Kinetic Characteristics of the Sequential Random Order

# Two-substrate Enzyme Mechanism

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The steady-state rate behaviour of random order two-substrate enzyme systems has been investigated and classified with respect to curve shapes obtained in Lineweaver-Burk plots. Relationships between curve shapes, rate constants, and substrate concentrations are examined, and it is shown that exhibition of linear reciprocal rate plots cannot be generally related to whether the mechanism is effectively ordered or not. Linear relationships are always obtained asymptotically at comparatively low substrate concentrations, and a general rate equation is derived for this asymptote case which, in fact, includes all cases of linear reciprocal rate behaviour inherent in the random order two-substrate mechanism.

Most enzymes catalyze bimolecular reactions.[1] For the corresponding two-substrate enzymatic reactions there are two general types of mechanism, the ping-pong mechanism where a product is released before both substrates have reacted with the enzyme, and the ternary complex (sequential) mechanism where the enzyme combines with both substrates before any products are formed.[2,3]

A main problem in the study of enzymes operating by sequential mechanisms has been to determine whether substrates add in compulsory or in random order to the enzyme. Using the notations of Dalziel [4] a random mechanism can be written as
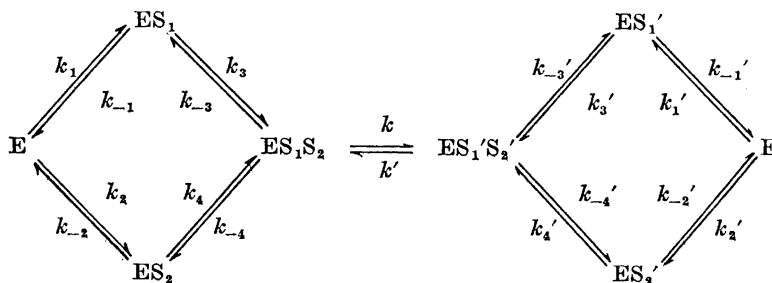
In a compulsory order mechanism ternary complexes are formed exclusively by one of the alternate pathways indicated in scheme (1).

Steady-state initial rate data are most commonly analyzed by means of reciprocal rate plots (Lineweaver-Burk plots).[5] Compulsory order mechanisms give rate equations of the usual hyperbolic (Briggs-Haldane [6]) type, and reciprocal plots are linear.[3] In the random order mechanism the steady-state reaction velocity is a more complex function of substrate concentrations. Dalziel has pointed out that both cases of substrate activation and inhibition are inherent in this mechanism which, in general, must be assumed to yield non-linear reciprocal rate plots (see eqn. (9) below).[4] It has, in fact, often been stated in the literature that random order mechanisms (with exception for the rapid equilibrium case [4]) always yield non-linear Lineweaver-Burk plots, and a large number of two-substrate enzyme systems have been claimed to operate by a compulsory order or a rapid equilibrium mechanism merely because they obey a Briggs-Haldane type of rate equation.[2,7]

The purpose of the present investigation is to determine the general kinetic characteristics of the random order mechanism (1) as they are manifested in Lineweaver-Burk plots, and particularly to define conditions under which linear relationships are obtained.

CHARACTERIZATION OF RECIPROCAL PLOTS FOR THE RANDOM ORDER MECHANISM

The reaction in mechanism (1) will only be considered in the forward direction, rate expressions for the reverse reaction being obtained by insertion or deletion of primes on rate constants and reactants. The steady-state rate equation (in absence of products) for mechanism (1) with respect to the first substrate S_1 is given by [4]

$$\frac{v}{c_E} = \frac{\alpha_2[S_1]^2 + \alpha_1[S_1]}{\beta_2[S_1]^2 + \beta_1[S_1] + \beta_0} \tag{2}$$

where v stands for the steady-state reaction velocity and c_E for the total concentration of enzyme. The coefficients α_i and β_i are functions of the concentration of the second substrate S_2

$$\alpha_2 = \alpha_{21}[S_2] \tag{3}$$

$$\alpha_1 = \alpha_{12}[S_2]^2 + \alpha_{11}[S_2] \tag{4}$$

$$\beta_2 = \beta_{21}[S_2] + \beta_{20} \tag{5}$$

$$\beta_1 = \beta_{12}[S_2]^2 + \beta_{11}[S_2] + \beta_{10} \tag{6}$$

$$\beta_0 = \beta_{02}[S_2]^2 + \beta_{01}[S_2] + \beta_{00} \tag{7}$$

The coefficients α_{ij} and β_{ij} are all positive and can be expressed in terms of rate constants in mechanism (1) as shown in Table 1. Thermodynamically, rate constants must satisfy the condition:

$$k_1 k_{-2} k_3 k_{-4} = k_{-1} k_2 k_{-3} k_4 \tag{8}$$

Table 1.

