# Model Studies of the Sequential Random-Order Two-Substrate Enzyme Mechanism

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Some previously not recognized kinetic properties of the ternary-complex mechanism shown in Scheme 1 are illustrated by example of a number of specified models. It is shown that reciprocally bilinear empirical rate equations of the Dalziel type (eqn. (5)) can be obtained also when rapid-equilibrium conditions are not prevalent. Such non-equilibrium cases may conform to Dalziel equations of the rapid-equilibrium or the compulsory-order type, but may also show a bilinear rate behaviour which cannot be described in terms of the relationships (Table 2) previously used for interpretation of empirical Dalziel coefficients.

More general relationships are required for description of the bilinear reciprocal rate behaviour inherent in the ternary-complex mechanism, and the superiority of the asymptote relationships (6) – (9) over those listed in Table 2 is demonstrated.

The random-order ternary-complex mechanism shown in Scheme 1 has been frequently discussed in relation to kinetic studies of a large number of enzymes catalyzing two-substrate reactions. The corresponding steady-state rate equation is of the second degree [1] with respect to both substrates,[2] but has been shown to reduce to the reciprocally bilinear Dalziel equation (5) under rapid-equilibrium conditions,[2,3] or when one of the binary enzyme-substrate complexes is non-existent (the compulsory-order case).[4]



$$ (1) $$
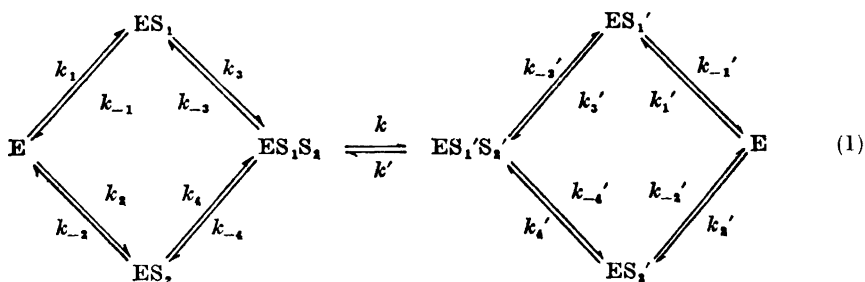
Interpretations of empirical Dalziel coefficients obtained for ternary-complex systems have usually been carried out exclusively in view of the relationships derived for the rapid-equilibrium and compulsory-order cases (Table 2). Recent investigations on the asymptotic properties of higher-degree reciprocal rate equations indicated, however, that also non-equilibrium random-order cases giving rise to an empirical rate equation of the Dalziel type might be inherent in the general ternary-complex mechanism.[5,6] The purpose of the present investigation is to establish the existence of such cases by examination of a number of specified model mechanisms based upon Scheme 1, and to describe and discuss some previously not recognized kinetic properties of the corresponding reactions.

## THEORETICAL

*Basic relationships for the ternary-complex mechanism.* The reaction mechanism given in Scheme 1 will only be considered in the forward direction, complementary relationships for the reverse reaction being obtained by insertion or deletion of primes on velocity constants and reactants.

The reciprocal rate equation in absence of products is given by [5]

$$y = \frac{z_1^2(\beta_{00}z_2^2 + \beta_{01}z_2 + \beta_{02}) + z_1(\beta_{10}z_2^2 + \beta_{11}z_2 + \beta_{12}) + \beta_{20}z_2^2 + \beta_{21}z_2}{\alpha_{11}z_1z_2 + \alpha_{12}z_1 + \alpha_{21}z_2} \tag{2}$$

where $y$ denotes the reciprocal molar enzymatic reaction velocity and $z_i = 1/[S_i]$ stands for reciprocal substrate concentrations. Coefficients $\alpha_{ij}$ and $\beta_{ij}$ are related to velocity constants in Scheme 1 as indicated in Table 1.

