MATCH
Communications in Mathematical and in Computer Chemistry

MATCH Commun. Math. Comput. Chem. **69** (2013) 215-248

ISSN 0340 - 6253

Initial Rate Equations in Four-Substrate Enzyme Reactions. Application to Discriminate between Some Mechanisms and Evaluate Global Kinetic Parameters

J.M. Yago[(a)], E. Arribas[(b)], C. Garrido-del Solo[(a)], F. Garcia-Sevilla[(c)], M. Garcia-Moreno[(a)] and R. Varon [(a)]

[(a)] Departamento de Química Física, Escuela de Ingenieros Industriales, Universidad de Castilla-La Mancha, Albacete, Spain[1]

[(b)] Applied Physics Department, Faculty of Computer Science Engineering, University of Castilla-La Mancha, Albacete, Spain

[(c)] Departamento de Ingenieria Electronica, Electrica, Automatica y Comunicaciones, Escuela de Ingenieros Industriales, Universidad de Castilla-La Mancha, Albacete, Spain

(Received May 15, 2012)

Abstract

There are no many studies on the kinetics of four-substrate enzyme reactions. The reasons may be the fewest known reactions and, on the other hand, the complexity of their theoretical kinetic analysis. Recently, contributions have been published that circumvent this last difficulty [1-5]. In addition, the few contributions found in literature about the kinetics of four-substrate enzyme species show and use the initial rate equations obtained under the total rapid equilibrium assumption. Furthermore the number of the reaction mechanisms which have been analyzed is very small. We have acquired the initial rate expressions for 13 four-substrate mechanisms, under the steady-

[1] Corresponding author, Prof. Ramón Varón, Departamento de Química Física, Escuela de Ingenieros Industriales, Campus Universitario, E -02071 Albacete, Spain. Tel.:+ 34 967599307; Fax: +34 967 599224; e-mail: ramon.varon@uclm.es

state strict conditions and using the rapid equilibrium assumption. We have used them to develop a method to discriminate between most of the mechanisms under study. This procedure consists of four steps and combines the rate equations of the mechanisms with the experimental rate graphs. This method also allows us to determine the binding order in some of them as well as to evaluate general kinetic parameters. Finally, a procedure to evaluate the global kinetic parameters involved in the rapid equilibrium rate constants is suggested.

INTRODUCTION

The literature on the kinetics of four-substrate enzyme-catalyzed reactions is considerably scarcer than for two or three-substrate ones. This may be due to the reduced number of reactions of this type known and the high complexity of their kinetic equations.

Although there are some studies that treat the steady-state kinetics of multi-substrate reactions, [6-9], there is only one, as far as we know, which describes the kinetics of four-substrate reactions under the steady state assumption [10]. This paper was motivated by two studies of the same authors about the enzyme carbamoyl phosphate synthetase [11,12].

In this contribution, the initial rate equations for several mechanisms are given or described. Thus, for quinternary complex mechanisms, the equations corresponding to an ordered mechanism and a Theorell- Chance type are given, however those corresponding to a random –order mechanism and to a random –order equilibrium mechanism are described. The authors state that is not possible to distinguish between these two last mechanisms on initial rate data alone. The reason is that although their rate equations are different, the plots of $1/v$ vs. $1/[X]$, where v is the initial rate and X one of the reactants, will be the same. Despite this, other techniques can be used for this purpose like product inhibition studies and saturation with a substrate. However, the expressions of the slopes and the intercepts of the straight lines of the plots of $1/v$ vs. $1/[X]$ are not given, except for the ordered mechanism.

Some mechanisms, in which no quinternary complexes are formed, are also studied, like a Ping – Pong mechanism and a Theorell – Chance for a substrate, whose equations are given.

The paper also includes a description of the equations of some mechanisms under the rapid equilibrium assumption.

Finally, the contribution includes a section devoted to show how product–inhibition studies allow sometimes to distinguish between some types of four-substrate mechanisms

The theoretical studies of Vrzhehch mentioned above [7-9] show that the kinetic behavior of multi-substrate systems can be described in terms of the interrelationships of the enzyme species. These interrelationships are named as scalar and vector.

Two enzyme species of an enzymatic reaction have a scalar interrelationship if there is a portion in the mechanism connecting them and consisting only of reversible steps. Whereas, two enzyme species of an enzymatic reaction have a vector interrelationship if in the direction of the reaction there is no irreversible step.

Depending on the type of interrelationships of the enzyme species of a mechanism, different plots of $1/v$ vs. 1/[X] are obtained. This can be used to distinguish among different types of mechanisms [8].

The few studies found in literature about the kinetics of four-substrate enzyme species [13,14] use the initial rate equations obtained under the total rapid equilibrium assumption. This also happens in many parts of the theoretical paper of Elliot and Tipton [10]. These equations are acquired assuming that all the reversible steps reach equilibrium almost from the beginning of the reaction. The reason could be that manual acquisition of the rapid equilibrium initial rate equations is easier than obtaining those corresponding to strict conditions. They are also simpler that these last ones. As a consequence of this, for many years it has been thought that the strict steady-state equations of an enzyme system might be too complex to be of practical interest [15].

Despite these advantages, the rapid equilibrium assumption is always a contrived simplification. Its application to an enzymatic system could lead us far from the real situation [16] (it may even place a limit on the applicability of the kinetic equations for some models, such as slow-binding reversible enzyme inhibition [17-19]. Nevertheless, the simplicity of the equations acquired under this assumption makes them very useful for complex reaction mechanisms. This is the reason for which we use them in this contribution although we have also acquired the steady-state strict equations.

This theoretical study has been motivated by three facts. First, the discovery in the last years of some four-substrate enzyme species and transporters of biological importance with unknown kinetics [20-22]. Secondly, the development of more powerful and flexible software for deriving enzyme rate expressions [23,24]. Finally, the lack of theoretical studies in the literature for many of the four-substrate reaction mechanisms.

- Sequential mechanisms
  - Ordered
    - Quad Quad (Mechanism 1)
  - Random
    - ABC (Mechanism 2)
    - AB (Mechanism 3)
    - BC (Mechanism 4)
    - CD (Mechanism 5)
- Ping Pong mechanisms
  - Bi Uni Uni Uni Uni Bi (Mechanism 6)
  - Hexa Uni (Mechanism 7)
  - Bi Random Uni Uni Uni Uni Bi Random (Mechanism 8)
  - Uni Uni Uni Uni Bi Bi (Mechanism 9)
  - Ter Uni Uni Uni Bi (Mechanism 10)
- Theorell-Chance mechanisms
  - Quad Quad (Mechanism 11)
  - Ter Uni Uni Ter (Mechanism 12)
  - Bi Uni Uni Uni Uni Bi (Mechanism 13)

Scheme 1

The objectives of this contribution are: 1) to acquire the initial steady-state rate equations for 13 four-substrate reaction mechanisms under both the strict conditions and the rapid equilibrium assumption. They have been selected for two reasons. Firstly, they correspond to different types of reaction mechanisms (ordered, random, Ping-Pong, Theorell – Chance). Thus, their study provides a wide view of the four-substrate enzyme kinetics. Secondly, most of them have never been analyzed before due to their complexity. The schemes and the equations of the different mechanisms are shown in Appendix A. The list of all of them is shown in Scheme 1 as well as the arbitrary number assigned to identify them in the paper; 2) To discriminate between the different mechanisms from their corresponding rapid equilibrium rate equations; 3) to suggest a way to determinate the binding order of the substrates in some the mechanisms studied; 4) To suggest a procedure to evaluate the global kinetic parameters involved in the rapid equilibrium rate equations of the mechanisms under study

1 MATERIAL AND METHODS

We have used the software WinStes developed by our research group [18] to acquire the initial rate equations for Mechanisms 1-13. The computer program WinStes was run on a PC compatible on a Pentium IV/3.2 GHz processor with 1 GB of RAM. This software is available to interested readers on *http://oretano.iele-ab.uclm.es/~fgarcia/WinStes/*. The general model for enzyme catalyzed reactions used by the software WinStes and for the kinetics analysis of Mechanisms 1-13 is that described in many other papers [16,23,25-27].

2 KINETIC ANALYSIS

The initial rate expressions for product P, v(P), i.e., the rate of product accumulation once the steady state is reached in the reaction mechanism, corresponding to each of the reaction mechanisms studied in this contribution have been acquired from the steady-state general model proposed by Varon *et al.* [20-22] using the software WinStes [18]. For every mechanism we have obtained two equations, given in Appendix A, one corresponding to the steady-state strict conditions and another under the rapid equilibrium assumption.

The rapid equilibrium assumption in a mechanism supposes that the first or pseudo-first order rate constants involved in one or more of its reversible steps of are much higher than the remaining ones, so that these steps reach the equilibrium from the onset of the reaction. If this assumption is not made, then the system is under strict conditions. As a simple example we will use the Michaelis-Menten mechanism (Scheme 2),

$$E + S \underset{k_{-1}}{\overset{k_1[S]}{\rightleftharpoons}} ES \xrightarrow{k_2} E + P$$

Scheme 2

where E is the free enzyme, S the substrate and P the reaction product. If we assume that the rate constants $k_1[S]_0$ and k_{-1} are much higher than k_2, the mechanism is in rapid equilibrium and the rate equation is:

$$v(P) = \frac{k_2 [E]_0 [S]_0}{K_1 + [S]_0} \qquad (1)$$

where K_1 is the dissociation equilibrium constant of the complex ES equal to k_{-1}/k_1. Under strict conditions, i.e. without any step in rapid equilibrium, the rate equation is:

$$v(P) = \frac{k_2 [E]_0 [S]_0}{K_m + [S]_0} \qquad (2)$$

where K_m is the Michaelis-Menten constant equal to $(k_{-1}+k_2)/k_1$. A complete analysis of strict and rapid equilibrium equations is carried out in Varon *et al.* [27]. In the acquisition of equations, we have supposed that the only species present at the onset of the reaction are the free enzyme E and the four substrates: A, B, C and D. Their initial concentrations are notated in the equations by $[E]_0$, [A], [B], [C] and [D], as the software WinStes requires. Under these conditions the rate expressions for all the products of the reaction mechanisms coincide with v(P).

