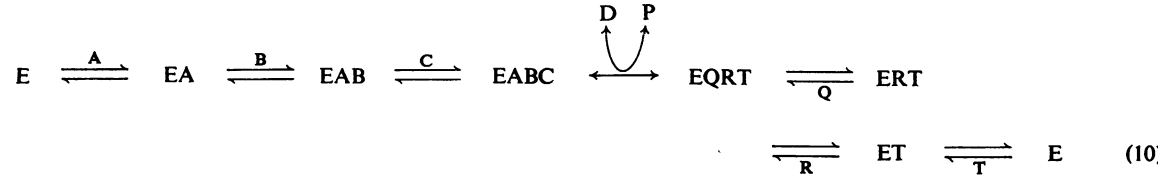
Compulsory-order binding of substrates. If the quinternary complex of substrates and enzyme is formed only by binding of the substrates to the enzyme in a compulsory order, e.g.:



(8)

This mechanism is not immediately distinguishable from a random-order equilibrium mechanism (see below) as the primary reciprocal plots (e.g. plots of $1/v$ against $1/A$ at various concentrations of B with C and D held constant) will be similar. Theoretically, as for three-substrate mechanisms (Dalziel, 1969), it is possible to distinguish the mechanisms by secondary, tertiary and quaternary plots of the slopes and intercepts, to demonstrate the lack of certain constants in the compulsory-order mechanism. However, in practice this will be even more difficult for a four-substrate mechanism than for a three-substrate mechanism (Dalziel, 1969) because of the relative insensitivity of such replots, thus making the mechanisms very difficult to distinguish on simple initial-rate data. As will be shown below, however, it is possible to distinguish these mechanisms by product-inhibition studies.

Another mechanism that would be practically indistinguishable from both above mechanisms on initial-rate data alone is the Theorell–Chance-type mechanism shown below.



(10)

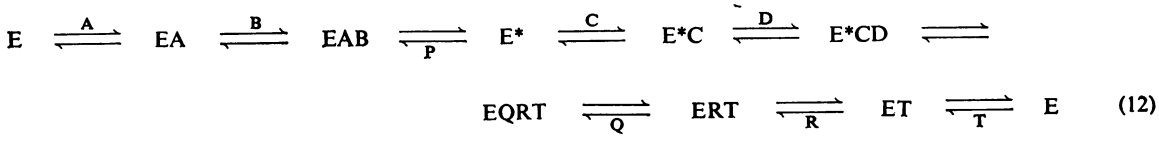
the steady-state derivation of the initial-rate equation will lead to the loss of three of the binary terms and two of the tertiary terms in eqn. (6), e.g. in the above case $K_s^A K_m^C = K_s^A K_m^D = K_s^B K_m^D = K_s^A K_{s'}^B K_m^D = K_s^A K_{s'}^C K_m^D = 0$, giving:

Although a quinternary complex must be formed its rate of breakdown through EQRT is very fast compared with any subsequent steps, thus making it kinetically insignificant (Theorell & Chance, 1951). This mechanism is described by an initial-rate equa-

$$v = \frac{V}{1 + \frac{K_m^A}{A} + \frac{K_m^B}{B} + \frac{K_m^C}{C} + \frac{K_m^D}{D} + \frac{K_s^A K_m^B}{AB} + \frac{K_{s'}^B K_m^C}{BC} + \frac{K_{s'}^C K_m^D}{CD} + \frac{K_s^A K_{s'}^B K_m^C}{ABC} + \frac{K_{s'}^B K_{s'}^C K_m^D}{BCD} + \frac{K_s^A K_{s'}^B K_{s'}^C K_m^D}{ABCD}} \quad (9)$$

tion that is identical in form with eqn. (9). However, this mechanism will again show a distinctive product-inhibition pattern.

It may be possible to form some idea of the order of binding of the substrates to the enzyme in the compulsory-order steady-state mechanism by use of the technique of saturation with a substrate (see, e.g. Frieden, 1959; Cleland, 1970). Saturation with a substrate will tend to make the step involving its binding to the enzyme essentially irreversible. Thus for an enzyme obeying the mechanism (8),



(12)

if a saturating concentration of B is present, terms in eqn. (9) with B in the denominator become small enough to be neglected, and so the equation becomes:

$$v = \frac{V}{1 + \frac{K_m^A}{A} + \frac{K_m^C}{C} + \frac{K_m^D}{D} + \frac{K_{s'}^C K_m^D}{CD}} \qquad (11)$$

Therefore the patterns obtained on reciprocal plots against $1/A$ at various fixed C or D concentrations will be parallel.

It will not always be possible to saturate with a substrate for a number of reasons, e.g. high substrate inhibition, insolubility of substrate, and of course the relative amount of the substrate needed will be dependent on the magnitude of the constant terms in the initial-rate equation. If saturation is possible it will only be able to distinguish those substrates that bind second and third from those that bind first and last, i.e. in mechanism (8) A and D are indistinguishable as are B and C, but A and D are distinguishable from B and C. However, combined with product-inhibition studies it is possible to obtain the total order of binding.

No quinternary complex

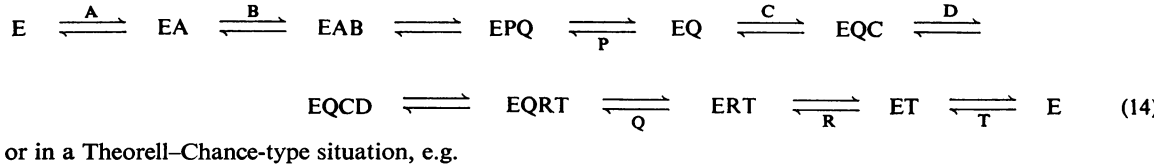
All four-substrate enzyme mechanisms in which no quinternary complex is formed are characterized by the occurrence of some parallel-line patterns on reciprocal plots at all concentrations of the other substrates. This is because product release makes a step essentially irreversible if initial rates are being considered, and thus the apparent K_s for the substrate that binds to the enzyme immediately before the product-release step becomes zero, e.g. the mechanism

(where E* is a free modified form of the enzyme) is described by the equation:

$$v = \frac{V}{1 + \frac{K_m^A}{A} + \frac{K_m^B}{B} + \frac{K_m^C}{C} + \frac{K_m^D}{D} + \frac{K_s^A K_m^B}{AB} + \frac{K_{s'}^C K_m^D}{CD}} \qquad (13)$$

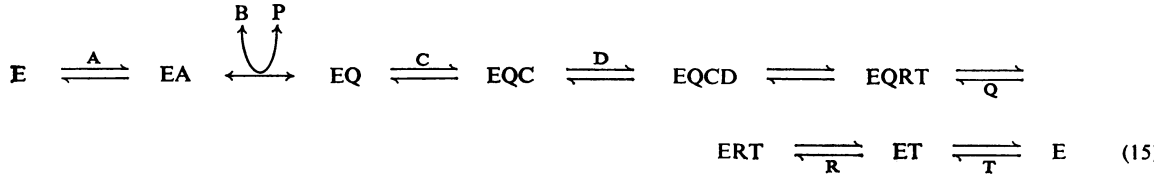
However, if the product-release step is made reversible by addition of the relevant product (P in the above example) the apparent K_s term that was previously zero ($K_{s'}^B$) becomes finite, and thus the parallel reciprocal plots will become intersecting.

If no quinternary complex is formed two situations may exist. There may be a situation analogous to the double-displacement (Ping-Pong) mechanism in which a free covalently modified form of the enzyme is formed [see mechanism (12) above], e.g. an acyl enzyme or a reduced flavoprotein. The other possibility is that product release may occur before all substrates are bound to the enzyme, but without the formation of a free modified enzyme species. The product release may occur as a separate step, e.g.



(14)

or in a Theorell–Chance-type situation, e.g.



(15)

Both mechanisms are also described by the initial-rate eqn. (13), but may theoretically be distinguished from the double-displacement-type mechanism by the absence of a free modified enzyme (although it may not always be easy to demonstrate the formation of a free modified enzyme in the latter case). The Theorell–Chance mechanism may, however, be distinguished from the other two by the product-inhibition patterns (see below).