$\alpha_{21} = k_1 k_3 k_4$

$\alpha_{12} = k_2 k_3 k_4$

$\alpha_{11} = k_1 k_{-2} k_3 + k_{-1} k_2 k_4$

$\beta_{21} = k_1 k_3 k_4 (A+B)$

$\beta_{20} = k_1 k_4 (1 + k_{-3} A)$

$\beta_{12} = k_2 k_3 k_4 (A+B)$

$\beta_{11} = k_3 k_4 + (k_1 k_{-2} k_3 + k_{-1} k_2 k_4)(A+B) + (k_1 k_3 k_{-4} + k_2 k_{-3} k_4) A$

$\beta_{10} = k_{-1} k_4 (1 + k_{-3} A) + k_1 k_{-2}(1 + k_{-3} A + k_{-4} A)$

$\beta_{02} = k_2 k_3 (1 + k_{-4} A)$

$\beta_{01} = k_{-1} k_2 (1 + k_{-3} A + k_{-4} A) + k_{-2} k_3 (1 + k_{-4} A)$

$\beta_{00} = k_{-1} k_{-2}(1 + k_{-3} A + k_{-4} A)$

$A = (k'_{-3} + k'_{-4} + k')/k(k'_{-3} + k'_{-4})$

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

Eqn. (2) can be recast in the reciprocal form

$$\frac{c_E}{v} = \frac{\beta_2 + \beta_1 [S_1]^{-1} + \beta_0 [S_1]^{-2}}{\alpha_2 + \alpha_1 [S_1]^{-1}} \qquad (9)$$

and it can be seen that c_E/v in general is a non-linear function of $1/[S_1]$. In order to study the shape of the curves described by eqn. (9) we introduce the notations $y = c_E/v$ and $x = 1/[S_1]$, when eqn. (9) transforms into

$$y(x) = \frac{\beta_2 + \beta_1 x + \beta_0 x^2}{\alpha_2 + \alpha_1 x} \qquad (10)$$

which may be written as

$$y(x) = \frac{\beta_1}{\alpha_1} - \frac{\alpha_2 \beta_0}{\alpha_1^2} + \frac{\beta_0}{\alpha_1} x + F(x) \qquad (11)$$

where

$$F(x) = \frac{\alpha_1^2 \beta_2 + \alpha_2^2 \beta_0 - \alpha_1 \alpha_2 \beta_1}{\alpha_1^2 (\alpha_2 + \alpha_1 x)} \qquad (12)$$

or

$$F(x) = \frac{\alpha_2 F(0)}{\alpha_2 + \alpha_1 x} \qquad (13)$$

$F(x)$ obviously approaches zero as x approaches infinity, and hence it follows from eqn. (11) that $y(x)$ has a linear asymptote given by

$$y_{as}(x) = \frac{\beta_1}{\alpha_1} - \frac{\alpha_2 \beta_0}{\alpha_1^2} + \frac{\beta_0}{\alpha_1} x \qquad (14)$$

$F(x)$ represents the difference between $y(x)$ and its linear asymptote, and is thus closely related to the curvature of the function $y(x)$; it may be observed that differentiation of eqn. (11) with respect to x yields

$$y'(x) = \frac{\beta_0}{\alpha_1} + F'(x) = \frac{\beta_0}{\alpha_1} - \frac{\alpha_1 \alpha_2 F(0)}{(\alpha_2 + \alpha_1 x)^2} \qquad (15)$$

$$y''(x) = F''(x) = \frac{2\alpha_1{}^2\alpha_2 F(0)}{(\alpha_2+\alpha_1 x)^3} \tag{16}$$