We have rearranged these equations for a better viewing, so that both the denominator and the numerator of the right side of each one of them are given, respectively, as sums of products of coefficients d_i (i = 1,2,...) and n_i (i = 1,2,...) multiplied (or not) by the concentrations of one or more of the substrates with different exponents. On the other hand, each of these coefficients d_i and n_i are products or sums of products of rate constants and can be obtained easily using the software WinStes. In writing this contribution we have omitted them to avoid an unnecessary excessive length of it.

Due to the complexity of the strict steady-state rate equations, we will use the rapid equilibrium ones in the rest of the paper. This option does not suppose any loss in the generality in the treatment, but makes it simpler.

All the rapid equilibrium equations corresponding to the Mechanisms 1-13 can be written as a function of the concentration of one of the substrates [X], in the following way:

$$\frac{v(P)}{[E]_0} = \frac{p_1[X]}{q_0 + q_1[X]} \tag{3}$$

where the expressions of the coefficients p_1, q_0 and q_1 may include the concentration of one or more of the rest of the substrates.

The Eq. (1) can also be written as:

$$\frac{[E]_0}{v(P)} = \alpha + \beta\frac{1}{[X]} \tag{4}$$

where α and β are:

$$\alpha = \frac{q_1}{p_1} \tag{5}$$

$$\beta = \frac{q_0}{p_1} \tag{6}$$

Notice that a plot of $[E]_0/v(P)$ *vs.* 1/[X], while the concentrations of the other three substrates remain constant, is a straight line with a slope β and an intercept α. The expressions of α and β for the Mechanisms 1 -13 are given in Appendix B for X = A, B, C and D.

3. RESULTS AND DISCUSSION

In this contribution we have obtained and described the equations for some four – substrate reaction mechanisms under the strict steady-state conditions and the rapid equilibrium assumption as well. This is the first time, as far as we know, that these expressions have been acquired for many of the Mechanisms 1-13. We have also rearranged them in a way [Eq. (2)] which allows comparing them. We have used for this purpose the equations acquired under the rapid equilibrium assumption because their simplicity. The slopes and the intercepts for the equations of the mechanisms under study are listed in Appendix B. The differences arising from these expressions suggest a procedure to distinguish among most of the Mechanisms 1-13, as well as to evaluate global kinetic parameters.

3.1 Experimental design and kinetic data analysis allowing distinguishing among most of the Mechanisms 1 -13.

The suggested method consists of the following four steps, which are summarized in Fig. 4. In the assays corresponding to each of the steps of the procedure, the initial concentration of the free enzyme, $[E]_0$, will be always the same for convenience.

Step 1. To obtain $v(P)/[E]_0$, varying the initial concentration of one of the substrates, X, while the concentrations of the other three substrates remain constant. The plot of $[E]_0/v(P)$ *vs.* $1/[X]$ will be a straight line according to Eq. (2). This procedure must be repeated at different constant concentrations of the other three substrates, different from X. We assume that the binding order of the substrates is not known. If this order were known for at least one of the substrates, the method will be simplified. Thus, a set of straight lines are originated when the values of $[E]_0/v(P)$ *vs.* $1/[X]$ are plotted. The slopes [Eq. (3)] and intercepts [Eq. (4)] of these lines can vary for Mechanisms 1-13 because of the different dependence of p_1, q_0 and q_1 with the initial concentrations of the substrates (see Appendix B). The different shape of these sets of straight lines is the first criterion that will allow us to distinguish among some the Mechanisms 1-13.

Despite of the number of expressions for α and β for each mechanism that appear in Appendix B, it is easy to see from them that there are only four possible types of sets of lines for the plot of $[E]_0/v(P)$ *vs.* $1/[X]$. These four possibilities are shown in Fig. 1.

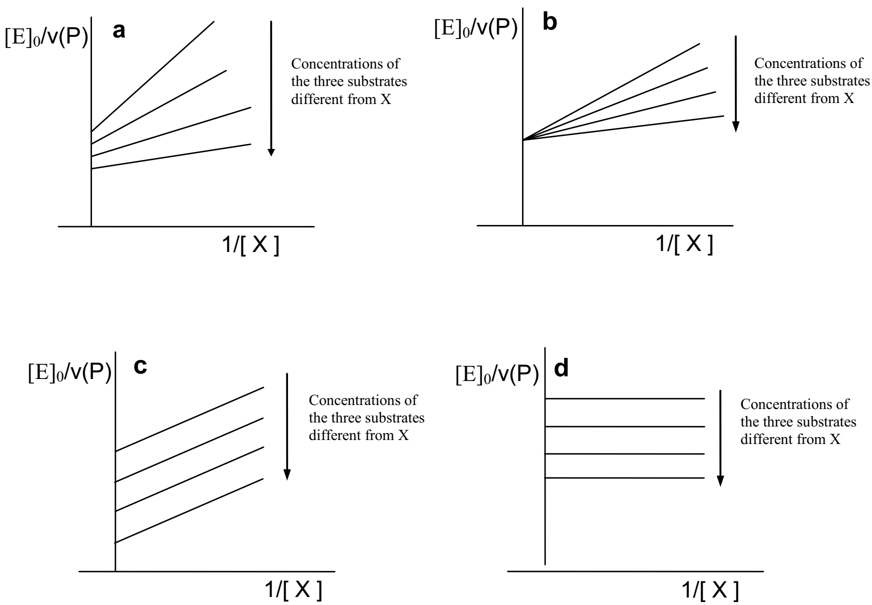

Figure 1 Schematic plots of Eq. (2). To obtain the four lines in Fig. 1(a), 1(b), 1(c) and 1(d), the concentration of substrate X has been varied for four constant concentrations of the remaining three substrates. The arrow points increasing concentrations of these three substrates. (a) set in which all the straight lines have different α and β. It appears in mechanisms in which the slope and the intercept of the lines depend on the concentration of one or more of the three substrates different from X. (b) all the lines share the same intercept but have different slope. Now β depends on the concentration of one or more of the mentioned three substrates meanwhile α is constant. It does not depend on the concentration of any substrate. (c) group of parallel straight lines with different intercept. Thus β does not depend on the concentration of any substrate while α has a dependence on some of them. (d) a parallel set of straight lines but with slope equal to zero.

The different types of sets of lines [Fig. 1(a)–1(d)] that are obtained for Mechanisms 1-13 with the procedure described above can be predicted from the expressions of α and β shown in Appendix B. The results are shown in Table 1.

Table 1. Types of plots $[E]_0/v(P)$ vs. $1/[X]$ (X = A, B, C and D) expected for Mechanisms 1-13 referred to the graphs in Fig. 1.

Mechanism	X	Fig. 1(a)	Fig. 1(b)	Fig. 1(c)	Fig. 1(d)	Mechanism	X	Fig. 1(a)	Fig. 1(b)	Fig. 1(c)	Fig. 1(d)
1	A	■				8	A	■			
	B	■					B	■			
	C	■					C			■	
	D		■				D			■	
2	A	■				9	A			■	
	B	■					B			■	
	C	■					C				■
	D		■				D				■
3	A	■				10	A	■			
	B	■					B	■			
	C	■					C	■			
	D		■				D			■	
4	A	■				11	A	■			
	B	■					B	■			
	C	■					C	■			
	D		■				D		■		
5	A	■				12	A	■			
	B	■					B	■			
	C	■					C	■			
	D	■					D			■	
6	A	■				13	A	■			
	B	■					B	■			
	C			■			C			■	
	D			■			D			■	
7	A			■							
	B			■							
	C			■							
	D			■							

As it can be seen in Table 1, there are only six different combinations of types of sets of lines. Thus, Mechanisms 1–13 can be classified in some of six different groups, that we name as Groups I–VI (Fig. 4), each of them characterized by one of the above mentioned combinations. Thus, after finishing this step, an unknown mechanism may be classified in one of the Groups I–VI. As Groups I, V and VI are made of only one mechanism, this step would allow to discriminate them from the rest of four- substrate mechanisms. However, this step does not allow to distinguish among the mechanisms included in Groups II–IV (see Fig. 4)

Step 2. The method in this step will lead to distinguish among some of the mechanisms of Group II, which cannot be discriminated in step 1. Once again it is necessary to obtain v(P)/ $[E]_0$, varying the initial concentration of one of the substrates, which will be called X. The plot of $[E]_0$/v(P) *vs.* 1/[X] will be a straight line according to Eq. (2), with an intercept α and a slope β. This procedure must be repeated at different initial concentrations of one of the remaining three substrates, which will be called Y, meanwhile the concentrations of the other two are the same in all the essays. As a result, a new set of straight lines is obtained, but different from those of Step 1. If this procedure is repeated but varying the substrates X and Y, a group of sets of lines will be acquired for each of the mechanisms of Group II.

The criterion used in this step to discriminate among these mechanisms will be the dependence of the slope of these lines with the concentration of substrate Y. The expressions of slope β, according to Eq. (3), vary for Mechanisms 1-13 and are given in Appendix B. From these expressions it can be seen that the plot of β *vs.* 1/[Y] for each of the mechanisms produces only two types of straight lines. The first of them is a straight line with intercept equal to 0 and positive slope. The second is a straight line with both intercept and slope positive. These two possibilities are represented in Fig. 2.