Table 1 shows the constants present in the initial-rate equations describing a number of plausible mechanisms obeying the steady-state assumption.

$$E \underset{}{\overset{A}{\rightleftharpoons}} EA \overset{B}{\rightleftharpoons} EAB \longrightarrow E + \text{products}$$

As already pointed out there are a number of examples of more than one mechanism being described by the same initial-rate equation. Also, in certain cases, although the initial-rate equations are different, the initial-rate data will not allow distinction of the mechanisms, e.g.:

$$E \overset{A}{\rightleftharpoons} EA \underset{P}{\rightleftharpoons} E^* \overset{B}{\rightleftharpoons} E^*B \overset{C}{\rightleftharpoons} E^*BC \overset{D}{\rightleftharpoons} E^*BCD \rightleftharpoons$$

$$EQRT \underset{Q}{\rightleftharpoons} ERT \underset{R}{\rightleftharpoons} ET \underset{T}{\rightleftharpoons} E \qquad (16)$$

is described by:

$$v = \frac{V}{1 + \frac{K_m^A}{A} + \frac{K_m^B}{B} + \frac{K_m^C}{C} + \frac{K_m^D}{D} + \frac{K_{s'}^B K_m^C}{BC} + \frac{K_{s'}^C K_m^D}{CD} + \frac{K_{s'}^B K_{s'}^C K_m^D}{BCD}} \qquad (17)$$

and the mechanism

$$E \overset{A}{\rightleftharpoons} EA \overset{B}{\rightleftharpoons} EAB \overset{C}{\rightleftharpoons} EABC \underset{P}{\rightleftharpoons} E^* \overset{D}{\rightleftharpoons} E^*D \rightleftharpoons$$

$$EQRT \underset{Q}{\rightleftharpoons} ERT \underset{R}{\rightleftharpoons} ET \underset{T}{\rightleftharpoons} E \qquad (18)$$

is described by:

$$v = \frac{V}{1 + \frac{K_m^A}{A} + \frac{K_m^B}{B} + \frac{K_m^C}{C} + \frac{K_m^D}{D} + \frac{K_s^A K_m^B}{AB} + \frac{K_s^B K_m^C}{BC} + \frac{K_s^A K_{s'}^B K_m^C}{ABC}} \qquad (19)$$

Eqns. (17) and (18) are obviously different, but they share the same form, and unless the substrate which binds first (or last) is known it is not possible to distinguish these mechanisms. However, as will be shown below these ambiguities in initial-rate data may be resolved by product-inhibition studies.

Equilibrium Mechanisms

As is the case with simpler multisubstrate systems, cases can exist in which all the substrates are bound to the enzyme in an equilibrium fashion, or in which part of the sequence is in equilibrium. This second case will be discussed below. The substrate binding may be totally random, totally ordered or one may have a system in which some of the substrates are bound in a random order whereas others are bound in a compulsory order. Dalziel (1969) has pointed out that it is unlikely that equilibrium binding of a substrate will apply under all conditions. If the binding of this substrate is followed by a step in which another substrate is bound, e.g. in the mechanism

the rate of breakdown of EA in the forward direction will depend on the product of a rate constant and the concentration of B. Thus it is unlikely that equilibrium binding for A will continue to hold at very high concentrations of B. However, such an equilibrium situation may well hold throughout the range of B concentrations used in kinetic studies (e.g. Rudolph & Fromm, 1973).

Quinternary complex mechanisms

Random-order binding of substrates. The equilibrium system that is most widely considered is that of totally random-order binding of the substrates. In the special case, where binding of one substrate does not affect the binding of any of the others, this mechanism is described by the equation:

Table 1. *Enzyme mechanisms involving four substrates*

Value of kinetic constants contained in eqn. (9). In all mechanisms K_m^A, K_m^B, and K_m^C are finite.

Mechanism	K_s^A	$K_{s'}^B$	$K_{s''}^C$
(*a*) $E \overset{A}{\rightleftharpoons} EA \overset{B}{\rightleftharpoons} EAB \overset{C}{\rightleftharpoons} EABC \overset{D}{\rightleftharpoons} EABCD \rightleftharpoons EPQRT \underset{P}{\rightleftharpoons} EQRT \underset{Q}{\rightleftharpoons} ERT \underset{R}{\rightleftharpoons} ET \underset{T}{\rightleftharpoons} E$	+	+	+
(*b*) $E \overset{A}{\rightleftharpoons} EA \overset{B}{\rightleftharpoons} EAB \overset{C}{\rightleftharpoons} EABC \overset{D\ P}{\longleftrightarrow} EQRT \underset{Q}{\rightleftharpoons} ERT \underset{R}{\rightleftharpoons} ET \underset{T}{\rightleftharpoons} E$	+	+	+
(*c*) $E \overset{A}{\rightleftharpoons} EA \overset{B}{\rightleftharpoons} EAB \overset{C\ P}{\longleftrightarrow} EQR \overset{D}{\rightleftharpoons} EQRD \rightleftharpoons EQRT \underset{Q}{\rightleftharpoons} ERT \underset{R}{\rightleftharpoons} ET \underset{T}{\rightleftharpoons} E$	+	+	0
(*d*) $E \overset{A}{\rightleftharpoons} EA \overset{B\ D}{\longleftrightarrow} EQ \overset{C}{\rightleftharpoons} EQC \overset{D}{\rightleftharpoons} EQCD \rightleftharpoons EQRT \underset{Q}{\rightleftharpoons} ERT \underset{R}{\rightleftharpoons} ET \underset{T}{\rightleftharpoons} E$	+	0	+
(*e*) $E \overset{A}{\rightleftharpoons} EA \underset{P}{\rightleftharpoons} E^* \overset{B}{\rightleftharpoons} E^*B \overset{C}{\rightleftharpoons} E^*BC \overset{D}{\rightleftharpoons} E^*BCD \rightleftharpoons EQRT \underset{Q}{\rightleftharpoons} ERT \underset{R}{\rightleftharpoons} ET \underset{T}{\rightleftharpoons} E$	0	+	+
(*f*) $E \overset{A}{\rightleftharpoons} EA \overset{B}{\rightleftharpoons} EAB \underset{P}{\rightleftharpoons} E^* \overset{C}{\rightleftharpoons} E^*C \overset{D}{\rightleftharpoons} E^*CD \rightleftharpoons EQRT \underset{Q}{\rightleftharpoons} ERT \underset{R}{\rightleftharpoons} ET \underset{T}{\rightleftharpoons} E$	+	0	+
(*g*) $E \overset{A}{\rightleftharpoons} EA \overset{B}{\rightleftharpoons} EAB \overset{C}{\rightleftharpoons} EABC \underset{P}{\rightleftharpoons} E^* \overset{D}{\rightleftharpoons} E^*D \rightleftharpoons EQRT \underset{Q}{\rightleftharpoons} ERT \underset{R}{\rightleftharpoons} ET \underset{T}{\rightleftharpoons} E$	+	+	0
(*h*) $E \overset{A}{\rightleftharpoons} EA \underset{P}{\rightleftharpoons} E^* \overset{B}{\rightleftharpoons} E^*B \underset{Q}{\rightleftharpoons} F \overset{C}{\rightleftharpoons} FC \overset{D}{\rightleftharpoons} FCD \rightleftharpoons ERT \underset{R}{\rightleftharpoons} ET \underset{T}{\rightleftharpoons} E$	0	0	+
(*i*) $E \overset{A}{\rightleftharpoons} EA \underset{P}{\rightleftharpoons} E^* \overset{B}{\rightleftharpoons} E^*B \overset{C}{\rightleftharpoons} E^*BC \underset{Q}{\rightleftharpoons} F \overset{D}{\rightleftharpoons} FD \rightleftharpoons ERT \underset{R}{\rightleftharpoons} ET \underset{T}{\rightleftharpoons} E$	0	+	0
(*j*) $E \overset{A}{\rightleftharpoons} EA \overset{B}{\rightleftharpoons} EAB \underset{P}{\rightleftharpoons} E^* \overset{C}{\rightleftharpoons} E^*C \underset{Q}{\rightleftharpoons} F \overset{D}{\rightleftharpoons} FD \rightleftharpoons ERT \underset{R}{\rightleftharpoons} ET \underset{T}{\rightleftharpoons} E$	+	0	0
(*k*) $E \overset{A}{\rightleftharpoons} EA \overset{B}{\rightleftharpoons} EAB \underset{P}{\rightleftharpoons} E^*Q \underset{Q}{\rightleftharpoons} E^* \overset{C}{\rightleftharpoons} E^*C \overset{D}{\rightleftharpoons} E^*CD \rightleftharpoons ERT \underset{R}{\rightleftharpoons} ET \underset{T}{\rightleftharpoons} E$	+	0	+
(*l*) $E \overset{A}{\rightleftharpoons} EA \overset{B}{\rightleftharpoons} EAB \overset{C}{\rightleftharpoons} EABC \underset{P}{\rightleftharpoons} E^*Q \underset{Q}{\rightleftharpoons} E^* \overset{D}{\rightleftharpoons} E^*D \rightleftharpoons ERT \underset{R}{\rightleftharpoons} ET \underset{T}{\rightleftharpoons} E$	+	+	0
(*m*) $E \overset{A}{\rightleftharpoons} EA \overset{B}{\rightleftharpoons} EAB \overset{C}{\rightleftharpoons} EABC \underset{P}{\rightleftharpoons} E^*QR \underset{Q}{\rightleftharpoons} E^*R \underset{R}{\rightleftharpoons} E^* \overset{D}{\rightleftharpoons} E^*D \rightleftharpoons ET \underset{T}{\rightleftharpoons} E$	+	+	0
(*n*) $E \overset{A}{\rightleftharpoons} EA \underset{P}{\rightleftharpoons} E^* \overset{B}{\rightleftharpoons} E^*B \overset{C}{\rightleftharpoons} E^*BC \underset{Q}{\rightleftharpoons} E^*R \underset{R}{\rightleftharpoons} F \overset{D}{\rightleftharpoons} FD \rightleftharpoons ET \underset{T}{\rightleftharpoons} E$	0	+	0
(*o*) $E \overset{A}{\rightleftharpoons} EA \overset{B}{\rightleftharpoons} EAB \underset{P}{\rightleftharpoons} E^* \overset{C}{\rightleftharpoons} E^*C \underset{Q}{\rightleftharpoons} E^*R \underset{R}{\rightleftharpoons} F \overset{D}{\rightleftharpoons} FD \rightleftharpoons ET \underset{T}{\rightleftharpoons} E$	+	0	0
(*p*) $E \overset{A}{\rightleftharpoons} EA \overset{B}{\rightleftharpoons} EAB \underset{P}{\rightleftharpoons} E^*Q \underset{Q}{\rightleftharpoons} E^* \overset{C}{\rightleftharpoons} E^*C \underset{R}{\rightleftharpoons} F \overset{D}{\rightleftharpoons} FD \rightleftharpoons ET \underset{T}{\rightleftharpoons} E$	+	0	0
(*q*) $E \overset{A}{\rightleftharpoons} EA \underset{P}{\rightleftharpoons} E^* \overset{B}{\rightleftharpoons} E^*B \underset{Q}{\rightleftharpoons} F \overset{C}{\rightleftharpoons} FC \underset{R}{\rightleftharpoons} F^* \overset{D}{\rightleftharpoons} F^*D \underset{T}{\rightleftharpoons} E$	0	0	0