Examination of eqns. (11)—(13) shows that $y(x)$ steadily approaches its linear asymptote without intersecting it, and the reciprocal plots described by eqn. (10) may be classified in three main groups with respect to the sign of $F(0)$. When $F(0)<0$ the curves are concave down (eqn. (16)) and approach the asymptote from below (type I). When $F(0)=0$ we have $F(x)=0$ according to eqn. (13), and $y(x)$ becomes identical with its linear asymptote (type II). When $F(0)>0$, finally, the curves are concave up and approach the asymptote from above, and for such type III curves we may distinguish between two cases depending on whether $y'(0)>0$ (IIIa) or $y'(0)<0$ (IIIb). In the latter case $y'(x)$ must become zero at some positive value of x as the asymptotic value of $y'(x)$ is positive (eqn. (15)), and $y(x)$ exhibits a minimum point. Defining M as

$$M = \frac{\beta_2}{\alpha_2} - \frac{\beta_1}{\alpha_1} \tag{17}$$

it follows from eqn. (12) that

$$F(0) = M + \frac{\alpha_2\beta_0}{\alpha_1{}^2} \tag{18}$$

and eqn. (15) becomes

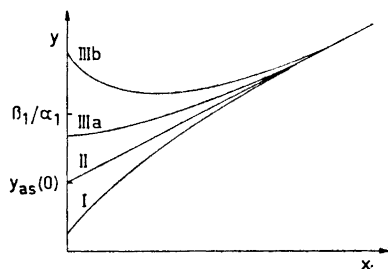$$y'(x) = \frac{\alpha_1\beta_0 x^2+2\alpha_2\beta_0 x-\alpha_1\alpha_2 M}{(\alpha_2+\alpha_1 x)^2} \tag{19}$$

Fig. 1. Classification of reciprocal rate curves with respect to the magnitude of $y(0)$ in relation to $y_{as}(0)$ and β_1/α_1.
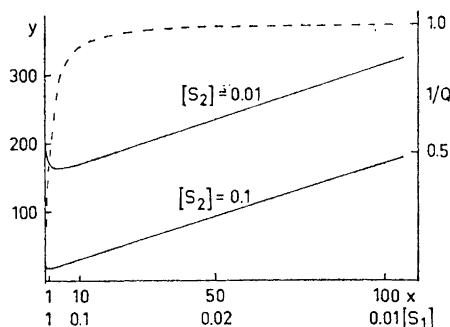

Fig. 2. Solid curves show reciprocal rate plots for a hypothetical enzyme obtained by putting all rate constants in mechanism (1) equal to unity with exception for $k_1=k_2=10^3$. Rate constants and substrate concentrations have not been given any dimensions, but any consistent set of dimensions will be acceptable. The dashed curve shows the variation of the flow ratio Q with $[S_1]$ at $[S_2]=0.01$.

Hence it follows that $M>0$ is a necessary and sufficient condition for type IIIb-kinetics, the minimum value of $y(x)$ being obtained for

$$x = \sqrt{\frac{\alpha_2^2}{\alpha_2^1} + \frac{\beta_2}{\beta_0} - \frac{\alpha_2\beta_1}{\alpha_1\beta_0}} - \frac{\alpha_2}{\alpha_1} \tag{20}$$

Typical shapes of the different curve types are indicated in Fig. 1. A type IIIb curve is also shown in Fig. 2.

RELATIONSHIPS BETWEEN CURVE SHAPES AND RATE CONSTANTS

Evaluation of M and $F(0)$ using eqns. (3)—(7) gives

$$M = \frac{m_0 + m_1[S_2]}{\alpha_{21}[S_2](\alpha_{11} + \alpha_{12}[S_2])} \tag{21}$$

$$F(0) = \frac{f_0 + f_1[S_2] + f_2[S_2]^2}{\alpha_{21}[S_2](\alpha_{11} + \alpha_{12}[S_2])^2} \tag{22}$$

where

$$
\begin{aligned}
m_0 &= \alpha_{11}\beta_{20} - \alpha_{21}\beta_{10} \\
m_1 &= \alpha_{11}\beta_{21} + \alpha_{12}\beta_{20} - \alpha_{21}\beta_{11} \\
f_0 &= \alpha_{21}^2\beta_{00} + \alpha_{11}m_0 \\
f_1 &= \alpha_{21}^2\beta_{01} + \alpha_{11}m_1 + \alpha_{12}m_0 \\
f_2 &= \alpha_{21}^2\beta_{02} + \alpha_{12}m_1
\end{aligned}
$$