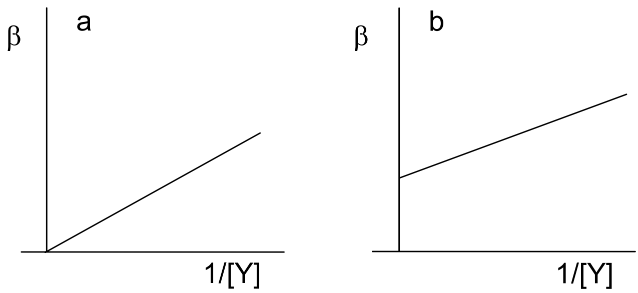

Figure 2. Schematic plots of the slope β *vs.* 1/[Y] of the straight lines obtained following the procedure explained in **4.1 Step 2**. (a) straight line with intercept equal to zero and positive slope. (b) straight line with both intercept and slope positive.

The different type of straight line [Fig. 2(a)–2(b)] that are obtained for Mechanisms 1–4 and 11, following the procedure previously described, can be also predicted from the expressions of the slopes given in Appendix B. The results are shown in Table 2, as well as the substrates X and Y of each essay.

As a consequence of the different sets of lines shown in Table 2, it is possible to distinguish between the Mechanisms 2 and 3. In addition, it is possible to discriminate between them and Mechanisms 1, 4 and 11. This three last ones show the same dependence of the slopes from the concentration of the different substrates. The reason is, as it can be seen in Appendix B, that their expressions for the slopes are exactly the same. Finally, the results in the discrimination among the mechanisms of Group II are shown in Fig. 4.

Table 2. Types of plots β vs. 1/[Y] (Y = A, B, C and D) expected for Mechanisms 1 – 4 and 11 referred to the graphs in Figure 2.

Mechanism	X	Y	Fig. 2(a)	Fig. 2(b)	Mechanism	X	Y	Fig. 2(a)	Fig. 2(b)
1	A	B	■		4	A	B	■	
	A	C	■			A	C	■	
	A	D	■			A	D	■	
	B	A		■		B	A		■
	B	C	■			B	C	■	
	B	D	■			B	D	■	
	C	A		■		C	A		■
	C	B		■		C	B		■
	C	D	■			C	D	■	
	D	A		■		D	A		■
	D	B		■		D	B		■
	D	C		■		D	C		■
2	A	B		■	11	A	B	■	
	A	C		■		A	C	■	
	A	D	■			A	D	■	
	B	A		■		B	A		■
	B	C		■		B	C	■	
	B	D	■			B	D	■	
	C	A		■		C	A		■
	C	B		■		C	B		■
	C	D	■			C	D	■	
	D	A		■		D	A		■
	D	B		■		D	B		■
	D	C		■		D	C		■
3	A	B		■					
	A	C	■						
	A	D	■						
	B	A		■					
	B	C	■						
	B	D	■						
	C	A		■					
	C	B		■					
	C	D	■						
	D	A		■					
	D	B		■					
	D	C	■						

Step 3. The purpose of this step is to discriminate between the Mechanisms 1, 4 and 11 of Group II. The method is exactly the same as it was described in Step 2, but taking the intercepts of the straight lines of the plots of $[E]_0/v(P)$ *vs.* 1/[X], when the concentration of only the remaining substrates, Y, was varied for a number of essays. The type of the plot of the intercept α *vs.* 1/[Y] will be the criterion that will lead to discriminate among the mechanisms mentioned above. These plots can be predicted from the expressions of the intercepts given in Appendix B. Only two different types of lines are actually obtained, those that are shown in Fig. 3; one straight line with both slope and intercept positive (Fig. 3(a)) and another parallel one to the horizontal axis and positive intercept (Fig. 3(b)).

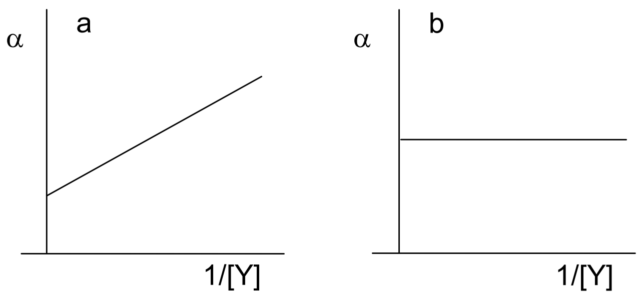

Figure 3. Schematic plots of the intercept α *vs.* 1/[Y] of the straight lines obtained following the procedure explained in **4.1 Step 3**. (a) straight line with both intercept and slope positive. (b) straight line with positive intercept and slope equal to zero.

In the same way as we did in Step 2, the different types of graphs [Fig. 3(a) – 3(b)] obtained for the intercepts of Mechanisms 1, 4 and 11 can be predicted from their expressions given in Appendix B. The results are shown in Table 3, giving also in all the cases the substrates X and Y of each essay.

Table 3. Types of plots α vs. 1/[Y] (Y = A, B, C and D) expected for Mechanisms 1, 4 and 11 referred to the graphs in Fig. 3.

Mechanism	X	Y	Fig. 3(a)	Fig. 3(b)	Mechanism	X	Y	Fig. 3(a)	Fig. 3(b)
	A	B	■			A	B	■	
	A	C	■			A	C	■	
	A	D	■			A	D	■	
	B	A		■		B	A		■
	B	C	■			B	C	■	
1	B	D	■		11	B	D	■	
	C	A		■		C	A		■
	C	B		■		C	B		■
	C	D	■			C	D	■	
	D	A		■		D	A		■
	D	B		■		D	B		■
	D	C		■		D	C		■
	A	B	■						
	A	C	■						
	A	D	■						
	B	A		■					
	B	C	■						
4	B	D	■						
	C	A	■						
	C	B		■					
	C	D		■					
	D	A		■					
	D	B		■					
	D	C		■					

The shapes of the graphs shown in Table 3 allow to discriminate Mechanism 4 from Mechanisms 1 and 11 (See Fig. 4). However it is not possible to distinguish between these two last mechanisms. The reason is the same as in Step 2. As can be seen in Appendix B, the expressions for their intercepts are similar.

Step 4. . In this step we will discriminate between the mechanisms of Group IV. In this case is not possible to follow the procedure described above in Step 2 because of the similarity of their expressions of the slope (see Appendix B), thus, we will follow the method explained in Step 3. The different types of graphs [Fig. 3(a) –3(b)] obtained for the intercepts of Mechanisms 6, 8 and 13 can also be predicted from their expressions given in Appendix B. The results are shown in Table 4.

Table 4. Types of plots α vs. 1/[Y] (Y = A, B, C and D) expected for Mechanisms 1, 4 and 11 referred to the graphs in Fig. 3.

Mechanism	X	Y	Fig. 3(a)	Fig. 3(b)	Mechanism	X	Y	Fig. 3(a)	Fig. 3(b)
	A	B	■			A	B	■	
	A	C	■			A	C	■	
	A	D	■			A	D	■	
	B	A		■		B	A		■
	B	C	■			B	C	■	
	B	D	■			B	D	■	
6	C	A	■		13	C	A	■	
	C	B	■			C	B	■	
	C	D	■			C	D	■	
	D	A	■			D	A	■	
	D	B	■			D	B	■	
	D	C	■			D	C	■	
	A	B	■						
	A	C	■						
	A	D	■						
	B	A	■						
	B	C	■						
8	B	D	■						
	C	A	■						
	C	B	■						
	C	D	■						
	D	A	■						
	D	B	■						
	D	C	■						

As it is shown in Table 3, there is only a small difference among the graphs of Mechanisms 6, 8 and 13. However, they could help to discriminate Mechanism 8 from the other two (See Fig. 4).

Unfortunately, the procedure described in Step 2 and 3 does not permit to distinguish between the Mechanisms of Group III. As can be seen in Appendix B, their slopes and intercepts expressions are very similar and the same types of graphs will be obtained for all of them.

3.2. Determination of the binding order of the substrates in some mechanisms

An indirect result of the procedure explained above is the determination of the binding order of the substrates in some of the mechanisms under study in this contribution. The differences in the graphs shown in Step 1, 2 and 3 could lead in some cases to know the first substrate to bind the free enzyme, the last one or indeed the whole binding order for all of them.

As an example, we will use Mechanisms 1, 4 and 11. The last substrate D to bind the enzyme species can be determined simply by the shape of the graph $[E]_0/v(P)$ *vs.* $1/[X]$ (Fig. 1). When X is A, B or C, the sets of lines correspond to Fig. 1(a). However if X is D, the set of lines is alike Fig. 1(b) (See Table 1). Once the last substrate to bind is known, the graphs β *vs.* $1/[Y]$ show the first one. In these three mechanisms when X is A and Y is B, C or D, the sets of lines obtained correspond in all the cases to Fig. 2.a (See Table 2). When X is B, C or D it does not happen. Thus, the first substrate to bind the enzyme species can be also determined.

3.3 Evaluation of global kinetic parameters

The expressions of Mechanisms 1-13 acquired using the software WinStes and the graphs obtained following the procedures described in the subsection 3.1, can be used to evaluate the global kinetic parameters, n_i/d_j, involved in the rapid equilibrium rate equations. Therefore it is possible to know the variation of the initial rate with the concentrations of the different substrates.

As an example, we will use Mechanism 4, which rate equation (A.8) given in Appendix A, can be rewritten for this task as:

$$\frac{v(P)}{[E]_0} = \frac{[A]\,[B]\,[C]\,[D]}{\frac{n_1}{d_1} + \frac{n_2}{d_1}[A] + \frac{n_3[A]\,[B]}{d_1} + \frac{n_4[A]\,[B]\,[C]}{d_1} + \frac{n_5[A]\,[B]\,[C]\,[D]}{d_1}} \quad (7)$$

In the following paragraphs a method is explained, using this example, to evaluate the coefficients n_i/d_1 (i = 1,2,3,4,5) involved in the equation. This procedure can be extrapolated, under the rapid equilibrium assumption, to the remaining mechanisms.