$$v=\frac{V}{1+\frac{K_s^A}{A}+\frac{K_s^B}{B}+\frac{K_s^C}{C}+\frac{K_s^D}{D}+\frac{K_s^AK_s^B}{AB}+\frac{K_s^AK_s^C}{AC}+\frac{K_s^AK_s^D}{AD}+\frac{K_s^BK_s^C}{BC}+\frac{K_s^BK_s^D}{BD}+\frac{K_s^CK_s^D}{CD}+\frac{K_s^AK_s^BK_s^C}{ABC}+\frac{K_s^AK_s^BK_s^D}{ABD}+\frac{K_s^AK_s^CK_s^D}{ACD}+\frac{K_s^BK_s^CK_s^D}{BCD}+\frac{K_s^AK_s^BK_s^CK_s^D}{ABCD}} \quad (20)$$

This mechanism will lead to reciprocal-plot patterns that all meet on the $1/s$ axis. However, if the binding of one substrate does affect the binding of the others, the form of the equation will remain the same but the value of some of the constants will alter, e.g.

$\frac{K_s^A}{A}$ will become $\frac{K_{BCDA}}{B}$;

$\frac{K_s^B}{B}$ will become $\frac{K_{ACDB}}{B}$ etc. and

$\frac{K_s^AK_s^BK_s^CK_s^D}{ABCD}$ will become

$$\frac{K_AK_{AB}K_{ABC}K_{ABCD}}{ABCD}\left(=\frac{K_{BA}K_BK_{ABC}K_{ABCD}}{ABCD}=\frac{K_{CA}K_{ACB}K_CK_{ABCD}}{ABCD}\text{ etc.}\right)$$

where $K_A=[E][A]/[EA]$, $K_{AB}=[EA][B]/[EAB]$, $K_{BCDA}=[EBCD][A]/[EABCD]$ etc. [see Dalziel (1969) for the equivalent equation for three substrates].

As stated above this mechanism is theoretically distinguishable from steady-state quinternary complex compulsory-order mechanisms, although in practice this distinction is not usually possible. If an attempt is made to saturate the enzyme with one substrate the mechanism will become effectively partially compulsory-order as the saturating substrate will successfully compete with the others to bind first to the enzyme. Unlike the steady-state compulsory-order mechanism parallel-line patterns on reciprocal plots will not be observed at saturating concentrations of the invariant substrates in any case.

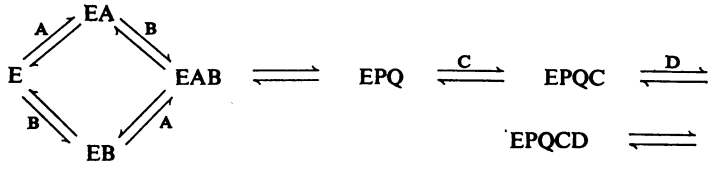
Hybrid Equilibrium and Steady-State Mechanisms

Cha (1968) has described a simple method for determination of initial-rate equations for enzyme mechanisms in which, although the overall reaction may be considered to be in a steady-state, certain steps are so rapid that they may be considered to be in equilibrium. Any of the steady-state mechanisms described above may be redescribed by using these criteria, but this treatment will be restricted to two of the more practically important cases, i.e. the existence of an equilibrium random-order sequence in a steady-state mechanism, and the situation where the first substrate binds in an equilibrium compulsory order.

Hybrid random–compulsory-order binding of substrates

There will be a large number of possible partially random four-substrate mechanisms, but this treatment will only consider the cases in which either the first two or the last two substrates bind randomly in an equilibrium segment of a compulsory-order mechanism.

For the mechanism:



(21)

if it is assumed that only the steps involving binding of A and B to the enzyme are in equilibrium, the initial-rate equation obtained by the method of Cha (1968) is

$$v=\frac{V}{1+\frac{K_m^A}{A}+\frac{K_m^B}{B}+\frac{K_m^C}{C}+\frac{K_m^D}{D}+\frac{K_s^AK_m^B}{AB}+\frac{K_s^AK_{m'}^C}{AC}+\frac{K_s^BK_{m'}^C}{BC}+\frac{K_{s'}^C(K_m^D+K_{m'}^D)}{CD}+\frac{K_s^AK_s^BK_{m'}^C}{ABC}+\frac{K_s^AK_{s'}^CK_{m'}^D}{ACD}+\frac{K_s^BK_{s'}^CK_{m'}^D}{BCD}+\frac{K_s^AK_s^BK_{s'}^CK_{m'}^D}{ABCD}} \quad (22)$$

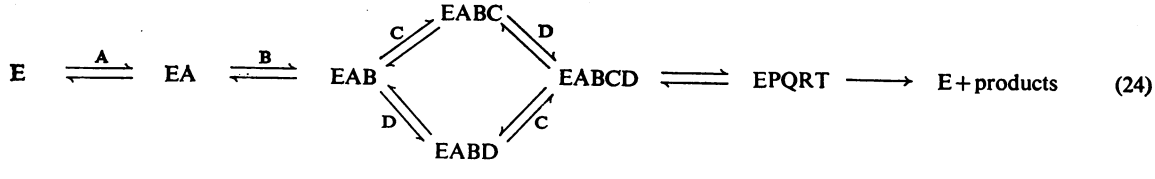
which is practically indistinguishable from eqn. (9) for the steady-state compulsory-order mechanism. If, however, the steady-state isomerization step EAB⇌ EPQ is removed (giving mechanism 7) we obtain a mechanism in which the equilibrium con-

ditions may not hold at all concentrations of C. Under conditions in which the equilibrium is maintained the initial-rate equation becomes:

$$v=\frac{V}{1+\frac{K_m^C}{C}+\frac{K_m^D}{D}+\frac{K_s^A K_m^C}{AC}+\frac{K_s^B K_m^C}{BC}+\frac{K_{s'}^C K_m^D}{CD}+\frac{K_s^A K_s^B K_m^C}{ABC}+\frac{K_s^A K_{s'}^C K_m^D}{ACD}+\frac{K_s^B K_{s'}^C K_m^D}{BCD}+\frac{K_s^A K_s^B K_{s'}^C K_m^D}{ABCD}} \quad (23)$$

This example illustrates the general point that although an isomerization step does not alter the form of a steady-state initial-rate equation [but does alter the absolute values of the constants (Plapp, 1973)], the form of the equation for a hybrid equilibrium and steady-state mechanism may be changed by an appropriate isomerization.