Using eqn. (8) and the expressions and notations given in Table 1 one obtains

$$m_0 = k_1 k_{-1} k_4^2 \ (k_2 - k_3 - k_3 k_{-3}A) \tag{23}$$

$$m_1 = k_1 k_3 k_4 \ (k_4(k_2 - k_3) - k_1 k_3 k_{-4}A) \tag{24}$$

$$f_0 = k_1 k_{-1} k_2 k_4^2 \ (k_{-1} k_4(k_2 - k_3) + k_1 k_{-2} k_3) \tag{25}$$

$$f_2 = k_1 k_2 k_3^2 k_4^2 \ (k_4(k_2 - k_3) + k_1 k_3) \tag{26}$$

$$f_1 = k_{-1}f_2/k_3 + k_3 f_0/k_{-1} \tag{27}$$

Factorisation of eqn. (22) using (27) yields

$$F(0) = \frac{(k_3[S_2] + k_{-1})(k_{-1}f_2[S_2] + k_3 f_0)}{k_{-1}k_3\alpha_{21}[S_2](\alpha_{11} + \alpha_{12}[S_2])^2} \tag{28}$$

and for classification of curves we have

$$\text{sign } (M) = \text{sign } (m_0 + m_1[S_2]) \tag{29}$$

$$\text{sign } (F(0)) = \text{sign } (k_{-1}f_2[S_2] + k_3 f_0) \tag{30}$$

Only the following relationships between curve shapes and rate constants are obvious and general:

a. Exhibition of type I kinetics ($F(0) < 0$) implies that $k_3 > k_2$; it follows from eqns. (25), (26), and (30) that f_0, f_2, and $F(0)$ are positive when $k_3 \leq k_2$.

b. Exhibition of type IIIb kinetics ($M > 0$) implies that $k_3 < k_2$; it follows from eqns. (23), (24), and (29) that m_0, m_1, and M are negative when $k_3 \geq k_2$.

RELATIONSHIPS BETWEEN CURVE SHAPES AND THE CONCENTRATION OF THE SECOND SUBSTRATE

It follows from eqns. (29) and (30) that the signs of M and $F(0)$ may vary with $[S_2]$, which means that different types of reciprocal plots with respect to one substrate may be obtained on variation of the concentration of the second substrate. The conditions leading to such transformations of the curve type are listed in Table 2. These conditions, being given in terms of the signs of m_0, m_1, f_0, and f_1, can readily be evaluated in terms of relationships between rate constants using eqns. (23)—(26). It may be noted that linear type II kinetics are obtained at one specific concentration of S_2, only, the concentration for which transformation between type I and IIIa kinetics takes place.

Table 2. Dependence of curve shapes on the concentration of the second substrate. Transformations between type IIIa and IIIb kinetics take place for $[S_2] = -m_0/m_1$, and between type I and IIIa kinetics for $[S_2] = -k_3 f_0/k_{-1} f_2$.

	Sign of			Change of curve type
m_0	m_1	f_0	f_2	as $[S_2]$ increases
+	+	+	+	IIIb (no change)
+	−	+	+	IIIb → IIIa
−	+	+	+	IIIa → IIIb
−	−	+	+	IIIa (no change)
−	−	+	−	IIIa → II → I
−	−	−	+	I → II → IIIa
−	−	−	−	I (no change)

The results listed in Table 2 are of great theoretical interest, since they demonstrate the complex kinetic behaviour which may be shown by enzyme systems operating by the random order mechanism (1). Not only are cases of apparent substrate activation and substrate inhibition inherent in rate eqn. (2), but also cases where the kinetic behaviour changes between apparent substrate activation and inhibition when the concentration of the second substrate is varied.