Firstly it is necessary to obtain the graphs α *vs.* 1/[Y] and β *vs.* 1/[Y] as it was explained in the subsection 4.1, Steps 2 and 3. Moreover, the rapid equilibrium rate equation of this mechanism, given in Appendix A, should be rearranged to suit Eq. (2). The expressions of their intercepts α and their slopes β are also given in Appendix B. Finally, the comparison between the values of the intercepts and slopes in the graphs with their expressions will allow us to evaluate global kinetic parameters of the mechanism.

The plot α *vs.* 1/[Y] when X = D and Y = A, B or C is a straight line with slope equal to zero (Table 3, Fig. 3(b)). The comparison of the intercept of this line allows us to get directly the ratio n_5/d_1 between two coefficients of the rate equation.

The plot α *vs.* 1/[Y] when X = C and Y = A is a straight line with both intercept and slope positive (Table 3, Fig. 3(a)). The slope of this line, according with the expression of α is equal to another kinetic parameter, n_4/d_1.

The plot of α *vs.* 1/[Y] when X = B and Y = C is a straight line with both intercept and slope positive (Table 3, Fig. 3(a)). The slope of this line is $n_3/(d_1[D])$. A plot of this slope versus 1/[D] is a straight line through the origin with slope equal to n_3/d_1.

The remaining expressions of the intercepts for Mechanism 4 are too complex to get any parameter as easily as it has just been described. Thus, we will use the graphs β *vs.* 1/[Y].

The plot of β *vs.* 1/[Y] when X = A and Y = B is a straight line with intercept equal to zero and positive slope (Table 2, Fig. 2(a)). The expression of this slope, as it is given in Appendix B, is $n_1/(d_1[C][D])$. In this case also, a plot of this slope versus 1/([C][D]) is a straight line through the origin with slope equal to n_1/d_1.

Finally, a plot of β *vs.* 1/[Y] when X = B and Y = A is a straight line with both intercept and slope positive (Table 2, Fig. 2(b)). The intercept of this line is equal to $n_2/(d_1[C][D])$. Thus, a plot of this intercept versus $1/([C][D])$ is a straight line through the origin with slope equal to n_2/d_1.

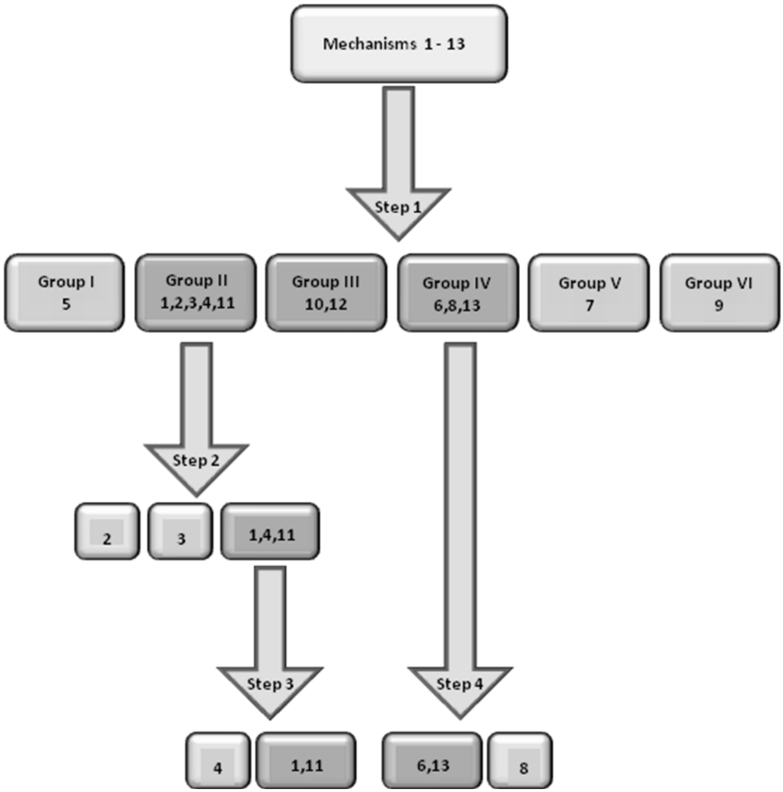

Figure 4. Schematic representation of the four steps of the method explained in the subsection 3.1 to discriminate between the different mechanisms. The big top box represents the whole group of four-substrate mechanisms under study. The darkest boxes contain reaction mechanisms which cannot be discriminated until that point of the

procedure. The clearest boxes contain those mechanisms that can be clearly distinguished in the corresponding step of the method.

4. CONCLUSIONS

In this contribution, we have acquired the initial rate expressions of a number of four – substrate reaction mechanisms under the strict steady-state conditions. This is the first time, to the best of our knowledge, that these equations are provided.

We have also obtained the initial rate expressions under the rapid equilibrium assumption for the same reaction mechanisms. These equations have been used to develop a kinetic analysis and to suggest a procedure to distinguish between most of them. Thus, seven of the mechanisms under study can be clearly indentified following the method described above. However, it appears three pairs of mechanisms which cannot be discriminated, even though it is possible to distinguish them from the rest. The initial rate expressions of these pairs (10,12), (1,11) and (6,13) are formally identical, varying only the expressions of the coefficients. Thus, they cannot be discriminate only by kinetic analysis.

Although there are some studies in the literature [5,8,11] about four–substrate mechanisms, this contribution provides, to the best of our knowledge, for the first time a procedure to distinguish among a number of them, including some which had never been studied before.

The procedure described in this contribution also allows determining the binding order of some of the substrates to the enzyme species in some cases.

Finally, the expressions acquired with the software and some values from the graphs, obtained in the method mentioned above, can be used to evaluate general kinetic parameters.

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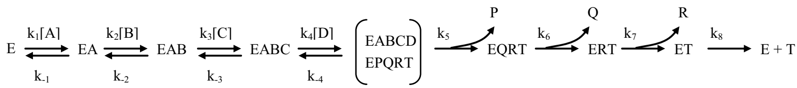
APPENDIX A

Reaction schemes and initial rate equations of the Mechanisms 1–13. The first equation corresponds to the strict steady- state conditions and the second one to the rapid equilibrium assumption.

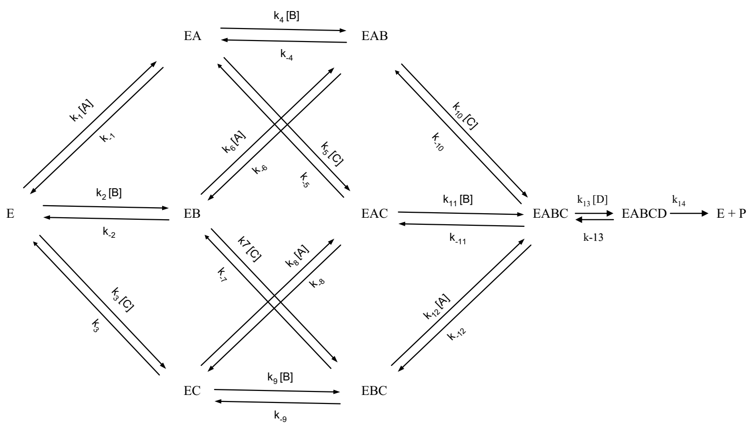
Mechanism 1



$$\frac{v(P)}{[E]_0}=\frac{d_1[A]\,[B]\,[C]\,[D]}{n_1+n_2[A]+n_3[D]+n_4[A]\,[B]+n_5[A]\,[D]+n_6[C]\,[D]+n_7[A]\,[B]\,[C]+n_8[A]\,[B]\,[D]+n_9[A]\,[C]\,[D]+ +n_{10}[B]\,[C]\,[D]+n_{11}[A]\,[B]\,[C]\,[D]} \quad (A.1)$$

$$\frac{v(P)}{[E]_0}=\frac{d_1[A]\,[B]\,[C]\,[D]}{n_1+n_2[A]+n_3[A]\,[B]+n_4[A]\,[B]\,[C]+n_5[A]\,[B]\,[C]\,[D]} \quad (A.2)$$