*Table 1.* Relationships between coefficients in eqn. (2) and velocity constants in the ternary-complex mechanism shown in Scheme 1. The table also gives numerical values (multiplied by $10^{-11}$) for coefficients in Model 2, defined in Table 3.

| General relationships | Model 2 |
|---|---|
| $\alpha_{21} = k_1k_3k_4$ | 400 000 |
| $\alpha_{12} = k_2k_3k_4$ | 400 000 |
| $\alpha_{11} = k_1k_{-2}k_3 + k_{-1}k_2k_4$ | 40 000 |
| $\beta_{21} = k_1k_3k_4(A+B)$ | 400 800 |
| $\beta_{20} = k_1k_4(1 + k_{-3}A)$ | 4 002 |
| $\beta_{12} = k_2k_3k_4(A+B)$ | 400 800 |
| $\beta_{11} = k_3k_4 + (k_1k_{-2}k_3 + k_{-1}k_2k_4)(A+B) + (k_1k_3k_{-4} + k_2k_{-3}k_4)A$ | 44 084 |
| $\beta_{10} = k_{-1}k_4(1 + k_{-3}A) + k_1k_{-2}(1 + k_{-3}A + k_{-4}A)$ | 80.03 |
| $\beta_{02} = k_2k_3(1 + k_{-4}A)$ | 400.2 |
| $\beta_{01} = k_{-1}k_2(1 + k_{-3}A + k_{-4}A) + k_{-2}k_3(1 + k_{-4}A)$ | 80.03 |
| $\beta_{00} = k_{-1}k_{-2}(1 + k_{-3}A + k_{-4}A)$ | 4.001 |

$$A = (k_{-3}' + k_{-4}' + k')/k(k_{-3}' + k_{-4}')$$
$$B = (k_{-1}'k_{-2}' + k_{-2}'k_{-3}' + k_{-1}'k_{-4}')/k_{-1}'k_{-2}'(k_{-3}' + k_{-4}')$$

The conditions (which will be referred to as rapid-equilibrium conditions)

$$k_{-1} \gg k_3[S_2] \tag{3}$$

$$k_{-2} \gg k_4[S_1] \tag{4}$$

imply that binary enzyme-substrate complexes are approximately in equilibrium with the free enzyme and substrates.[2,7] Dalziel has shown that under these conditions eqn. (2) reduces to the bilinear relationship

$$y_D = \phi_0 + \phi_1 z_1 + \phi_2 z_2 + \phi_{12} z_1 z_2 \tag{5}$$

where coefficients are related to velocity constants in Scheme 1 as indicated in Table 2 (the rapid-equilibrium case).

*Table 2.* Relationships between Dalziel coefficients and velocity constants in Scheme 1 in the rapid-equilibrium and compulsory-order cases. The notations $R = 1/(k_{-3} + k_{-4})$ and $K_i = k_{-i}/k_i$ are used. $A$ and $B$ are defined in Table 1. In the compulsory-order case we have $k_{-4} = k_{-4}' = 0$.

| Coefficient | Rapid-equilibrium case | Compulsory-order case |
|---|---|---|
| $\phi_0$ | $A + B$ | $A + B$ |
| $\phi_1$ | $K_4(A + R)$ | $1/k_1$ |
| $\phi_2$ | $K_3(A + R)$ | $K_3(A + R)$ |
| $\phi_{12}$ | $K_1 K_3(A + R)$ | $K_1 K_3(A + R)$ |

Eqn. (5) is also known to be obtained for a compulsory-order mechanism,[4] in which only one of the two possible routes for formation of the ternary enzyme-substrate complex from free enzyme is available. Relationships for Dalziel coefficients obtained in the compulsory-order case under the assumption that $ES_2$ and $ES'_2$ are non-existent are given in Table 2.

It follows from the asymptotic properties of higher-degree reciprocal rate equations [6] that eqn. (2) may approach and, from a statistical point of view, be experimentally indistinguishable from a bilinear relationship of the Dalziel type without fulfilling requirements of mathematical identity (*cf.* data given in Table 4). Empirical Dalziel coefficients for such asymptote cases are related to coefficients in eqn. (2) through [5]

$$\phi_{12} = \beta_{00}/\alpha_{11} \tag{6}$$

$$\phi_1 = (\beta_{01} - \alpha_{12} a_{12})/\alpha_{11} \tag{7}$$

$$\phi_2 = (\beta_{10} - \alpha_{21} a_{12})/\alpha_{11} \tag{8}$$

$$\phi_0 = (\beta_{11} - \alpha_{21}\phi_1 - \alpha_{12}\phi_2)/\alpha_{11} \tag{9}$$

Even though there are two possible routes for ternary-complex formation in the generalized Scheme 1, it might happen that one of the routes predominates at certain combinations of substrate concentrations, making the reaction effectively ordered. The quotient $Q$ between the reaction flow *via* $ES_1$ and the flow *via* $ES_2$ is in absence of products given by [5]

$$Q = \frac{k_1 k_3 (k_{-2} + k_4 [S_1])}{k_2 k_4 (k_{-1} + k_3 [S_2])} \tag{10}$$

For an effectively compulsory-order mechanism in which $ES_2$ does not contribute significantly to the reaction rate we have $Q \gg 1$ over the entire range of substrate concentrations used, while a mechanism where $Q = 1$ for at least one combination of substrate concentrations within the range tested will be considered as effectively random-order.