In the case of a mechanism in which the last two substrates, C and D, bind randomly in equilibrium:



(24)

the mechanism will also be described by an initial-rate equation practically indistinguishable from eqn. (9):

$$v=\frac{V}{1+\frac{K_m^A}{A}+\frac{K_m^B}{B}+\frac{K_m^C}{C}+\frac{K_m^D}{D}+\frac{K_s^A K_m^B}{AB}+\frac{K_{s'}^C K_m^D}{CD}+\frac{K_s^B K_{s'}^C K_m^D}{BCD}+\frac{K_s^A K_{s'}^B K_{s'}^C K_m^D}{ABCD}} \quad (25)$$

It therefore may not be possible to distinguish compulsory-order steady-state, random-order equilibrium and partially random mechanisms on the basis of initial-rate data. A distinction may be possible with the use of other techniques, e.g. product-inhibition, isotope-exchange or substrate-binding studies.

Compulsory-order binding of substrates

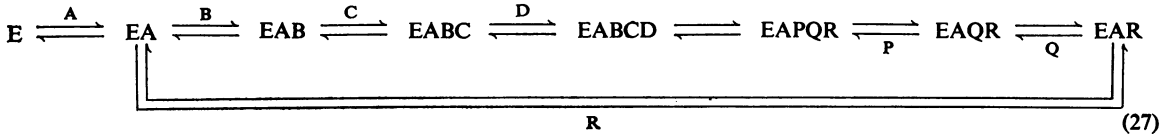
Any of the steady-state mechanisms listed in Table 1 may be adapted to accommodate the binding of A to the enzyme being in equilibrium. In all cases K_m^A becomes negligible while equilibrium conditions hold unless the binding of A is followed by a steady-state product-release step. Thus the only difference in the initial-rate equations will be the loss of the K_m^A/A term, since the determined value of K_m^A becomes equal to half the enzyme concentration (Tipton, 1974). For compulsory-order mechanism (8) the equilibrium binding of A will give the initial-rate equation:

$$v=\frac{V}{1+\frac{K_m^B}{B}+\frac{K_m^C}{C}+\frac{K_m^D}{D}+\frac{K_s^A K_m^B}{AB}+\frac{K_{s'}^B K_m^C}{BC}+\frac{K_{s'}^C K_m^D}{CD}+\frac{K_s^A K_{s'}^B K_m^C}{ABC}+\frac{K_{s'}^B K_{s'}^C K_m^D}{BCD}+\frac{K_s^A K_{s'}^B K_{s'}^C K_m^D}{ABCD}} \quad (26)$$

In this case the equilibrium conditions may break down at higher concentrations of B and indeed must do so when $B=\infty$ and under these conditions eqn. (9) will be obeyed.

Probably the most common examples of equilibrium binding in a compulsory-order mechanism involve activator rather than substrate binding. Examples of this include the compulsory binding of Mg^{2+} ions to a number of enzymes before the binding of the other substrates (see e.g. Cleland, 1970; Morrison & Ebner, 1971; Warren & Tipton, 1974). The pattern obtained on reciprocal plots against $1/B$ at various free A concentrations will be one in which the lines appear to meet on the $1/v$ axis.

In such a mechanism it is probably that the activator does not leave the enzyme between the cycles, e.g.



(27)

Such a mechanism will give an initial-rate equation that is identical with eqn. (26) but in this case equilibrium conditions for A binding will hold at all concentrations of B since steady-state analysis of eqn. (27) shows the binding of A to remain at equilibrium.

Product inhibition

Product-inhibition studies can provide the information necessary to distinguish between enzyme mechanisms that are not unique on initial-rate data alone. The presence of any inhibitor of an enzyme reaction may have one of three possible effects on reciprocal plots: (a) it may increase a constant that appears only in the slope term, thus causing competitive inhibition; (b) it may alter a constant that appears only in the intercept term, giving uncompetitive inhibition or (c) it may alter constants that appear in both the slope and intercept terms, giving mixed or non-competitive inhibition.

The effects of products as inhibitors of enzyme reactions are best explained by the use of selected examples. Table 2 shows the intercept and slope terms for the various reciprocal plots that may be obtained with the compulsory-order quinternary-complex steady-state mechanism (8). If T binds only to the same enzyme form (E) as A, it will increase the amount of A required to half-saturate the enzyme. The constants K_m^A and K_s^A will both be increased by a factor of $(1+T/K_i^T)$ where K_i^T is the apparent dissociation constant of the ET complex. When $1/v$ is plotted against $1/A$ only the slope term is affected (competitive inhibition), but both slope and intercept terms are affected if $1/B$, $1/C$ or $1/D$ is plotted (mixed inhibition).

R binds to the enzyme complex ET and will thus decrease V by a factor of $(1+R/K_i^R)$. It will also have the effect of pulling the reaction over in the direction of the enzyme–substrate complexes, thus decreasing K_m^A, K_m^B, K_m^C and K_m^D by the same factor, $(1+R/K_i^R)$. The constants K_s^A, $K_{s'}^B$ and $K_{s'}^C$ will not be affected as they all represent dissociation constants at limiting conditions when the next substrate to bind tends to zero (Tipton, 1974), and so cannot be affected by an inhibitor binding to the enzyme after that substrate. It may be seen that in all the slope terms the effects of R on V and K_m will cancel out, but this will not be the case with the intercept terms, thus giving uncompetitive inhibition with respect to all substrates. The same considerations may also be applied to Q.

At first sight product P may appear similar to Q and R. However, as well as having the same effects as Q and R, P has the further effect of being capable of partly reversing the reaction and thus displacing

Table 2. *Slope and intercept terms for reciprocal plots against each substrate for mechanism (a) (Table 1)*

	Slope	Intercept
$\frac{1}{A}$	$\frac{1}{V}\left(K_m^A+\frac{K_s^AK_m^B}{B}+\frac{K_s^AK_{s'}^BK_m^C}{BC}+\frac{K_s^AK_{s'}^BK_{s'}^CK_m^D}{BCD}\right)$	$\frac{1}{V}\left(1+\frac{K_m^B}{B}+\frac{K_m^C}{C}+\frac{K_m^D}{D}+\frac{K_{s'}^BK_m^C}{BC}+\frac{K_{s'}^CK_m^D}{CD}+\frac{K_{s'}^BK_{s'}^CK_m^D}{BCD}\right)$
$\frac{1}{B}$	$\frac{1}{V}\left(K_m^B+\frac{K_s^AK_m^B}{A}+\frac{K_{s'}^BK_m^C}{C}+\frac{K_s^AK_{s'}^BK_m^C}{AC}+\frac{K_{s'}^BK_{s'}^CK_m^D}{CD}+\frac{K_s^AK_{s'}^BK_{s'}^CK_m^D}{ACD}\right)$	$\frac{1}{V}\left(1+\frac{K_m^A}{A}+\frac{K_m^C}{C}+\frac{K_m^D}{D}+\frac{K_{s'}^CK_m^D}{CD}\right)$
$\frac{1}{C}$	$\frac{1}{V}\left(K_m^C+\frac{K_{s'}^BK_m^C}{B}+\frac{K_{s'}^CK_m^D}{D}+\frac{K_s^AK_{s'}^BK_m^C}{AB}+\frac{K_{s'}^BK_{s'}^CK_m^D}{BD}+\frac{K_s^AK_{s'}^BK_{s'}^CK_m^D}{ABD}\right)$	$\frac{1}{V}\left(1+\frac{K_m^A}{A}+\frac{K_m^B}{B}+\frac{K_m^D}{D}+\frac{K_s^AK_m^B}{AB}\right)$
$\frac{1}{D}$	$\frac{1}{V}\left(K_m^D+\frac{K_{s'}^CK_m^D}{C}+\frac{K_{s'}^BK_{s'}^CK_m^D}{BC}+\frac{K_s^AK_{s'}^BK_{s'}^CK_m^D}{ABC}\right)$	$\frac{1}{V}\left(1+\frac{K_m^A}{A}+\frac{K_m^B}{B}+\frac{K_m^C}{C}+\frac{K_s^AK_m^B}{AB}+\frac{K_{s'}^BK_m^C}{BC}+\frac{K_s^AK_{s'}^BK_m^C}{ABC}\right)$

Table 3. *Slope and intercept terms for reciprocal plots against each substrate for mechanism (f) (Table 1)*

	Slope	Intercept
$\frac{1}{A}$	$\frac{1}{V}\left(K_m^A+\frac{K_s^A K_m^B}{B}\right)$	$\frac{1}{V}\left(1+\frac{K_m^B}{B}+\frac{K_m^C}{C}+\frac{K_m^D}{D}+\frac{K_s^C K_m^D}{CD}\right)$
$\frac{1}{B}$	$\frac{1}{V}\left(K_m^B+\frac{K_s^A K_m^B}{A}\right)$	$\frac{1}{V}\left(1+\frac{K_m^A}{A}+\frac{K_m^C}{C}+\frac{K_m^D}{D}+\frac{K_s^C K_m^D}{CD}\right)$
$\frac{1}{C}$	$\frac{1}{V}\left(K_m^C+\frac{K_s^C K_m^D}{D}\right)$	$\frac{1}{V}\left(1+\frac{K_m^A}{A}+\frac{K_m^B}{B}+\frac{K_m^D}{D}+\frac{K_s^A K_m^B}{AB}\right)$
$\frac{1}{D}$	$\frac{1}{V}\left(K_m^D+\frac{K_s^C K_m^D}{C}\right)$	$\frac{1}{V}\left(1+\frac{K_m^A}{A}+\frac{K_m^B}{B}+\frac{K_m^C}{C}+\frac{K_s^A K_m^B}{AB}\right)$

Table 4. *Product-inhibition patterns for mechanisms involving four substrates and three products*

Mechanisms as in Table 1 but without product R. C, competitive; M, mixed; U, uncompetitive inhibition.