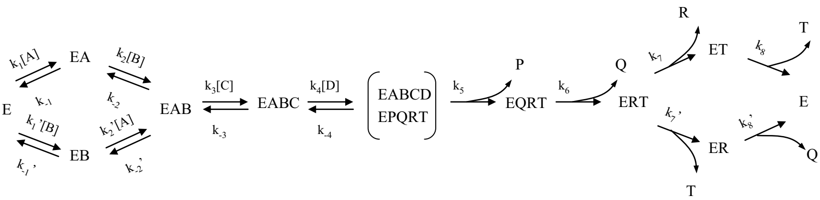
Mechanism 2



$$\frac{v(P)}{[E]_0}=\frac{\begin{array}{l}d_1[A][B][C][D]+d_2[A]^2[B][C][D]+d_3[A][B]^2[C][D]+d_4[A][B][C]^2[D]+\\ d_5[A]^2[B]^2[C][D]+d_6[A]^3[B][C][D]+d_7[A][B]^3[C][D]+d_8[A]^2[B][C]^2[D]+\\ d_9[A][B]^2[C]^2[D]+d_{10}[A][B][C]^3[D]+d_{11}[A]^3[B]^2[C][D]+d_{12}[A]^2[B]^3[C][D]+\\ d_{13}[A]^4[B][C][D]+d_{14}[A]^2[B]^2[C]^2[D]+d_{15}[A]^3[B][C]^2[D]+d_{16}[A]^2[B][C]^3[D]+\\ d_{17}[A][B]^3[C]^2[D]+d_{18}[A][B]^4[C][D]+d_{19}[A][B]^2[C]^3[D]+d_{20}[A][B][C]^4[D]+\\ d_{21}[A]^4[B]^2[C][D]+d_{22}[A]^3[B]^3[C][D]+d_{23}[A]^3[B]^2[C]^2[D]+d_{24}[A]^2[B]^3[C]^2[D]+\\ d_{25}[A]^4[B][C]^2[D]+d_{26}[A]^3[B][C]^3[D]+d_{27}[A]^2[B]^2[C]^3[D]+d_{28}[A]^2[B]^4[C][D]+\\ d_{29}[A][B]^4[C]^2[D]+d_{30}[A][B]^3[C]^3[D]+d_{31}[A]^2[B][C]^4[D]+d_{32}[A][B]^2[C]^4[D]\end{array}}{\begin{array}{l}n_0+n_1[A]+n_2[B]+n_3[C]+n_4[D]+n_5[A][B]+n_6[A][C]+n_7[A][D]+n_8[A]^2+n_9[B][C]+\\ n_{10}[B][D]+n_{11}[B]^2+n_{12}[C][D]+n_{13}[C]^2+n_{14}[A][B][C]+n_{15}[A][B][D]+n_{16}[A]^2[B]+\\ n_{17}[A][B]^2+n_{18}[A][C][D]+n_{19}[A]^2[C]+n_{20}[A][C]^2+n_{21}[A]^2[D]+n_{22}[A]^3+n_{23}[B][C][D]+\\ n_{24}[B]^2[C]+n_{25}[B][C]^2+n_{26}[B]^2[D]+n_{27}[B]^3+n_{28}[C]^2[D]+n_{29}[C]^3+n_{30}[A][B][C][D]+\\ n_{31}[A]^2[B][C]+n_{32}[A][B]^2[C]+n_{33}[A]^2[B][D]+n_{34}[A][B]^2[D]+n_{35}[A]^2[B]^2+n_{36}[A]^3[B]+\\ n_{37}[A][B]^3+n_{38}[A][B][C]^2+n_{39}[A]^2[C][D]+n_{40}[A][C]^2[D]+n_{41}[A]^2[C]^2+n_{42}[A]^3[C]+\\ n_{43}[A][C]^3+n_{44}[A]^3[D]+n_{45}[A]^4+n_{46}[B]^2[C][D]+n_{47}[B][C]^2[D]+n_{48}[B]^2[C]^2+n_{49}[B]^3[C]+\\ n_{50}[B]^3[D]+n_{51}[B]^4+n_{52}[B][C]^3+n_{53}[C]^3[D]+n_{54}[C]^4+n_{55}[A]^2[B][C][D]+n_{56}[A][B]^2[C][D]+\\ n_{57}[A]^2[B]^2[C]+n_{58}[A]^2[B]^2[D]+n_{59}[A]^3[B][C]+n_{60}[A]^3[B][D]+n_{61}[A]^3[B]^2+n_{62}[A][B]^3[C]+\\ n_{63}[A][B]^3[D]+n_{64}[A]^2[B]^3+n_{65}[A]^4[B]+n_{66}[A][B][C]^2[D]+n_{67}[A]^2[B][C]^2+n_{68}[A][B]^2[C]^2+\\ n_{69}[A]^2[C]^2[D]+n_{70}[A]^3[C][D]+n_{71}[A]^3[C]^2+n_{72}[A]^4[C]+n_{73}[A][B][C]^3+n_{74}[A][C]^3[D]+\\ n_{75}[A]^2[C]^3+n_{76}[A]^4[D]+n_{77}[B]^2[C]^2[D]+n_{78}[B]^3[C][D]+n_{79}[B]^3[C]^2+n_{80}[A][B]^4+n_{81}[B]^4[C]+\\ n_{82}[B]^4[D]+n_{83}[B][C]^3[D]+n_{84}[B]^2[C]^3+n_{85}[A][C]^4+n_{86}[B][C]^4+n_{87}[C]^4[D]+n_{88}[A]^2[B]^2[C][D]+\\ n_{89}[A]^3[B][C][D]+n_{90}[A]^3[B]^2[C]+n_{91}[A]^3[B]^2[D]+n_{92}[A][B]^3[C][D]+n_{93}[A]^2[B]^3[C]+\\ n_{94}[A]^2[B]^3[D]+n_{95}[A]^4[B][C]+n_{96}[A]^4[B][D]+n_{97}[A]^4[B]^2+n_{98}[A]^3[B]^3+n_{99}[A]^2[B][C]^2[D]+\\ n_{100}[A][B]^2[C]^2[D]+n_{101}[A]^2[B]^2[C]^2+n_{102}[A]^3[B][C]^2+n_{103}[A][B]^3[C]^2+n_{104}[A]^3[C]^2[D]+\\ n_{105}[A]^4[C][D]+n_{106}[A]^4[C]^2+n_{107}[A][B][C]^3[D]+n_{108}[A]^2[B][C]^3+n_{109}[A]^2[C]^3[D]+\\ n_{110}[A]^3[C]^3+n_{111}[A][B]^2[C]^3+n_{112}[B]^3[C]^2[D]+n_{113}[A][B]^4[C]+n_{114}[A][B]^4[D]+n_{115}[A]^2[B]^4+\\ n_{116}[B]^4[C][D]+n_{117}[B]^4[C]^2+n_{118}[B]^2[C]^3[D]+n_{119}[B]^3[C]^3+n_{120}[A][B][C]^4+n_{121}[A][C]^4[D]+\\ n_{122}[A]^2[C]^4+n_{123}[B][C]^4[D]+n_{124}[B]^2[C]^4+n_{125}[A]^3[B]^2[C][D]+n_{126}[A]^2[B]^3[C][D]+\\ n_{127}[A]^4[B][C][D]+n_{128}[A]^4[B]^2[C]+n_{129}[A]^4[B]^2[D]+n_{130}[A]^3[B]^3[C]+n_{131}[A]^3[B]^3[D]+\\ n_{132}[A]^2[B]^2[C]^2[D]+n_{133}[A]^3[B][C]^2[D]+n_{134}[A]^3[B]^2[C]^2+n_{135}[A]^2[B]^3[C]^2+n_{136}[A]^4[B][C]^2+\\ n_{137}[A]^4[C]^2[D]+n_{138}[A]^2[B][C]^3[D]+n_{139}[A]^3[B][C]^3+n_{140}[A]^3[C]^3[D]+n_{141}[A]^2[B]^2[C]^3+\\ n_{142}[A][B]^3[C]^2[D]+n_{143}[A][B]^4[C][D]+n_{144}[A]^2[B]^4[C]+n_{145}[A]^2[B]^4[D]+n_{146}[A][B]^4[C]^2+\\ n_{147}[B]^4[C]^2[D]+n_{148}[A][B]^2[C]^3[D]+n_{149}[A][B]^3[C]^3+n_{150}[B]^3[C]^3[D]+n_{151}[A][B][C]^4[D]+\\ n_{152}[A]^2[B][C][D]^4+n_{153}[A]^2[C]^4[D]+n_{154}[A][B]^2[C]^4+n_{155}[B]^2[C]^4[D]+n_{156}[A]^4[B]^2[C][D]+\\ n_{157}[A]^3[B]^3[C][D]+n_{158}[A]^3[B]^2[C]^2[D]+n_{159}[A]^2[B]^3[C]^2[D]+n_{160}[A]^4[B][C]^2[D]+\\ n_{161}[A]^3[B][C]^3[D]+n_{162}[A]^2[B]^2[C]^3[D]+n_{163}[A]^2[B]^4[C][D]+n_{164}[A][B]^4[C]^2[D]+\\ n_{165}[A][B]^3[C]^3[D]+n_{166}[A]^2[B][C]^4[D]+n_{167}[A][B]^2[C]^4[D]\end{array}} \tag{A.3}$$

$$\frac{v(P)}{[E]_0}=\frac{d_1[A][B][C][D]}{n_1+n_2[C]+n_3[B]+n_4[A]+n_5[B][C]+n_6[A][C]+n_7[A][B]+n_8[A][B][C]+[A][B][C][D]} \tag{A.4}$$

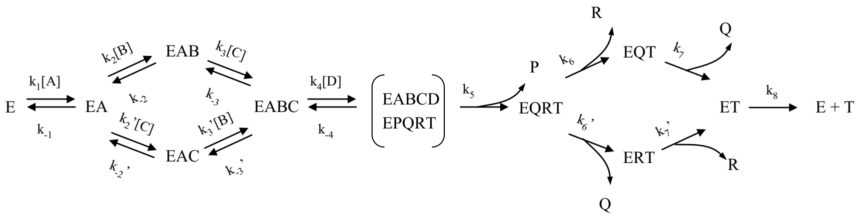
Mechanism 3



$$\frac{v(P)}{[E]_0} = \frac{d_1[A][B][C][D] + d_2[A]^2[B][C][D] + d_3[A][B]^2[C][D]}{\begin{array}{l} n_1 + n_2[A] + n_3[B] + n_4[D] + n_5[A][B] + n_6[A][D] + n_7[A]^2 + n_8[B][D] + n_9[B]^2 + \\ n_{10}[C][D] + n_{11}[A][B][C] + n_{12}[A][B][D] + n_{13}[A]^2[B] + n_{14}[A][C][D] + n_{15}[A]^2[D] + \\ n_{16}[B][C][D] + n_{17}[A][B]^2 + n_{18}[B]^2[D] + n_{19}[A][B][C][D] + n_{20}[A]^2[B][C] + \\ n_{21}[A]^2[B][D] + n_{22}[A]^2[C][D] + n_{23}[A][B]^2[C] + n_{24}[A][B]^2[D] + n_{25}[B]^2[C][D] + \\ n_{26}[A]^2[B][C][D] + n_{27}[A][B]^2[C][D] \end{array}} \quad (A.5)$$

$$\frac{v(P)}{[E]_0} = \frac{d_1[A][B][C][D]}{n_1 + n_2[B] + n_3[A] + n_4[A][B] + n_5[A][B][C] + n_6[A][B][C][D]} \quad (A.6)$$