*Restrictions imposed on model mechanisms.* Basic data for the model mechanisms considered in the present investigation are given in Table 3. Substrate

*Table 3.* Choice of velocity constants in Scheme 1 for model mechanisms considered in the present investigation. The restrictions $A = 0.002$, $B = 1$ and $k = 1000$ have been imposed on all models. The theoretical rate eqn. (2) for each model conforms within the indicated maximum relative error to a Dalziel equation defined by eqns. $(5) - (9)$, and within 0.5 % to eqn. (11). The empirical rate behaviour is thus identical (within the usual experimental precision) for all models, and is illustrated in Figs. 1 and 2.

| | Model number | | | | | |
| --- | --- | --- | --- | --- | --- | --- |
| | 1 | 2 | 3 | 4 | 5 | 6 |
| $k_1$ | $10^5$ | $10^5$ | $10^9$ | $10^2$ | $5 \times 10$ | $2 \times 10^2$ |
| $k_2$ | $10^5$ | $5 \times 10^3$ | $10^3$ | $3 \times 10^{-1}$ | $5 \times 10$ | $2 \times 10^{-3}$ |
| $k_3$ | $2 \times 10^5$ | $2 \times 10^8$ | $10^9$ | $3 \times 10^2$ | $10^2$ | $2 \times 10^4$ |
| $k_4$ | $2 \times 10^5$ | $2 \times 10^5$ | $10^3$ | $10^2$ | $10^2$ | $2 \times 10^2$ |
| $k_{-1}$ | $10^4$ | $10^4$ | $10^8$ | $10$ | $10$ | $2 \times 10$ |
| $k_{-2}$ | $10^4$ | $5 \times 10^2$ | $10^2$ | $10$ | $10$ | $2 \times 10$ |
| $k_{-3}$ | $10^6$ | $10^9$ | $5 \times 10^9$ | $10^3$ | $10^9$ | $5 \times 10^9$ |
| $k_{-4}$ | $10^6$ | $10^6$ | $5 \times 10^3$ | $10^0$ | $10^0$ | $5 \times 10^2$ |
| Maximum error | 0 | $2 \times 10^{-4}$ | $2 \times 10^{-3}$ | $3 \times 10^{-5}$ | 0 | $10^{-7}$ |

concentrations and velocity constants have not been given any dimensions, but any consistent set of dimensions will do. The thermodynamic identity $k_1 k_{-2} k_3 k_{-4} = k_{-1} k_2 k_{-3} k_4$ is valid for all of the models and, for simplicity, concentrations of both substrates are assumed to be varied over identical ranges (1 to 0.01).

Models 1 and 5 give theoretical rate equations that are identical with the Dalziel eqn. (5). Other models have been selected under the restriction $(y - y_D)/y < 0.2$ % over the postulated range of substrate concentrations, where $y$ is given by eqn. (2) and $y_D$ by eqns. $(5) - (9)$. This means that the theoretical rate equation for all models is experimentally indistinguishable (within the usual precision of steady-state kinetic data) from the bilinear asymptotic Dalziel equation.

The models in Table 3 are used to describe characteristics of Dalziel equations for the forward reaction, and relative magnitudes of velocity constants with primes have not been explicitly stated. Such constants only affect magnitudes of the quantities $A$ and $B$ defined in Table 1, and for simplicity the restrictions $k = 10^3$, $k'/(k_{-3}' + k_{-4}') = 1$ (whence $A = 0.002$) and $B = 1$ have been imposed on all models. It then follows that $\phi_0 \approx B = 1$, *i.e.* some step of

product dissociation from the enzyme is assumed to be rate-limiting. Interesting properties of the models are not directly related to these restrictions. Models showing the same kinetic properties can easily be selected when $B$ or $k'/(k_{-3}' + k_{-4}')$ are chosen large or small in comparison to unity, and when $A > B$ (indicating that interconversion of ternary complexes is assumed to be rate-limiting).