		Substrate			
Mechanism	Product	A	B	C	D
(*a*)	P	M	M	M	M
	Q	U	U	U	U
	T	C	M	M	M
(*b*)	P	M	M	M	C
	Q	U	U	U	U
	T	C	M	M	M
(*c*)	P	M	M	C	C
	Q	U	U	U	M
	T	C	M	M	U
(*d*)	P	M	C	C	M
	Q	U	U	M	M
	T	C	M	U	U
(*e*)	P	M	C	M	M
	Q	U	M	M	M
	T	C	U	U	U
(*f*)	P	M	M	C	M
	Q	U	U	M	M
	T	C	M	U	U
(*g*)	P	M	M	M	C
	Q	U	U	U	M
	T	C	M	M	U
(*h*)	P	M	C	U	U
	Q	U	M	C	M
	T	C	U	M	M
(*i*)	P	M	C	M	U
	Q	U	M	M	C
	T	C	U	U	M
(*j*)	P	M	M	C	U
	Q	U	U	M	C
	T	C	M	U	M
(*k*)	P	M	M	U	U
	Q	U	U	C	M
	T	C	M	M	M
(*l*)	P	M	M	M	U
	Q	U	U	U	C
	T	C	M	M	M

D from the enzyme. This will tend to increase K_m^D, leading to an alteration of all the slope as well as intercept terms, thus making inhibition by P mixed with respect to all substrates.

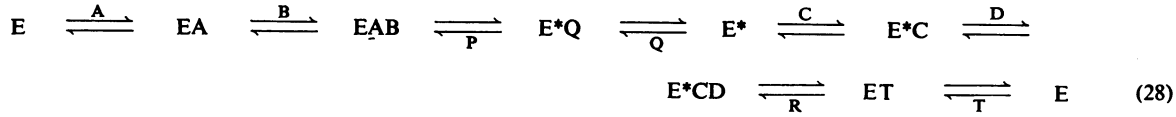
By inspection of Table 2, it is obvious that some inhibition patterns will differ at saturating concentrations of substrates, i.e. at saturating A, product T will not inhibit as terms containing K_s^A and K_m^A will become negligible; T will become uncompetitive with respect to C at saturating B, and uncompetitive with respect to D at saturating B or C, as the terms containing K_s^A in the slope terms become negligible (but K_m^A/A in the intercept term is unaffected). Similarly P will be uncompetitive with respect to A at saturating B, C or D; uncompetitive with respect to B at saturating C or D, and uncompetitive with respect to C at saturating D; as in all those situations the terms containing K_m^D in the slope terms become negligible.

The second example illustrates product-inhibition patterns for a double-displacement type of mechanism (12). As in the previous example T will increase K_s^A and K_m^A, and inspection of Table 3 shows that T will be competitive with respect to A, mixed with respect to B and uncompetitive with respect to C and D. Product R will again decrease V and all K_m terms and so will be uncompetitive with respect to all substrates. Product Q will decrease the same constants as R, but will also be able to displace D by reversal of the reaction leading to an increase in K_m^D. Thus Q will be mixed with respect to C and D, but uncompetitive with respect to A and B as there is no K_m^D in the relevant slope terms.

The effects of P will be more complex as not only will it increase $K_{s'}^C$ and K_m^C by binding to the same form of the enzyme (E*) as C, but it will also make the step between the binding of B and C reversible. The slope and intercept terms will thus become similar to those in the previous example (compulsory-order quinternary complex) and the inhibition with respect to A, B and D will be mixed.

Tables 4 and 5 show the inhibition patterns

obtained with four-substrate enzyme reactions having three or four products. It can be seen that most of four-substrate mechanisms have unambiguous product-inhibition patterns. Only in certain cases where the mechanism is symmetrical will some ambiguity exist, e.g. in mechanism (10) with the Theorell–Chance step it is not immediately possible to distinguish A from D, B from C, P from T or R from Q (although it may be possible to show by separate techniques which substrate binds first to the enzyme, thus fixing the order of the rest). The double-displacement-type mechanisms



and

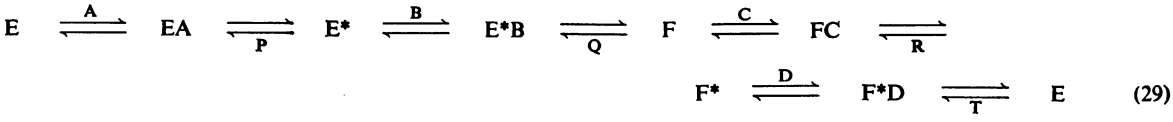


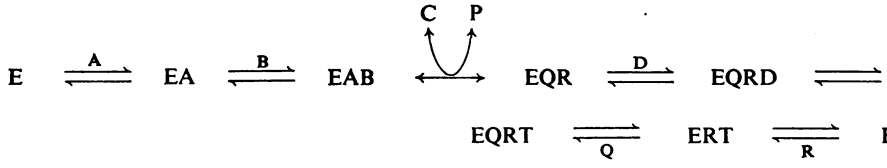
Table 5. *Product-inhibition patterns for mechanisms involving four substrates and four products*

Mechanisms as in Table 1. C, competitive; M, mixed; U, uncompetitive inhibition.

Mechanism	Product	Substrate A	B	C	D
(*a*)	P	M	M	M	M
	Q	U	U	U	U
	R	U	U	U	U
	T	C	M	M	M
(*b*)	P	M	M	M	C
	Q	U	U	U	U
	R	U	U	U	U
	T	C	M	M	M
(*c*)	P	M	M	C	C
	Q	U	U	U	U
	R	U	U	U	U
	T	C	M	M	U
(*d*)	P	M	C	C	M
	Q	U	U	M	M
	R	U	U	U	U
	T	C	M	U	U
(*e*)	P	M	C	M	M
	Q	U	M	M	M
	R	U	U	U	U
	T	C	U	U	U
(*f*)	P	M	M	C	M
	Q	U	U	M	M
	R	U	U	U	U
	T	C	M	U	U
(*g*)	P	M	M	M	C
	Q	U	U	U	M
	R	U	U	U	U
	T	C	M	M	U
(*h*)	P	M	C	U	U
	Q	U	M	C	M
	R	U	U	M	M
	T	C	U	U	U
(*i*)	P	M	C	M	U
	Q	U	M	M	C
	R	U	U	U	M
	T	C	U	U	M
(*j*)	P	M	M	C	U
	Q	U	U	M	C
	R	U	U	U	M
	T	C	M	U	U
(*k*)	P	M	M	U	U
	Q	U	U	C	M
	R	U	U	M	M
	T	C	M	U	U
(*l*)	P	M	M	M	U
	Q	U	U	U	C
	R	U	U	U	M
	T	C	M	M	U
(*m*)	P	M	M	M	U
	Q	U	U	U	U
	R	U	U	U	C
	T	C	M	M	M
(*n*)	P	M	C	M	U
	Q	U	M	M	U
	R	U	U	U	C
	T	C	U	U	M
(*o*)	P	M	M	C	U
	Q	U	U	M	U
	R	U	U	U	C
	T	C	M	U	M
(*p*)	P	M	M	U	U
	Q	U	U	C	U
	R	U	U	M	C
	T	C	M	U	M
(*q*)	P	M	C	U	U
	Q	U	M	C	U
	R	U	U	M	C
	T	C	U	U	M

also present problems as it must be decided which is the unmodified form of the enzyme, and then which substrate binds to that form in order to establish the order of binding.