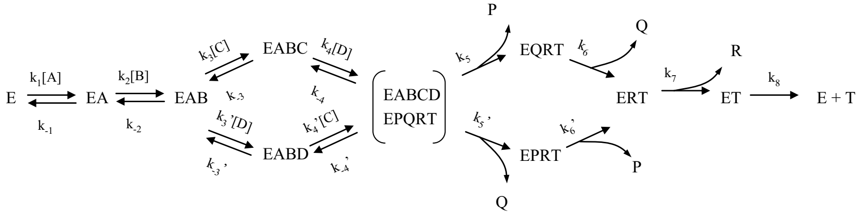
Mechanism 4



$$\frac{v(P)}{[E]_0} = \frac{d_1[A][B][C][D] + d_2[A][C]^2[D] + d_3[A][B][C]^2[D] + d_4[A][C]^3[D]}{\begin{array}{l} n_1 + n_2[A] + n_3[D] + n_4[C] + n_5[A][B] + n_6[A][C] + n_7[A][D] + n_8[C][D] + \\ n_9[A][B][C] + n_{10}[A][B][D] + n_{11}[A][C]^2 + n_{12}[A][C][D] + n_{13}[C]^2[D] + \\ n_{14}[B][C][D] + n_{15}[A][B][C][D] + n_{16}[A][B][C]^2 + n_{17}[A][C]^2[D] + n_{18}[A][C]^3 + \\ n_{19}[B][C]^2[D] + n_{20}[C]^3[D] + n_{21}[A][B][C]^2[D] + n_{22}[A][C]^3[D] \end{array}} \quad (A.7)$$

$$\frac{v(P)}{[E]_0} = \frac{d_1[A][B][C][D]}{n_1 + n_2[A] + n_3[A][B] + n_4[A][B][C] + n_5[A][B][C][D]} \quad (A.8)$$

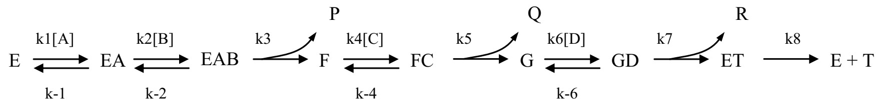
Mechanism 5



$$\frac{v(P)}{[E]_0}=\frac{d_1[A][B][C][D]+d_2[A][B][C]^2[D]+d_3[A][B][C][D]^2}{\begin{array}{l}n_1+n_2[A]+n_3[C]+n_4[D]+n_5[A][B]+n_6[A][C]+n_7[A][D]+n_8[C][D]+\\ n_9[A][B][C]+n_{10}[A][B][D]+n_{11}[A][C][D]+n_{12}[C]^2[D]+n_{13}[C][D]^2+\\ n_{14}[B][C][D]+n_{15}[A][B][C][D]+n_{16}[A][B][C]^2+n_{17}[A][B][D]^2+\\ n_{18}[A][C]^2[D]+n_{19}[A][C][D]^2+n_{20}[B][C]^2[D]+n_{21}[B][C][D]^2+n_{22}[A][B][C]^2[D]\end{array}} \qquad \text{(A.9)}$$

$$\frac{v(P)}{[E]_0}=\frac{d_1[A][B][C][D]}{n_1+n_2[A]+n_3[A][B]+n_4[A][B][D]+n_5[A][B][C]+n_6[A][B][C][D]} \qquad \text{(A.10)}$$

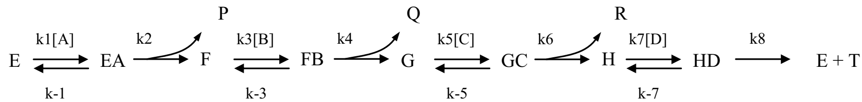
Mechanism 6



$$\frac{v(P)}{[E]_0}=\frac{d_1[A][B][C][D]}{n_1[C][D]+n_2[A][B][C]+n_3[A][B][D]+n_4[A][C][D]+n_5[B][C][D]+n_6[A][B][C][D]} \qquad \text{(A.11)}$$

$$\frac{v(P)}{[E]_0}=\frac{d_1[A][B][C][D]}{n_1[C][D]+n_2[A][B][C]+n_3[A][B][D]+n_4[A][C][D]+n_5[A][B][C][D]} \qquad \text{(A.12)}$$

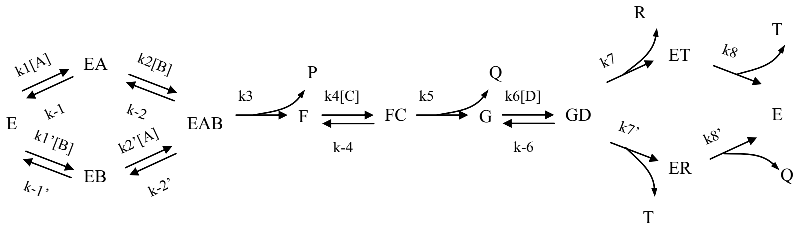
Mechanism 7



$$\frac{v(P)}{[E]_0}=\frac{d_1[A][B][C][D]}{n_1[A][B][C]+n_2[A][B][D]+n_3[A][C][D]+n_4[B][C][D]+n_5[A][B][C][D]} \qquad \text{(A.13)}$$

$$\frac{v(P)}{[E]_0}=\frac{d_1[A][B][C][D]}{n_1[A][B][C]+n_2[A][B][D]+n_3[A][C][D]+n_4[B][C][D]+n_5[A][B][C][D]} \qquad \text{(A.14)}$$

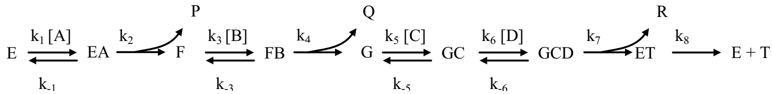
Mechanism 8



$$\frac{v(P)}{[E]_0}=\frac{d_1[A][B][C][D]+d_2[A]^2[B][C][D]+d_3[A][B]^2[C][D]}{\begin{array}{l}n_1[C][D]+n_2[A][B][C]+n_3[A][B][D]+n_4[A][C][D]+n_5[B][C][D]+n_6[A][B][C][D]+\\ n_7[A]^2[B][C]+n_8[A]^2[B][D]+n_9[A]^2[C][D]+n_{10}[A][B]^2[C]+n_{11}[A][B]^2[D]+\\ n_{12}[B]^2[C][D]+n_{13}[A]^2[B][C][D]+n_{14}[A][B]^2[C][D]\end{array}} \tag{A.15}$$

$$\frac{v(P)}{[E]_0}=\frac{d_1[A][B][C][D]}{n_1[C][D]+n_2[A][B][C]+n_3[A][B][D]+n_4[A][C][D]+n_5[B][C][D]+n_6[A][B][C][D]} \tag{A.16}$$

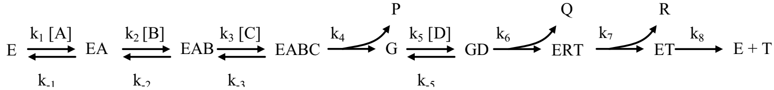
Mechanism 9



$$\frac{v(P)}{[E]_0}=\frac{d_1[A][B][C][D]}{n_1[A][B]+n_2[A][B][C]+n_3[A][B][D]+n_4[A][C][D]+n_5[B][C][D]+n_6[A][B][C][D]} \tag{A.17}$$

$$\frac{v(P)}{[E]_0}=\frac{d_1[A][B][C][D]}{n_1[A][C][D]+n_2[B][C][D]+n_3[A][B][C][D]} \tag{A.18}$$

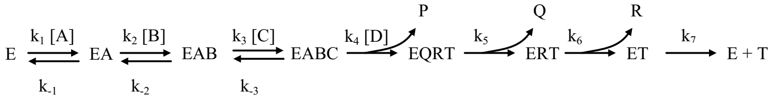
Mechanism 10



$$\frac{v(P)}{[E]_0}=\frac{d_1[A][B][C][D]}{\begin{array}{l}n_1[D]+n_2[A][D]+n_3[C][D]+n_4[A][B][C]+n_5[A][B][D]+n_6[A][C][D]+n_7[B][C][D]+\\ n_8[A][B][C][D]\end{array}} \tag{A.19}$$

$$\frac{v(P)}{[E]_0}=\frac{d_1[A][B][C][D]}{n_1[D]+n_2[A][D]+n_3[A][B][C]+n_4[A][B][D]+n_5[A][B][C][D]} \tag{A.20}$$

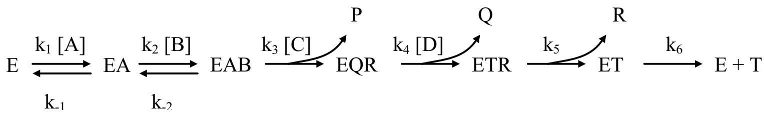
Mechanism 11



$$\frac{v(P)}{[E]_0} = \frac{d_1[A]\,[B]\,[C]\,[D]}{n_1 + n_2[A] + n_3[D] + n_4[A]\,[B] + n_5[A]\,[D] + n_6[C]\,[D] + n_7[A]\,[B]\,[C] + n_8[A]\,[B]\,[D] + n_9[A]\,[C]\,[D] + n_{10}[B]\,[C]\,[D] + n_{11}[A]\,[B]\,[C]\,[D]} \quad \text{(A.21)}$$

$$\frac{v(P)}{[E]_0} = \frac{d_1[A]\,[B]\,[C]\,[D]}{n_1 + n_2[A] + n_3[A]\,[B] + n_4[A]\,[B]\,[C] + n_5[A]\,[B]\,[C]\,[D]} \quad \text{(A.22)}$$