Examination of eqns. (12) and (28) shows that $F(x)$ approaches zero for large values of $[S_2]$ as well as of x. Non-linearity in reciprocal plots with respect to one substrate will, therefore, in general be most easily demonstrated using high concentrations of this substrate (x small) and low concentrations of the second substrate. Reciprocal plots will always tend to be linear when the second substrate is present in "saturating" amounts. This can be illustrated by example of the "model enzyme" described in Fig. 2, which gives significantly curved reciprocal plots with respect to S_1 when $[S_2]$ equals 0.01, but apparently linear plots when $[S_2]$ is increased to 0.1.

RELATIONSHIPS BETWEEN CURVE SHAPES AND UTILIZATION OF ALTERNATE PATHWAYS

The quotient Q between the reaction flow *via* ES_1 and the flow *via* ES_2 in mechanism (1) is given by

$$Q = \frac{k_1[E][S_1] - k_{-1}[ES_1]}{k_2[E][S_2] - k_{-2}[ES_2]} \tag{31}$$

whence for the steady-state solution [7,8]

$$Q = \frac{k_1 k_3 (k_{-2} + k_4[S_1])}{k_2 k_4 (k_{-1} + k_3[S_2])} \tag{32}$$

It can be seen that Q approaches zero for large values of $[S_2]$, and the exhibition of linear reciprocal plots with respect to S_1 at high concentrations of S_2 appears to be a consequence of the fact that the mechanism becomes effectively ordered, reaction taking place almost exclusively by the pathway involving ES_2.

Such a correspondence between linear reciprocal plots and a preferred pathway is, however, not general. As was shown above, linear type II kinetics are obtained for $[S_2] = -k_3 f_0 / k_{-1} f_2$. Substituting this into eqn. (32) and rearranging using eqns. (25) and (26) we get

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

Since no restrictions have been imposed on the rate constants in the type II case, Q may attain any value including unity. This means that linear reciprocal rate equations may be obtained also when both pathways to the ternary complex $ES_1 S_2$ are equally favoured.

Similar results are obtained on examination of the asymptotically linear part of reciprocal plots and can, for instance, be illustrated by the above model enzyme. The dashed curve in Fig. 2 shows the variation of $1/Q$ with x for $[S_2] = 0.01$. It can be seen that the reaction is perfectly random ($Q \approx 1$) under conditions giving rise to the linear part of the $y(x)$-curve, while the reaction becomes effectively ordered ($1/Q \approx 0$) at high concentrations of S_1, where deviations from the linear kinetics characteristic of an ordered mechanism are as largest. The conclusion must be that there are no general relationships between curve shapes and the flow ratio Q in a random order mechanism.

REDUCTION OF THE GENERAL RATE EQUATION AT COMPARATIVELY LOW SUBSTRATE CONCENTRATIONS

The possibility of detecting curvature in reciprocal rate plots is dependent upon the experimental precision; initial rate determinations are often subjected to a very pronounced statistical variation. Since the difference $F(x)$ between $y(x)$ and its linear asymptote decreases with increasing values of $x = 1/[S_1]$, there will always be a limiting value of $[S_1]$ below which curvature cannot be

experimentally detected. At such "comparatively low" substrate concentrations $y(x)$ will become indistinguishable from its linear asymptote $y_{as}(x)$ as given by eqn. (14).

Due to the symmetry of mechanism (1) y as a function of $1/[S_2]$ should similarly become linear at comparatively low concentrations of S_2. This can be demonstrated by examination of $y_{as}(x)$. The coefficient β_0/α_1 can be expressed as a function of $z = 1/[S_2]$ using eqns. (4) and (7):

$$\frac{\beta_0}{\alpha_1} = \frac{\beta_{02} + \beta_{01}z + \beta_{00}z^2}{\alpha_{12} + \alpha_{11}z} \qquad (33)$$

Eqn. (33) is exactly analogous to eqn. (10), and it follows that β_0/α_1 approaches a linear asymptote for large values of z. Comparison with eqn. (14) shows that

$$\left(\frac{\beta_0}{\alpha_1}\right)_{as} = \frac{\beta_1}{\alpha_{11}} - \frac{\alpha_{12}\beta_{00}}{\alpha_{11}^2} + \frac{\beta_{00}}{\alpha_{11}}z \qquad (34)$$