One interesting point that has arisen out of this study is the inhibition of the Theorell–Chance-type mechanisms (15) and (30):



(30)

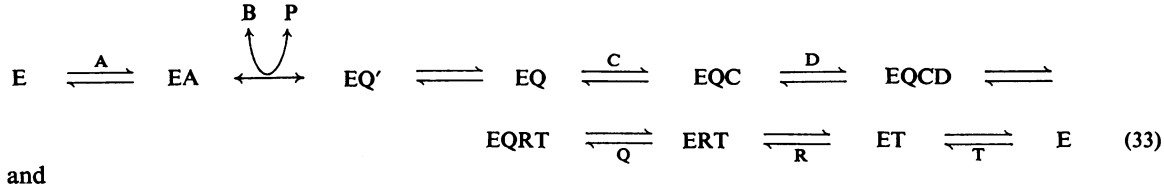
As these mechanisms are written product P will be competitive with respect to both B and C in mechanism (15) and competitive with respect to both C and D in mechanism (30). The rate equations in the presence of P become:

$$v=\frac{V}{1+\frac{K_m^A}{A}+\frac{K_m^B}{B}+\frac{K_m^C}{C}+\frac{K_m^D}{D}+\frac{K_s^AK_m^B}{AB}+\frac{K_{s'}^BK_m^C\cdot P}{BC}+\frac{K_{s'}^CK_m^D}{CD}+\frac{K_s^AK_{s'}^BK_m^C\cdot P}{ABC}+\frac{K_{s'}^BK_{s'}^CK_m^D\cdot P}{BCD}+\frac{K_s^AK_{s'}^BK_{s'}^CK_m^D\cdot P}{ABCD}} \quad (31)$$

and

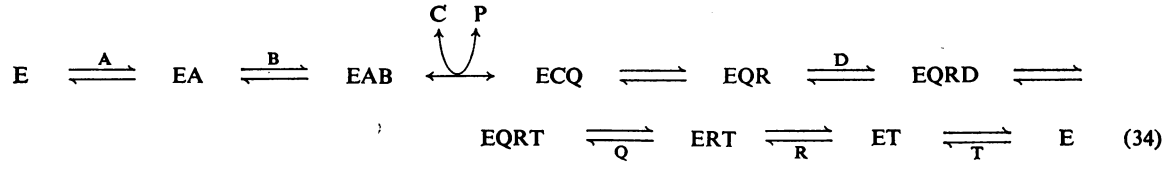
$$v=\frac{V}{1+\frac{K_m^A}{A}+\frac{K_m^B}{B}+\frac{K_m^C}{C}+\frac{K_m^D}{D}+\frac{K_s^AK_m^B}{AB}+\frac{K_{s'}^BK_m^C}{BC}+\frac{K_{s'}^CK_m^D\cdot P}{CD}+\frac{K_s^AK_{s'}^BK_m^C}{ABC}+\frac{K_{s'}^BK_{s'}^CK_m^D\cdot P}{BCD}+\frac{K_s^AK_{s'}^BK_{s'}^CK_m^D\cdot P}{ABCD}} \quad (32)$$

respectively. Neither equation shows the normal form in that no constant is multiplied by $(1+P/K_i^P)$. In eqn. (31) it may be noted that K_s^B also is equal to $1/K_{s',i}^P$ and in eqn. (32) $K_{s'}^C$ is equalt o $1/K_{s',i}^P$ (where $K_{s',i}^P$ is the apparent dissociation constant of the enzyme–P complex). If, however, a steady-state isomerization step occurs after the Theorell–Chance step, i.e. mechanisms (15) and (30) become respectively:



(33)

and



(34)

The form of the initial-rate equations will not be altered but the product-inhibition patterns will. Eqns. (31) and (32) now become respectively (35) and (36).

$$v=\frac{V}{1+\frac{K_m^A}{A}+\frac{K_m^B}{B}\left(1+\frac{P}{K_i^P}\right)+\frac{K_m^C}{C}+\frac{K_m^D}{D}+\frac{K_s^AK_m^B}{AB}\left(1+\frac{P}{K_i^P}\right)+\frac{K_{s'}^BK_m^C\cdot P}{BC}+\frac{K_{s'}^CK_m^D}{CD}+\frac{K_s^AK_{s'}^BK_m^C\cdot P}{ABC}+\frac{K_{s'}^BK_{s'}^CK_m^D\cdot P}{BCD}+\frac{K_s^AK_{s'}^BK_{s'}^CK_m^D\cdot P}{ABCD}} \quad (35)$$

and

$$v=\frac{V}{1+\frac{K_m^A}{A}+\frac{K_m^B}{B}+\frac{K_m^C}{C}\left(1+\frac{P}{K_i^P}\right)+\frac{K_m^D}{D}+\frac{K_s^AK_m^B}{AB}+\frac{K_{s'}^BK_m^C}{BC}\left(1+\frac{P}{K_i^P}\right)+\frac{K_{s'}^CK_m^D\cdot P}{CD}+\frac{K_s^AK_{s'}^BK_m^C}{ABC}\left(1+\frac{P}{K_i^P}\right)+\frac{K_{s'}^BK_{s'}^CK_m^D\cdot P}{BCD}+\frac{K_s^AK_{s'}^BK_{s'}^CK_m^D\cdot P}{ABCD}} \quad (36)$$

Thus in mechanism (33) P is competitive with respect to B and mixed with respect to C, and in mechanism (34) it is competitive with respect to C and mixed with respect to D. These therefore are more examples of an isomerization step affecting the kinetic mechanism.

It is not possible to predict product-inhibition patterns for the equilibrium random-order mechanism beyond the statement that each product will be competitive with respect to one substrate, and may be mixed, non-competitive or competitive with respect to the other three. Further predictions may be made as to which products will be competitive to which substrate by looking for structural similarities between substrates and products (cf. Tipton, 1974).

If it is assumed that product release is in a compulsory-order steady-state manner then equilibrium steps in a steady-state mechanism will have little effect on the product-inhibition patterns. All patterns will be the same as that for the compulsory-order quinternary-complex steady-state mechanism unless the first substrate binds in equilibrium. In mechanism (21) where A and B bind randomly to the enzyme in equilibrium the last product to leave the enzyme (T) will be competitive with respect to both substrates. If, in any compulsory-order mechanism, substrate A binds to the enzyme in equilibrium then similarly T will be competitive with respect to both A and B. However, if A is an activator and does not leave the enzyme so that T binds not to the free enzyme but to the EA complex then T will be uncompetitive with respect to A but competitive with respect to B.

Much care must be taken when interpreting the results of product-inhibition studies as the patterns described above take no account of any dead-end complexes which might be formed between the product and enzyme. Often the situation exists where a product is a close analogue of a substrate (e.g. ADP and ATP) and may compete with the substrate for the same binding site to form a dead-end complex. Again taking the steady-state compulsory-order quinternary-complex mechanism (8) as an example, and making the assumption that product R is a close analogue of B, it is probable that the dead-end complex EAR will be formed. Thus as well as decreasing K_m^A, K_m^B, K_m^C, K_m^D and V, the product R will increase K_m^B and $K_{s'}^B$ by $(1+R/K_i^R)$. Inhibition by R will therefore be mixed with respect to all substrates as all slope terms contain K_m^B or $K_{s'}^B$. Similarly if T were an analogue of D then K_m^B as well as K_s^A and K_m^A will be increased by $(1+T/K_i^T)$ making inhibition by T mixed with respect to all substrates. These effects may usually be predicted from the analogy between substrate and product and should not cause too much confusion.

Mechanisms Involving Two Molecules of One Substrate

All the mechanisms that have been discussed may readily be modified to cover mechanisms involving three substrates in which two molecules of one substrate are used. For example the initial-rate equation for a compulsory-order steady-state quinternary-complex mechanism in which two molecules of one substrate bind first and last

$$E \overset{A}{\rightleftharpoons} EA \overset{B}{\rightleftharpoons} EAB \overset{C}{\rightleftharpoons} EABC \overset{A}{\rightleftharpoons} EABCA \rightleftharpoons EPQRT \longrightarrow E+\text{products} \qquad (37)$$

may be derived from eqn. (9) by substituting A for D and $K_m^{A'}$ for K_m^D:

$$v=\frac{V}{1+\frac{K_m^A+K_m^{A'}}{A}+\frac{K_m^B}{B}+\frac{K_m^C}{C}+\frac{K_s^AK_m^B}{AB}+\frac{K_m^{A'}K_{s'}^C}{AC}+\frac{K_{s'}^BK_m^C}{BC}+\frac{K_s^AK_{s'}^BK_m^C+K_m^{A'}K_{s'}^BK_{s'}^C}{ABC}+\frac{K_s^AK_m^{A'}K_{s'}^BK_{s'}^C}{A^2BC}} \qquad (38)$$

The equation contains an A^2 term and so reciprocal plots against $1/A$ will be non-linear (although the non-linearity may only be seen at low substrate concentrations). This non-linearity will only be seen if the two molecules bind to enzyme species that are reversibly connected (Cleland, 1963*a*); in the above example at a saturating concentration of B (i.e. at such a high concentration of B that all terms containing its concentration become negligible, cf. Cleland, 1970) when the step from EA to EAB becomes essentially irreversible, eqn. (38) becomes:

$$v=\frac{V}{1+\frac{K_m^A+K_m^{A'}}{A}+\frac{K_m^C}{C}+\frac{K_m^{A'}K_{s'}^C}{AC}} \qquad (39)$$