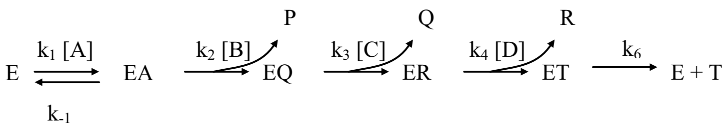
Mechanism 12



$$\frac{v(P)}{[E]_0} = \frac{d_1[A]\,[B]\,[C]\,[D]}{n_1[D] + n_2[A]\,[D] + n_3[C]\,[D] + n_4[A]\,[B]\,[C] + n_5[A]\,[B]\,[D] + n_6[A]\,[C]\,[D] + n_7[B]\,[C]\,[D] + n_8[A]\,[B]\,[C]\,[D]} \quad \text{(A.23)}$$

$$\frac{v(P)}{[E]_0} = \frac{d_1[A]\,[B]\,[C]\,[D]}{n_1[D] + n_2[A]\,[D] + n_3[A]\,[B]\,[C] + n_4[A]\,[B]\,[D] + n_5[A]\,[B]\,[C]\,[D]} \quad \text{(A.24)}$$

Mechanism 13



$$\frac{v(P)}{[E]_0} = \frac{d_1[A]\,[B]\,[C]\,[D]}{n_1[C]\,[D] + n_2[A]\,[B]\,[C] + n_3[A]\,[B]\,[D] + n_4[A]\,[C]\,[D] + n_5[B]\,[C]\,[D] + n_6[A]\,[B]\,[C]\,[D]} \quad \text{(A.25)}$$

$$\frac{v(P)}{[E]_0} = \frac{d_1[A]\,[B]\,[C]\,[D]}{n_1[C]\,[D] + n_2[A]\,[B]\,[C] + n_3[A]\,[B]\,[D] + n_4[A]\,[C]\,[D] + n_5[A]\,[B]\,[C]\,[D]} \quad \text{(A.26)}$$

APPENDIX B

Expressions for α and β corresponding to Eqs. (2)–(4) for Mechanisms 1–13. X is the substrate which concentration is varied in the plot $[E]_0/v(P)$ *vs.* $1/[X]$.

Mechanism 1

X	α	β
A	$\frac{1}{[D]}\left\{\frac{1}{[C]}\left(\frac{n_2}{d_1}\frac{1}{[B]}+\frac{n_3}{d_1}\right)+\frac{n_4}{d_1}\right\}+\frac{n_5}{d_1}$	$\frac{n_1}{d_1}\frac{1}{[B][C][D]}$
B	$\frac{1}{[D]}\left(\frac{n_3}{d_1}\frac{1}{[C]}+\frac{n_4}{d_1}\right)+\frac{n_5}{d_1}$	$\frac{1}{[C]}\frac{1}{[D]}\left(\frac{n_1}{d_1}\frac{1}{[A]}+\frac{n_2}{d_1}\right)$
C	$\frac{n_4}{d_1}\frac{1}{[D]}+\frac{n_5}{d_1}$	$\frac{1}{[D]}\left\{\frac{1}{[B]}\left(\frac{n_1}{d_1}\frac{1}{[A]}+\frac{n_2}{d_1}\right)+\frac{n_3}{d_1}\right\}$
D	$\frac{n_5}{d_1}$	$\frac{1}{[C]}\left\{\frac{1}{[B]}\left(\frac{n_1}{d_1}\frac{1}{[A]}+\frac{n_2}{d_1}\right)+\frac{n_3}{d_1}\right\}+\frac{n_4}{d_1}$

Mechanism 2

X	α	β
A	$\frac{1}{[D]}\left\{\frac{1}{[B]}\left(\frac{n_4}{d_1}\frac{1}{[C]}+\frac{n_6}{d_1}\right)+\frac{n_7}{d_1}\frac{1}{[C]}+\frac{n_8}{d_1}\right\}+\frac{1}{d_1}$	$\frac{1}{[D]}\left\{\frac{1}{[B]}\left(\frac{n_1}{d_1}\frac{1}{[C]}+\frac{n_2}{d_1}\right)+\frac{n_3}{d_1}\frac{1}{[C]}+\frac{n_5}{d_1}\right\}$
B	$\frac{1}{[D]}\left\{\frac{1}{[A]}\left(\frac{n_3}{d_1}\frac{1}{[C]}+\frac{n_5}{d_1}\right)+\frac{n_7}{d_1}\frac{1}{[C]}+\frac{n_8}{d_1}\right\}+\frac{1}{d_1}$	$\frac{1}{[D]}\left\{\frac{1}{[A]}\left(\frac{n_1}{d_1}\frac{1}{[C]}+\frac{n_2}{d_1}\right)+\frac{n_4}{d_1}\frac{1}{[C]}+\frac{n_6}{d_1}\right\}$
	$\frac{1}{[D]}\left\{\frac{1}{[A]}\left(\frac{n_2}{d_1}\frac{1}{[B]}+\frac{n_5}{d_1}\right)+\frac{n_6}{d_1}\frac{1}{[B]}+\frac{n_8}{d_1}\right\}+\frac{1}{d_1}$	$\frac{1}{[D]}\left\{\frac{1}{[A]}\left(\frac{n_1}{d_1}\frac{1}{[B]}+\frac{n_3}{d_1}\right)+\frac{n_4}{d_1}\frac{1}{[B]}+\frac{n_7}{d_1}\right\}$
D	$\frac{1}{d_1}$	$\frac{1}{[A]}\left\{\frac{1}{[B]}\left(\frac{n_1}{d_1}\frac{1}{[C]}+\frac{n_2}{d_1}\right)+\frac{n_3}{d_1}\frac{1}{[C]}+\frac{n_5}{d_1}\right\}+\frac{1}{[B]}\left(\frac{n_4}{d_1}\frac{1}{[C]}+\frac{n_6}{d_1}\right)+\frac{n_7}{d_1}\frac{1}{[C]}+\frac{n_8}{d_1}$

Mechanism 3

X	α	β
A	$\frac{1}{[D]}\left\{\frac{1}{[C]}\left(\frac{n_3}{d_1}\frac{1}{[B]}+\frac{n_4}{d_1}\right)+\frac{n_5}{d_1}\right\}+\frac{n_6}{d_1}$	$\frac{1}{[C]}\frac{1}{[D]}\left(\frac{n_1}{d_1}\frac{1}{[B]}+\frac{n_2}{d_1}\right)$
B	$\frac{1}{[D]}\left\{\frac{1}{[C]}\left(\frac{n_2}{d_1}\frac{1}{[A]}+\frac{n_4}{d_1}\right)+\frac{n_5}{d_1}\right\}+\frac{n_6}{d_1}$	$\frac{1}{[C]}\frac{1}{[D]}\left(\frac{n_1}{d_1}\frac{1}{[A]}+\frac{n_3}{d_1}\right)$
C	$\frac{n_5}{d_1}\frac{1}{[D]}+\frac{n_6}{d_1}$	$\frac{1}{[D]}\left\{\frac{1}{[A]}\left(\frac{n_1}{d_1}\frac{1}{[B]}+\frac{n_2}{d_1}\right)+\frac{n_3}{d_1}\frac{1}{[B]}+\frac{n_4}{d_1}\right\}$
D	$\frac{n_6}{d_1}$	$\frac{1}{[C]}\left\{\frac{1}{[A]}\left(\frac{n_1}{d_1}\frac{1}{[B]}+\frac{n_2}{d_1}\right)+\frac{n_3}{d_1}\frac{1}{[B]}+\frac{n_4}{d_1}\right\}+\frac{n_5}{d_1}$

Mechanism 4

X	α	β
A	$\frac{1}{[D]}\left\{\frac{1}{[C]}\left(\frac{n_2}{d_1}\frac{1}{[B]}+\frac{n_3}{d_1}\right)\right\}+\frac{n_5}{d_1}$	$\frac{n_1}{d_1}\frac{1}{[B][C][D]}$
B	$\frac{1}{[D]}\left(\frac{n_3}{d_1}\frac{1}{[C]}+\frac{n_4}{d_1}\right)+\frac{n_5}{d_1}$	$\frac{1}{[C]}\frac{1}{[D]}\left(\frac{n_1}{d_1}\frac{1}{[A]}+\frac{n_2}{d_1}\right)$
C	$\frac{n_4}{d_1}\frac{1}{[A]}+\frac{n_5}{d_1}$	$\frac{1}{[D]}\left\{\frac{1}{[B]}\left(\frac{n_1}{d_1}\frac{1}{[A]}+\frac{n_2}{d_1}\right)+\frac{n_3}{d_1}\right\}$
D	$\frac{n_5}{d_1}$	$\frac{1}{[C]}\left\{\frac{1}{[B]}\left(\frac{n_1}{d_1}\frac{1}{[A]}+\frac{n_2}{d_1}\right)+\frac{n_3}{d_1}\right\}+\frac{n_4}{d_1}$

Mechanism 5

X	α	β
A	$\frac{1}{[D]}\left\{\frac{1}{[C]}\left(\frac{n_2}{d_1}\frac{1}{[B]}+\frac{n_3}{d_1}\right)+\frac{n_5}{d_1}\right\}+\frac{n_4}{d_1}\frac{1}{[C]}+\frac{n_6}{d_1}$	$\frac{n_1}{d_1}\frac{1}{[B][C][D]}$
B	$\frac{1}{[D]}\left(\frac{n_3}{d_1}\frac{1}{[C]}+\frac{n_5}{d_1}\right)+\frac{n_4}{d_1}\frac{1}{[C]}+\frac{n_6}{d_1}$	$\frac{1}{[C]}\frac{1}{[D]}\left(\frac{n_1}{d_1}\frac{1}{[A]}+\frac{n_2}{d_1}\right)$
C	$\frac{n_5}{d_1}\frac{1}{[D]}+\frac{n_6}{d_1}$	$\frac{1}{[D]}\left\{\frac{1}{[B]}\left(\frac{n_1}{d_1}\frac{1}{[A]}+\frac{n_2}{d_1}\right)+\frac{n_3}{d_1}\right\}+\frac{n_4}{d_1}$
D	$\frac{n_4}{d_1}\frac{1}{[C]}+\frac{n_6}{d_1}$	$\frac{1}{[C]}\left\{\frac{1}{[B]}\left(\frac{n_1}{d_1}\frac{1}{[A]}+\frac{n_2}{d_1}\right)+\frac{n_3}{d_1}\right\}+\frac{n_5}{d_1}$