It can, similarly, be shown that $y_{as}(0)$ becomes an asymptotically linear function of z when z is large, and for comparatively low concentrations of both of the substrates (x and z large) eqn. (10) reduces to a Dalziel [4] type of rate equation:

$$y(x,z) = \phi_0 + \phi_1 x + \phi_2 z + \phi_{12}xz \qquad (35)$$

where (cf. eqn. (34))

$$\phi_{12} = \beta_{00}/\alpha_{11} \qquad (36)$$

$$\phi_1 = (\beta_{01} - \alpha_{12}\phi_{12})/\alpha_{11} \qquad (37)$$

$$\phi_2 = (\beta_{10} - \alpha_{21}\phi_{12})/\alpha_{11} \qquad (38)$$

$$\phi_0 = (\beta_{11} - \alpha_{11}\phi_1 - \alpha_{12}\phi_2)/\alpha_{11} \qquad (39)$$

Conditions justifying application of the above "low concentration assumption" used for reduction of the general reciprocal rate eqn. (10) to the linear forms (14) and (35)—(39) cannot be stated explicitly in terms of rate constants, as the actual experimental precision will determine whether curvature can be detected or not.

DISCUSSION

As was mentioned above, it has often been uncritically assumed that reciprocal rate plots for the random order mechanism always are non-linear when the rapid equilibrium assumption cannot be applied. More critical authors [8,9] have emphasized that linear relationships may be inherent in rate eqn. (10), but Reiner [8] stated that eqn. (10) will be linear only by an outside chance (type II kinetics), and that it will almost always be possible to discriminate between the ordered case and the random case. Wratten and Cleland [9] reached the same conclusions, and added that appreciable contribution from both of the alternate pathways to the ternary enzyme-substrate complex will lead to detectable curvature of reciprocal plots.

The present investigation has confirmed that a linear reciprocal rate equation with respect to one substrate only can be obtained at one specific concentration of the second substrate, but has shown that linearity in reciprocal rate equations cannot be generally related to the utilization of a preferred pathway. Furthermore, attention has been drawn to the important fact that reciprocal rate plots may appear linear (at least asymptotically) also when the rate equation is non-linear.

Substrate concentrations can, for practical reasons (recording of reaction rates, solubility, *etc.*), only be varied over a limited range, *e.g.* by a factor of 10^3. This range must be considered as narrow in view of the wide range of possible values of rate constants, and initial rate determinations can only be assumed to give information of a limited part of the reciprocal rate curve. As illustrated by the model enzyme described in Fig. 2, the experimental picture obtained will be entirely dependent upon the relative magnitude of the substrate concentrations tested. The model enzyme shows typical IIIb-kinetics in the "normal" range indicated in Fig. 2, exhibits permanent substrate inhibition [10] in a high concentration range ($[S_1] > 0.5$), and apparently obeys Briggs-Haldane kinetics when being observed in a low concentration range ($[S_1] < 0.1$).

It might thus well happen that all substrate concentrations within the range available for experimental variation should be considered as comparatively low (particularly when relationships between substrate concentrations and rate constants are such that the enzyme operates in the proximity of type II kinetics). Under such conditions the non-linear rate eqn. (10) for a random order mechanism becomes experimentally indistinguishable from the bilinear Dalziel eqn. (35), which is known to govern the kinetics of compulsory order and rapid equilibrium mechanisms,[3] and it will not be possible to discriminate between the different cases by initial rate studies. The mere exhibition of linear Lineweaver-Burk plots with respect to both substrates can certainly not be taken as evidence for elimination of a random order mechanism.

The "low concentration assumption" introduced above for derivation of eqns. (35)—(39) may appear similar to the rapid equilibrium assumption as stated by Dalziel,[4] but is less restrictive. Reciprocal plots for the random mechanism may be apparently linear at much higher concentrations than required to justify a rapid equilibrium assumption. Eqns. (35)—(39) are, consequently, more general than the corresponding expressions derived by equilibrium analysis, and include any bilinear kinetic behaviour inherent in the random order mechanism.

A complete analysis of the conditions under which a two-substrate enzyme system operating by a random order mechanism may conform to a Dalziel type of rate equation has apparently not been presented previously. The general bilinear relationships (eqns. (35)—(39)) derived in the present investigation may, therefore, be of great value for studies and discussions of such enzyme systems.

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