Similarly at saturating C concentrations the A^2 term disappears from eqn. (38). As stated above, it is often not possible to saturate the enzyme with a substrate but the above effect may also be seen by the occurrence of linear intercept plots of reciprocal plots against $1/B$ or $1/C$ at a number of A concentrations. A product-release step will also be an essentially irreversible step so the steady-state mechanism

Table 6. *Enzyme mechanisms involving three substrates*

Kinetic constants contained in initial-rate equations. All contain K_m^A; $K_m^{A'}$;

Mechanism

(i) $E \overset{A}{\rightleftharpoons} EA \overset{A}{\rightleftharpoons} EAA \overset{B}{\rightleftharpoons} EAAB \overset{C}{\rightleftharpoons} EAABC \rightleftharpoons EPQRT \underset{P}{\rightleftharpoons} EQRT \underset{Q}{\rightleftharpoons}$
$ERT \underset{R}{\rightleftharpoons} ET \underset{T}{\rightleftharpoons} E$

(ii) $E \overset{A}{\rightleftharpoons} EA \overset{B}{\rightleftharpoons} EAB \overset{A}{\rightleftharpoons} EAAB \overset{C}{\rightleftharpoons} EAABC \rightleftharpoons EPQRT \underset{P}{\rightleftharpoons} EQRT \underset{Q}{\rightleftharpoons}$
$ERT \underset{R}{\rightleftharpoons} ET \underset{T}{\rightleftharpoons} E$

(iii) $E \overset{A}{\rightleftharpoons} EA \overset{B}{\rightleftharpoons} EAB \overset{C}{\rightleftharpoons} EABC \overset{A}{\rightleftharpoons} EAABC \rightleftharpoons EPQRT \underset{P}{\rightleftharpoons} EQRT \underset{Q}{\rightleftharpoons}$
$ERT \underset{R}{\rightleftharpoons} ET \underset{T}{\rightleftharpoons} E$

(iv) $E \overset{B}{\rightleftharpoons} EB \overset{A}{\rightleftharpoons} EBA \overset{A}{\rightleftharpoons} EBAA \overset{C}{\rightleftharpoons} EBAAC \rightleftharpoons EPQRT \underset{P}{\rightleftharpoons} EQRT \underset{Q}{\rightleftharpoons}$
$ERT \underset{R}{\rightleftharpoons} ET \underset{T}{\rightleftharpoons} E$

(v) $E \overset{B}{\rightleftharpoons} EB \overset{A}{\rightleftharpoons} EBA \overset{C}{\rightleftharpoons} EBAC \overset{A}{\rightleftharpoons} EBAAC \rightleftharpoons EPQRT \underset{P}{\rightleftharpoons} EQRT \underset{Q}{\rightleftharpoons}$
$ERT \underset{R}{\rightleftharpoons} ET \underset{T}{\rightleftharpoons} E$

(vi) $E \overset{B}{\rightleftharpoons} EB \overset{C}{\rightleftharpoons} EBC \overset{A}{\rightleftharpoons} EBCA \overset{A}{\rightleftharpoons} EBCAA \rightleftharpoons EPQRT \underset{P}{\rightleftharpoons} EQRT \underset{Q}{\rightleftharpoons}$
$ERT \underset{R}{\rightleftharpoons} ET \underset{T}{\rightleftharpoons} E$

(vii) $E \overset{A}{\rightleftharpoons} EA \overset{A}{\rightleftharpoons} EAA \overset{B}{\rightleftharpoons} EAAB \overset{C\ P}{\rightleftharpoons} EQRT \underset{Q}{\rightleftharpoons} ERT \underset{R}{\rightleftharpoons} ET \underset{T}{\rightleftharpoons} E$

(viii) $E \overset{A}{\rightleftharpoons} EA \overset{B}{\rightleftharpoons} EAB \overset{C}{\rightleftharpoons} EABC \overset{A\ P}{\rightleftharpoons} EQRT \underset{Q}{\rightleftharpoons} ERT \underset{R}{\rightleftharpoons} ET \underset{T}{\rightleftharpoons} E$

(ix) $E \overset{A}{\rightleftharpoons} EA \underset{P}{\rightleftharpoons} E^* \overset{A}{\rightleftharpoons} E^*A \overset{B}{\rightleftharpoons} E^*AB \overset{C}{\rightleftharpoons} E^*ABC \rightleftharpoons EQRT \underset{Q}{\rightleftharpoons}$
$ERT \underset{R}{\rightleftharpoons} ET \underset{T}{\rightleftharpoons} E$

(x) $E \overset{A}{\rightleftharpoons} EA \overset{A}{\rightleftharpoons} EAA \underset{P}{\rightleftharpoons} E^* \overset{B}{\rightleftharpoons} E^*B \overset{C}{\rightleftharpoons} E^*BC \rightleftharpoons EQRT \underset{Q}{\rightleftharpoons}$
$ERT \underset{R}{\rightleftharpoons} ET \underset{T}{\rightleftharpoons} E$

(xi) $E \overset{A}{\rightleftharpoons} EA \overset{A}{\rightleftharpoons} EAA \overset{B}{\rightleftharpoons} EAAB \underset{P}{\rightleftharpoons} E^* \overset{C}{\rightleftharpoons} E^*C \rightleftharpoons EQRT \underset{Q}{\rightleftharpoons}$
$ERT \underset{R}{\rightleftharpoons} ET \underset{T}{\rightleftharpoons} E$

(xii) $E \overset{A}{\rightleftharpoons} EA \underset{P}{\rightleftharpoons} E^* \overset{B}{\rightleftharpoons} E^*B \overset{C}{\rightleftharpoons} E^*BC \overset{A}{\rightleftharpoons} E^*ABC \rightleftharpoons EQRT \underset{Q}{\rightleftharpoons}$
$ERT \underset{R}{\rightleftharpoons} ET \underset{T}{\rightleftharpoons} E$

(xiii) $E \overset{A}{\rightleftharpoons} EA \overset{B}{\rightleftharpoons} EAB \underset{P}{\rightleftharpoons} E^* \overset{C}{\rightleftharpoons} E^*C \overset{A}{\rightleftharpoons} E^*AC \rightleftharpoons EQRT \underset{Q}{\rightleftharpoons}$
$ERT \underset{R}{\rightleftharpoons} ET \underset{T}{\rightleftharpoons} E$

(xiv) $E \overset{A}{\rightleftharpoons} EA \overset{B}{\rightleftharpoons} EAB \overset{C}{\rightleftharpoons} EAC \underset{P}{\rightleftharpoons} E^* \overset{A}{\rightleftharpoons} E^*A \rightleftharpoons EQRT \underset{Q}{\rightleftharpoons}$
$ERT \underset{R}{\rightleftharpoons} ET \underset{T}{\rightleftharpoons} E$

with two molecules of one substrate being utilized

K_m^B; and K_m^C. $K_m^{A'} = K_m$ for second molecule of A to bind to the enzyme.

$K_s^A K_m^{A'}$; $K_{s'}^{A'} K_m^B$; $K_{s'}^B K_m^C$; $K_s^A K_{s'}^{A'} K_m^B$; $K_{s'}^{A'} K_{s'}^B K_m^C$; $K_s^A K_{s'}^{A'} K_{s'}^B K_m^C$

$K_s^A K_m^B$; $K_m^{A'} K_{s'}^B$; $K_{s'}^{A'} K_m^C$; $K_s^A K_m^{A'} K_{s'}^B$; $K_{s'}^{A'} K_{s'}^B K_m^C$; $K_s^A K_{s'}^{A'} K_{s'}^B K_m^C$

$K_s^A K_m^B$; $K_m^{A'} K_{s'}^C$; $K_{s'}^B K_m^C$; $K_s^A K_{s'}^B K_m^C$; $K_m^{A'} K_{s'}^B K_{s'}^C$; $K_s^A K_m^{A'} K_{s'}^B K_{s'}^C$

$K_m^A K_s^B$; $K_{s'}^{A'} K_m^C$; $K_s^A K_m^{A'}$; $K_s^A K_m^{A'} K_s^B$; $K_s^A K_{s'}^{A'} K_m^C$; $K_s^A K_{s'}^{A'} K_s^B K_m^C$

$K_m^A K_s^B$; $K_s^A K_m^C$; $K_m^{A'} K_{s'}^C$; $K_{s'}^A K_s^B K_m^C$; $K_s^A K_m^{A'} K_{s'}^C$; $K_{s'}^A K_m^{A'} K_s^B K_{s'}^C$