Mechanism 6

X	α	β
A	$\frac{n_2}{d_1}\frac{1}{[D]}+\frac{n_3}{d_1}\frac{1}{[C]}+\frac{n_4}{d_1}\frac{1}{[B]}+\frac{n_5}{d_1}$	$\frac{n_1}{d_1}\frac{1}{[B]}$
B	$\frac{n_2}{d_1}\frac{1}{[D]}+\frac{n_3}{d_1}\frac{1}{[C]}+\frac{n_5}{d_1}$	$\frac{n_1}{d_1}\frac{1}{[A]}+\frac{n_4}{d_1}$
C	$\frac{1}{[B]}\left(\frac{n_1}{d_1}\frac{1}{[A]}+\frac{n_4}{d_1}\right)+\frac{n_2}{d_1}\frac{1}{[D]}+\frac{n_5}{d_1}$	$\frac{n_3}{d_1}$
D	$\frac{1}{[B]}\left(\frac{n_1}{d_1}\frac{1}{[A]}+\frac{n_4}{d_1}\right)+\frac{n_3}{d_1}\frac{1}{[C]}+\frac{n_5}{d_1}$	$\frac{n_2}{d_1}$

Mechanism 7

X	α	β
A	$\frac{n_1}{d_1}\frac{1}{[D]}+\frac{n_2}{d_1}\frac{1}{[C]}+\frac{n_3}{d_1}\frac{1}{[B]}+\frac{n_5}{d_1}$	$\frac{n_4}{d_1}$
B	$\frac{n_1}{d_1}\frac{1}{[D]}+\frac{n_2}{d_1}\frac{1}{[C]}+\frac{n_4}{d_1}\frac{1}{[A]}+\frac{n_5}{d_1}$	$\frac{n_3}{d_1}$
C	$\frac{n_1}{d_1}\frac{1}{[D]}+\frac{n_3}{d_1}\frac{1}{[B]}+\frac{n_4}{d_1}\frac{1}{[A]}+\frac{n_5}{d_1}$	$\frac{n_2}{d_1}$
D	$\frac{n_2}{d_1}\frac{1}{[C]}+\frac{n_3}{d_1}\frac{1}{[B]}+\frac{n_4}{d_1}\frac{1}{[A]}+\frac{n_5}{d_1}$	$\frac{n_1}{d_1}$

Mechanism 8

X	α	β
A	$\frac{n_2}{d_1}\frac{1}{[D]}+\frac{n_3}{d_1}\frac{1}{[C]}+\frac{n_4}{d_1}\frac{1}{[B]}+\frac{n_6}{d_1}$	$\frac{n_1}{d_1}\frac{1}{[B]}+\frac{n_5}{d_1}$
B	$\frac{n_2}{d_1}\frac{1}{[D]}+\frac{n_3}{d_1}\frac{1}{[C]}+\frac{n_5}{d_1}\frac{1}{[A]}+\frac{n_6}{d_1}$	$\frac{n_1}{d_1}\frac{1}{[A]}+\frac{n_4}{d_1}$
C	$\frac{1}{[A]}\left(\frac{n_1}{d_1}\frac{1}{[B]}+\frac{n_5}{d_1}\right)+\frac{n_2}{d_1}\frac{1}{[D]}+\frac{n_4}{d_1}\frac{1}{[B]}+\frac{n_6}{d_1}$	$\frac{n_3}{d_1}$
D	$\frac{1}{[A]}\left(\frac{n_1}{d_1}\frac{1}{[B]}+\frac{n_5}{d_1}\right)+\frac{n_3}{d_1}\frac{1}{[C]}+\frac{n_4}{d_1}\frac{1}{[B]}+\frac{n_6}{d_1}$	$\frac{n_2}{d_1}$

Mechanism 9

X	α	β
A	$\frac{n_1}{d_1}\frac{1}{[B]}+\frac{n_3}{d_1}$	$\frac{n_2}{d_1}$
B	$\frac{n_2}{d_1}\frac{1}{[A]}+\frac{n_3}{d_1}$	$\frac{n_1}{d_1}$
C	$\frac{n_1}{d_1}\frac{1}{[B]}+\frac{n_2}{d_1}\frac{1}{[A]}+\frac{n_3}{d_1}$	0
D	$\frac{n_1}{d_1}\frac{1}{[B]}+\frac{n_2}{d_1}\frac{1}{[A]}+\frac{n_3}{d_1}$	0

Mechanism 10

X	α	β
A	$\frac{1}{[C]}\left(\frac{n_2}{d_1}\frac{1}{[B]}+\frac{n_4}{d_1}\right)+\frac{n_3}{d_1}\frac{1}{[D]}+\frac{n_5}{d_1}$	$\frac{n_1}{d_1}\frac{1}{[B][C]}$
B	$\frac{n_3}{d_1}\frac{1}{[D]}+\frac{n_4}{d_1}\frac{1}{[C]}+\frac{n_5}{d_1}$	$\frac{1}{[C]}\left(\frac{n_1}{d_1}\frac{1}{[A]}+\frac{n_2}{d_1}\right)$
C	$\frac{n_3}{d_1}\frac{1}{[D]}+\frac{n_5}{d_1}$	$\frac{1}{[B]}\left(\frac{n_1}{d_1}\frac{1}{[A]}+\frac{n_2}{d_1}\right)+\frac{n_4}{d_1}$
D	$\frac{1}{[C]}\left\{\frac{1}{[B]}\left(\frac{n_1}{d_1}\frac{1}{[A]}+\frac{n_2}{d_1}\right)+\frac{n_4}{d_1}\right\}+\frac{n_5}{d_1}$	$\frac{n_3}{d_1}$

Mechanism 11

X	α	β
A	$\frac{1}{[D]}\left\{\frac{1}{[C]}\left(\frac{n_2}{d_1}\frac{1}{[B]}+\frac{n_3}{d_1}\right)+\frac{n_4}{d_1}\right\}+\frac{n_5}{d_1}$	$\frac{n_1}{d_1}\frac{1}{[B][C][D]}$
B	$\frac{1}{[D]}\left(\frac{n_3}{d_1}\frac{1}{[C]}+\frac{n_4}{d_1}\right)+\frac{n_5}{d_1}$	$\frac{1}{[C]}\frac{1}{[D]}\left(\frac{n_1}{d_1}\frac{1}{[A]}+\frac{n_2}{d_1}\right)$
C	$\frac{n_4}{d_1}\frac{1}{[D]}+\frac{n_5}{d_1}$	$\frac{1}{[D]}\left\{\frac{1}{[B]}\left(\frac{n_1}{d_1}\frac{1}{[A]}+\frac{n_2}{d_1}\right)+\frac{n_3}{d_1}\right\}$
D	$\frac{n_5}{d_1}$	$\frac{1}{[C]}\left\{\frac{1}{[B]}\left(\frac{n_1}{d_1}\frac{1}{[A]}+\frac{n_2}{d_1}\right)+\frac{n_3}{d_1}\right\}+\frac{n_4}{d_1}$

Mechanism 12

X	α	β
A	$\frac{1}{[C]}\left(\frac{n_2}{d_1}\frac{1}{[B]}+\frac{n_4}{d_1}\right)+\frac{n_3}{d_1}\frac{1}{[D]}+\frac{n_5}{d_1}$	$\frac{n_1}{d_1}\frac{1}{[B][C]}$
B	$\frac{n_3}{d_1}\frac{1}{[D]}+\frac{n_4}{d_1}\frac{1}{[C]}+\frac{n_5}{d_1}$	$\frac{1}{[C]}\left(\frac{n_1}{d_1}\frac{1}{[A]}+\frac{n_2}{d_1}\right)$
C	$\frac{n_3}{d_1}\frac{1}{[D]}+\frac{n_5}{d_1}$	$\frac{1}{[B]}\left(\frac{n_1}{d_1}\frac{1}{[A]}+\frac{n_2}{d_1}\right)+\frac{n_4}{d_1}$
D	$\frac{1}{[C]}\left\{\frac{1}{[B]}\left(\frac{n_1}{d_1}\frac{1}{[A]}+\frac{n_2}{d_1}\right)+\frac{n_4}{d_1}\right\}+\frac{n_5}{d_1}$	$\frac{n_3}{d_1}$

Mechanism 13

X	α	β
A	$\frac{n_2}{d_1}\frac{1}{[D]}+\frac{n_3}{d_1}\frac{1}{[C]}+\frac{n_4}{d_1}\frac{1}{[B]}+\frac{n_5}{d_1}$	$\frac{n_1}{d_1}\frac{1}{[B]}$
B	$\frac{n_2}{d_1}\frac{1}{[D]}+\frac{n_3}{d_1}\frac{1}{[C]}+\frac{n_5}{d_1}$	$\frac{n_1}{d_1}\frac{1}{[A]}+\frac{n_4}{d_1}$
C	$\frac{1}{[B]}\left(\frac{n_1}{d_1}\frac{1}{[A]}+\frac{n_4}{d_1}\right)+\frac{n_2}{d_1}\frac{1}{[D]}+\frac{n_5}{d_1}$	$\frac{n_3}{d_1}$
D	$\frac{1}{[B]}\left(\frac{n_1}{d_1}\frac{1}{[A]}+\frac{n_4}{d_1}\right)+\frac{n_3}{d_1}\frac{1}{[C]}+\frac{n_5}{d_1}$	$\frac{n_2}{d_1}$