In order to ascertain that none of the four terms in the Dalziel equation is negligibly small over the entire range of substrate concentrations considered, velocity constants have been chosen to satisfy the relationships $\phi_1 \approx \phi_2 = 0.01\phi_0$ and $\phi_{12} = 0.001\phi_0$. This means that all models approximately conform to the empirical Dalziel equation

$$y = 1 + 0.01z_1 + 0.01z_2 + 0.001z_1z_2 \tag{11}$$

Differences between reaction velocities calculated from eqns. (2) and (11), respectively, are actually less than 0.5 %, for which reason Lineweaver-Burk plots and replots of slopes and intercepts will be closely similar to (practically indistinguishable from) those shown in Figs. 1 and 2 for all of the models.
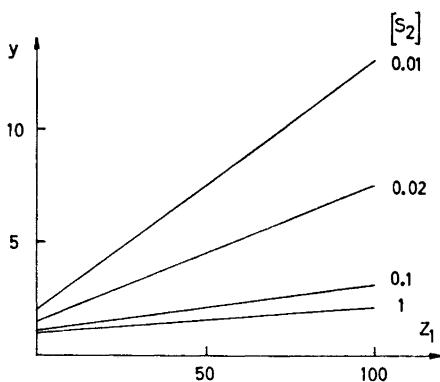


*Fig. 1.* Lineweaver-Burk plots with respect to $S_1$ at different constant concentrations of $S_2$, calculated from eqn. (11) which within a maximum error of 0.5 % describes the rate behaviour of all model mechanisms defined in Table 3.
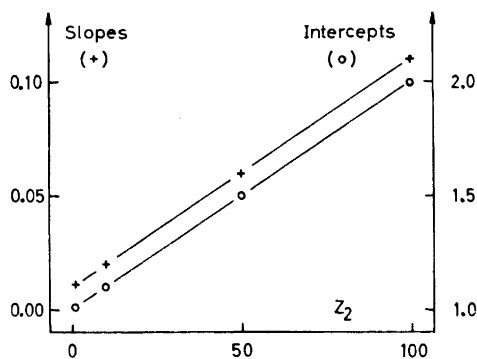
*Fig. 2.* Replots of slopes and intercepts of the Lineweaver-Burk plots in Fig. 1, as a function of $z_2 = 1/[S_2]$.

## RESULTS

In Model 1 magnitudes of velocity constants in the ternary-complex mechanism (Scheme 1) have been symmetrically chosen in order to ascertain that both routes for ternary-complex formation are utilized; since $k_1 = k_2$, $k_3 = k_4$, and $k_{-1} = k_{-2}$ the flow ratio $Q$ defined by eqn. (10) equals unity when substrate concentrations are of equal magnitude, and the mechanism is effectively random-order. Calculated values of coefficients in eqn. (2) for Model 1 are listed in Table 1, and it can easily be shown that the theoretical rate equation is mathematically identical with its asymptotic Dalziel equation

$$y = 1.002 + 0.010005z_1 + 0.010005z_2 + 0.00100025z_1z_2$$

It has been generally accepted that in effectively random-order cases of the ternary-complex mechanism the second-degree eqn. (2) only reduces to a bilinear Dalziel equation under rapid-equilibrium conditions,[8-10] and calculation of the rapid-equilibrium equation for Model 1, using the relationships given in Table 2, seems to support this idea in giving

$$y_{r.e.} = 1.002 + 0.0100025z_1 + 0.0100025z_2 + 0.00100025z_1z_2$$

Deviations from the theoretical rate equation are evidently insignificant, being zero in $\phi_0$ and $\phi_{12}$ and less than 0.03 % in $\phi_1$ and $\phi_2$. For comparison, it may be observed that the compulsory-order relationships give $\phi_1 = 10^{-5}$, which is erroneous by three orders of magnitude.

Despite the fact that Model 1 is effectively random-order and conforms to a Dalziel equation of the rapid-equilibrium type, the model does not operate under rapid-equilibrium conditions (3) and (4). Both $k_3[S_2]$ and $k_4[S_1]$ can even be larger than $k_{-1}$ and $k_{-2}$, respectively; the former quantities vary between $2 \times 10^3$ and $2 \times 10^5$, while $k_{-1} = k_{-2} = 10^4$. It may be concluded that rapid-equilibrium conditions are required neither for linearization of eqn. (2), nor for obtaining a rapid-equilibrium type of Dalziel equation. Examination of Table 3, in fact, shows that $k_3 > k_{-1}$ and $k_4 > k_{-2}$ for all of the models considered in the present investigation, i.e. all models represent non-equilibrium cases over the postulated ranges of substrate concentrations.