$K_m^A K_s^C$; $K_{s'}^A K_m^{A'}$; $K_s^B K_m^C$; $K_{s'}^A K_m^{A'} K_{s'}^C$; $K_m^A K_s^B K_{s'}^C$; $K_{s'}^A K_m^{A'} K_s^B K_{s'}^C$

$K_s^A K_m^{A'}$; $K_{s'}^{A'} K_m^B$; $K_{s'}^B K_m^C$; $K_s^A K_{s'}^{A'} K_m^B$; $K_{s'}^{A'} K_{s'}^B K_m^C$; $K_s^A K_{s'}^{A'} K_{s'}^B K_m^C$

$K_s^A K_m^B$; $K_m^{A'} K_{s'}^C$; $K_{s'}^B K_m^C$; $K_s^A K_{s'}^B K_m^C$; $K_m^{A'} K_{s'}^B K_{s'}^C$; $K_s^A K_m^{A'} K_{s'}^B K_m^C$

$K_{s'}^{A'} K_m^B$; $K_{s'}^B K_m^C$; $K_{s'}^A K_{s'}^B K_m^C$

$K_s^A K_m^{A'}$; $K_{s'}^B K_m^C$

$K_s^A K_m^{A'}$; $K_{s'}^{A'} K_m^B$; $K_s^A K_{s'}^{A'} K_m^B$

$K_m^{A'} K_{s'}^C$; $K_{s'}^B K_m^C$; $K_m^{A'} K_{s'}^B K_{s'}^C$

$K_s^A K_m^B$; $K_m^{A'} K_{s'}^C$

$K_s^A K_m^B$; $K_{s'}^B K_m^C$; $K_s^A K_{s'}^B K_m^C$

$$E \underset{}{\overset{A}{\rightleftharpoons}} EA \overset{B}{\rightleftharpoons} EAB \underset{P}{\rightleftharpoons} E^* \overset{C}{\rightleftharpoons} E^*C \overset{A}{\rightleftharpoons}$$

$$E^*CA \rightleftharpoons E^*QRT \longrightarrow E + \text{products} \qquad (40)$$

will be described by the initial-rate equation

$$v = \frac{V}{1 + \frac{K_m^A + K_m^{A'}}{A} + \frac{K_m^B}{B} + \frac{K_m^C}{C} + \frac{K_s^A K_m^B}{AB} + \frac{K_m^{A'} K_{s'}^C}{AC}} \qquad (41)$$

which will give linear reciprocal plots against $1/A$. Table 6 lists the initial-rate equations for a number of plausible mechanisms involving three substrates of which two molecules of one are utilized. Table 7 shows the predicted linearity and non-linearity of the reciprocal plots and intercept replots for the mechanisms in Table 6.

Product-inhibition patterns may be predicted in exactly the same way as stated above. They may also be derived by inspection of Tables 4 and 5 with one substrate replaced by a second molecule of another substrate. If this latter method is used it must be noted that if inhibition by a product is competitive with respect to one molecule of the substrate that binds twice and mixed with respect to the other molecule then the overall effect will be one of competitive inhibition.

Table 7. *Linearity of reciprocal plots for systems obeying the mechanisms in Table 6 involving two molecules of one substrate*

L, linear; N, non-linear.

Mechanism	Reciprocal plots against A	Plot of $1/A$ against intercepts from reciprocal plots	
		against B	against C
(i)	N	N	N
(ii)	N	L	N
(iii)	N	L	L
(iv)	N	N	N
(v)	N	N	L
(vi)	N	N	N
(vii)	N	N	N
(viii)	N	L	L
(ix)	L	L	L
(x)	N	N	N
(xi)	N	N	N
(xii)	L	L	L
(xiii)	L	L	L
(xiv)	L	L	L

Table 8. *Product-inhibition patterns for mechanisms in Table 6 involving two molecules of one substrate*

C, competitive; M, mixed; U, uncompetitive inhibition.

Mechanism	Product	Substrate			Mechanism	Product	Substrate		
		A	B	C			A	B	C
(i)	P	M	M	M	(viii)	P	C	M	M
	Q	U	U	U		Q	U	U	U
	R	U	U	U		R	U	U	U
	T	C	M	M		T	C	M	M
(ii)	P	M	M	M	(ix)	P	C	M	M
	Q	U	U	U		Q	M	M	M
	R	U	U	U		R	U	U	U
	T	C	M	M		T	C	U	U
(iii)	P	M	M	M	(x)	P	M	C	M
	Q	U	U	U		Q	U	M	M
	R	U	U	U		R	U	U	U
	T	C	M	M		T	C	U	U
(iv)	P	M	M	M	(xi)	P	M	M	C
	Q	U	U	U		Q	U	U	M
	R	U	U	U		R	U	U	U
	T	M	C	M		T	C	M	U
(v)	P	M	M	M	(xii)	P	M	C	M
	Q	U	U	U		Q	M	M	M
	R	U	U	U		R	U	U	U
	T	M	C	M		T	C	U	U
(vi)	P	M	M	M	(xiii)	P	M	M	C
	Q	U	U	U		Q	M	U	M
	R	U	U	U		R	U	U	U
	T	M	C	M		T	C	M	U
(vii)	P	M	M	C	(xiv)	P	C	M	M
	Q	U	U	U		Q	M	U	U
	R	U	U	U		R	U	U	U
	T	C	M	M		T	C	M	M

Table 8 lists the product-inhibition patterns for the mechanisms in Table 6.

There is one further complication which is that if two molecules of one substrate are utilized it is possible that two molecules of one product may be released. When considering inhibition by this product the effects on slope and intercept terms are additive. As with the two molecules of one substrate, non-linear inhibition will only be observed if the two molecules of the same product bind to reversibly connected forms of the enzyme.

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References

Alberty, R. A. (1953) *J. Amer. Chem. Soc.* **75**, 1928–1932
Cha, S. (1968) *J. Biol. Chem.* **243**, 820–825
Cleland, W. W. (1963*a*) *Biochim. Biophys. Acta* **67**, 104–172
Cleland, W. W. (1963*b*) *Biochim. Biophys. Acta* **67**, 173–187
Cleland, W. W. (1970) *Enzymes* **2**, 1–66
Dalziel, K. (1957) *Acta. Chem. Scand.* **11**, 1706–1723
Dalziel, K. (1969) *Biochem. J.* **114**, 547–556
Elliott, K. R. F. & Tipton, K. F. (1974*a*) *Biochem. J.* **141**, 807–816
Elliott, K. R. F. & Tipton, K. F. (1974*b*) *Biochem. J.* **141**, 817–824
Frieden, C. (1959) *J. Biol. Chem.* **234**, 2891–2896
Fromm, H. J. (1967) *Biochim. Biophys. Acta* **139**, 221–230
King, E. L. & Altman, C. (1956) *Phys. Chem.* **60**, 1375–1378
Knappe, J., Ringelmann, E. & Lynen, F. (1959) *Biochem. Z.* **332**, 195–213
Lagerkvist, U. (1958) *J. Biol. Chem.* **233**, 143–149
Lazzarini, R. A. & Atkinson, D. E. (1961) *J. Biol. Chem.* **236**, 3330–3335
Metzenberg, R. L., Hall, L. M., Marshall, M. & Cohen, P. P. (1957) *J. Biol. Chem.* **229**, 1019–1025
Morrison, J. F. & Ebner, K. E. (1971) *J. Biol. Chem.* **246**, 3977–3984
Palmer, J. M. & Wedding, R. T. (1966) *Biochim. Biophys. Acta* **113**, 167–174
Pettersson, G. (1972) *Biochim. Biophys. Acta* **276**, 1–11
Plapp, B. V. (1973) *Arch. Biochem. Biophys.* **156**, 112–114
Preiss, J. & Handler, P. (1958) *J. Biol. Chem.* **233**, 493–500
Rudolph, F. B. & Fromm, H. J. (1973) *J. Theor. Biol.* **39**, 363–371
Theorell, H. & Chance, B. (1951) *Acta. Chem. Scand.* **5**, 1127–1144
Tipton, K. F. (1974) in *Companion to Biochemistry* (Bull, A., Lagnado, J. R., Thomas, J. O. & Tipton, K. F., eds.), pp. 227–251, Longmans, London
Utter, M. F. & Keech, D. B. (1960) *J. Biol. Chem.* **235**, PC17–PC18
Vol'kenshtein, M. V. & Gol'dshtein, B. N. (1966) *Biokhimiya* **31**, 473–478
Warren, G. B. & Tipton, K. F. (1974) *Biochem. J.* **139**, 311–320
Wong, J. T.-F. & Hanes, C. S. (1962) *Can. J. Biochem. Physiol.* **40**, 763–804