Non-equilibrium mechanisms conforming to a rapid-equilibrium type of rate equation must not necessarily be symmetrical to become effectively random-order. This is shown by Model 2 where the flow ratio approximately equals unity when, for instance, $[S_1] = 0.02$ and $[S_2] = 0.4$. The theoretical reciprocal rate equation for Model 2 is non-linear with respect to both $z_1$ and $z_2$ but, as shown in Table 4, deviations from the Dalziel equation calculated using the rapid-equilibrium relationships in Table 2 (or using the more precise asymptote relationships (6) − (9)) are insignificant on the 0.1 % level of precision.

Table 4. Theoretical reciprocal reaction velocity ($y$; calculated from eqn. (2)) for Model 2 as a function of reciprocal substrate concentrations. Reaction velocities obtained using the asymptote Dalziel equation ($y_{as}$; calculated from eqns. (5) − (9)) and the rapid-equilibrium Dalziel equation ($y_{r.e.}$; calculated using the relationships in Table 2) are shown for comparison.

| $z_1$ | $z_2$ | $y$ | $y_{as}$ | $y_{r.e.}$ |
|---|---|---|---|---|
| 1 | 1 | 1.02301 | 1.02301 | 1.02300 |
| 1 | 10 | 1.12201 | 1.12201 | 1.12200 |
| 1 | 100 | 2.11201 | 2.11201 | 2.11200 |
| 10 | 1 | 1.12209 | 1.12210 | 1.12200 |
| 10 | 10 | 1.30210 | 1.30210 | 1.30200 |
| 10 | 100 | 3.10210 | 3.10210 | 3.10200 |
| 100 | 1 | 2.11260 | 2.11300 | 2.11200 |
| 100 | 10 | 3.10286 | 3.10300 | 3.10200 |
| 100 | 100 | 13.00299 | 13.00300 | 13.00201 |

In Model 3, the flow ratio $Q$ varies between $10^5$ and $10^7$, being equal to $10^6$ when substrate concentrations are of equal magnitude. This means that the reaction flow *via* $ES_2$ is completely negligible over the considered range of substrate concentration, and Model 3 represents an effectively compulsory-order non-equilibrium mechanism. Nevertheless, the rate behaviour can be excellently described by (and will be experimentally indistinguishable from) a Dalziel equation of the rapid-equilibrium type. For example, the rapid-equilibrium value of $\phi_1$ agrees within $10^{-5}$ % with the value calculated using the asymptote relationships ($\phi_1 \approx 10^{-2}$), while the compulsory-order value is of an entirely different order of magnitude ($\phi_1 = 10^{-9}$).

Non-equilibrium mechanisms can also give rise to a Dalziel rate equation of the compulsory-order type, where $\phi_1$ is given by $1/k_1$ instead of by $K_4(A+R)$ (see Table 2). Model 4 illustrates this fact in giving a rate equation which deviates less than 0.003 % from the asymptotic Dalziel equation, and where Dalziel coefficients for the asymptote deviate less than 0.3 % from the values predicted by the compulsory-order relationships. In particular, we have $\phi_1 \approx 1/k_1 = 10^{-2} \gg K_4(A+R) \approx 3 \times 10^{-5}$.

Models $1-4$ conform either to a rapid-equilibrium or to a compulsory-order type of Dalziel equation, *i.e.* interpretations of Dalziel coefficients for these models can be carried out in terms of previously described relationships (Table 2). Model 5, however, gives a rate equation which is mathematically identical with its bilinear asymptote

$$y = 1.002 + 0.01002z_1 + 0.01002z_2 + 0.001004z_1z_2$$

while the corresponding rapid-equilibrium and compulsory-order Dalziel equations become

$$y_{\text{r.e.}} = 1.002 + 0.00502z_1 + 0.00502z_2 + 0.001004z_1z_2$$

$$y_{\text{c.o.}} = 1.002 + 0.02z_1 + 0.00502z_2 + 0.001004z_1z_2$$

As shown in Fig. 3, neither of the latter relationships can be reliably used for description and interpretation of the rate behaviour of Model 5. Deviations in $\phi_1$ and $\phi_2$ are most significant, both from a theoretical and from a practical point of view.
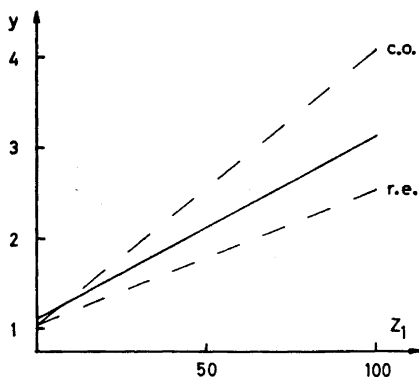


*Fig. 3.* Lineweaver-Burk plot with respect to $S_1$ at $[S_2] = 0.1$ for Model 5. The full line represents the theoretical rate behaviour according to eqn. (2), while dashed lines have been calculated using the rapid-equilibrium (r.e.) and compulsory-order (c.o.) relationships given in Table 2.

Calculation of the flow ratio shows that Model 5 represents an effectively random-order mechanism ($Q = 1$ when $[S_1] = [S_2]$). Model 6, on the other hand, is effectively compulsory-order ($Q > 10^3$) and conforms within $10^{-5}$ % to a Dalziel equation where coefficients are given by the asymptote relationships (6)−(9), $i.e.$ where $\phi_1 \approx 0.01$. Also in this case rapid-equilibrium and compulsory-order relationships are inadequate for interpretation of empirical Dalziel coefficients in predicting that $\phi_1 \approx 0.005$.

## DISCUSSION

It has been generally accepted that enzymes operating by the ternary-complex mechanism shown in Scheme 1 only will conform to a bilinear Dalziel equation under rapid-equilibrium conditions, or when there is an obligatory order of combination of substrates to the enzyme. The results described above, however, establish that an empirical rate equation of the Dalziel type can be obtained also when substrate concentrations are too high to fulfill rapid-equilibrium requirements, and irrespective of whether the reaction is effectively ordered or not. Under restrictive conditions the rate equation may even become mathematically identical with a Dalziel equation (Models 1 and 5), but of more general interest are the models illustrating cases where deviations from a bilinear reciprocal rate behaviour exist but are too small to be experimentally detected, $i.e.$ where the theoretical reciprocal rate equation can be approximated by its asymptotic Dalziel equation.

Such non-equilibrium cases cannot be considered as being more restrictive than, for instance, rapid-equilibrium cases. It may be observed that a number of "unnecessary" restrictions have been imposed on the models described in the present investigation (mainly restrictions concerning the magnitude of $A$, $B$, $\phi_0$, and $k$, but also restrictions for the range of substrate concentrations considered and the relative magnitude of Dalziel coefficients), and models showing the same basic kinetic properties can easily be constructed under the same or less restrictive conditions using other combinations of velocity constants. Non-equilibrium cases might, consequently, be equally frequent as rapid-equilibrium cases, and should be considered in the diagnostic interpretation of empirical Dalziel equations obtained in ternary-complex systems.

As was mentioned in the introduction, such interpretations have usually been carried out exclusively in view of the relationships listed in Table 2. The diagnostic value of such an approach is, obviously, dependent upon whether or not these relationships can be applied to all possible bilinear cases (including non-equilibrium cases) inherent in the general ternary-complex mechanism. Models 1−3 examplify that non-equilibrium mechanisms may give rise to Dalziel equations of the rapid-equilibrium type, and Model 4 shows that a compulsory-order equation can also be obtained. Models 5 and 6, however, represent mechanisms where both rapid-equilibrium and compulsory-order relationships fail to give an adequate description of the enzymatic rate behaviour, errors in predicted magnitudes of $\phi_1$ and $\phi_2$ being in the order of 100 %. For these reasons, it may be concluded that the relationships in

Table 2 are insufficient for interpretation of Dalziel coefficients in the general case. Evaluation and interpretation of Dalziel equations obtained for enzyme systems operating by a ternary-complex mechanism cannot be reliably carried out exclusively in view of the well-known relationships (Table 2) derived for the rapid-equilibrium and compulsory-order cases.

The asymptote relationships (6) − (9), on the other hand, fulfill requirements of general applicability as they have been shown to include any (exact or approximate) bilinear rate behaviour inherent in the ternary-complex mechanism.[5] Models 5 and 6, and to some extent Model 1 and the results listed in Table 4, illustrate the superiority of the asymptote relationships over those listed in Table 2.

In view of the biochemical importance of ternary-complex mechanisms, it is of great interest to obtain detailed and conclusive information about the conditions under which the general rate equation (1) becomes indistinguishable from those obtained for the rapid-equilibrium and the compulsory-order cases. As pointed out by Cleland,[11] this is a formidable task, but the results described in the present investigation further emphasize the importance of the problem and the need for a more general analysis of relationships between Dalziel coefficients and velocity constants in Scheme 1. These problems can probably be tackled by examination of the asymptote eqns. (6) − (9), and such work is in progress.

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