

KINETIC ANALYSIS OF INHIBITOR ACTIONS ON ENZYMES¹

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ABSTRACT - A set of data on a given peroxidase inhibition by quecetin, showing an unusually high experimental error, was used to demonstrate how data, seemingly unsuitable for graphical analysis, may still provide useful information on the inhibition mechanism. The most reliable model turned out to be a mixed non-competitive inhibition. The present statistical procedure has proved (i) to be a simple, general and unequivocal way to carry on kinetic analysis of enzymatic reactions under inhibition action, based only on consecutive linear regressions (no previous assumption about the inhibition mechanism is required); (ii) that experimental errors can play an essential role on the final decision about the inhibition mechanism and (iii) to be able to show how close to the Michaelis-Menten mechanism the kinetic model actually is. Therefore all proposed inhibition mechanisms were subjected to statistical judgement.

Index terms: Michaelis-Menten, kinetics, inhibition.

ANÁLISE CINÉTICA DAS AÇÕES DOS INIBIDORES SOBRE AS ENZIMAS

RESUMO - Um conjunto de dados, com alta dispersão experimental, de uma reação de inibição de uma peroxidase por quecetina foi usado para demonstrar o modo pelo qual é possível, através de manipulação gráfica e de análise de regressão linear, obter resultados estatisticamente interpretáveis sobre o mecanismo da inibição enzimática. O modelo cinético mais provável da reação foi estabelecido como sendo o de uma inibição não-competitiva mista. O procedimento estatístico aqui proposto (i) revelou-se relativamente simples, geral e método não-ambíguo de análise numérica em cinética enzimática envolvendo inibição, baseado em regressões lineares consecutivas, e que não requer, necessariamente, o estabelecimento prévio de uma hipótese do tipo de inibição; (ii) pôs em evidência a influência dos erros experimentais na decisão sobre o mecanismo da reação de inibição e (iii) permitiu verificar a validade do modelo cinético e em qual extensão é explicado pela equação de Michaelis-Menten. Por isso, todos os métodos de inibição propostos foram submetidos a julgamento estatístico.

Termos para indexação: Michaelis-Menten, cinética, inibição.

INTRODUCTION

Experimentally, kinetic studies on enzyme reactions that follow Michaelis-Menten (MM) formalism require quite a few prerequisites, such as: (i) enzyme integrity during experiments, (ii) a much

higher equilibrium formation rate between enzyme and substrate than that of product formation, (iii) well defined stoichiometry, (iv) only one substrate molecule to be bound to the activity center at a time, and (v) full reversibility of enzyme-ligand association (Keleti, 1986); also (vi) any ligand excess effects should be avoided. Moreover, it seems also suitable to measure activities in the steady-state. Previous tests should be carried out to establish an optimal range of inhibitor and substrate concentrations (Brune & Fabris, 1988).

These restrictions, though of relatively easy experimental control, make the kinetic parameter determinations somewhat artificial (Hill et al., 1977). Decisions within established reaction mechanisms are usually a matter of intuition, statistical standard

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deviation analysis and biases (Keleti, 1983). On the other hand, the knowledge of kinetic parameters allows comparisons and standardization of enzyme reactions and also speculations about their molecular mechanisms.

A widely applied technique for parameter evaluations through plotting reaction rate *versus* substrate concentration is the Lineweaver-Burk (LB) graph, which helps distinguish neatly among alternatives of the substrate-inhibitor-enzyme binding nature (Engel, 1977), though other linearization methods are also used for this purpose (Cleland, 1967; Metzler, 1977). Since there are unavoidable errors to be taken into account, the observed results may become graphically blurred (Cornish-Bowden, 1981; Duggleby, 1981; Prats & Rodriguez, 1992). Eventually it turns out to be just a matter of personal judgement whether or not a putative reaction model is likely to occur (Mannervik, 1981, 1982).

Even though academic or commercial fitting computer routines based on non-linear algorithms are available for many general statistical purposes, kinetic parameters of enzyme reactions following MM kinetics are more commonly estimated through well-known linear numerical techniques, e.g., that of LB plots. Even in the presence of a second ligand, the kinetic parameters may be evaluated numerically by linear statistical methods. This is also valid for equilibrium constants of reactions involving a modified enzyme molecule.

The initial enzymatic reaction



has an equilibrium constant given by

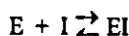
$$K_S = \frac{[E][S]}{[ES]}$$

where E, S and ES represent, respectively, the unengaged enzyme, substrate and enzyme-substrate complex, while brackets stand for their related concentrations.

If the enzyme is previously coupled to an inhibitor, I, the new equilibrium constant is

$$K_S^* = \frac{[EI][S]}{[ESI]}$$

Similarly, the equilibrium



has an equilibrium constant given by

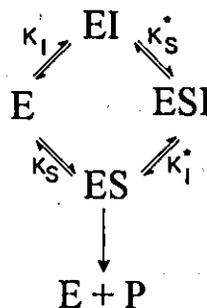
$$K_I = \frac{[E][I]}{[EI]}$$

and, by analogy,

$$K_I^* = \frac{[ES][I]}{[ESI]}$$

So far, the scientific literature has not clearly explained how to ascertain best estimated values of kinetic parameters and their related statistical errors in a broad sense, disregarding the previous knowledge of any assumed inhibition action (Krantz, 1992). Particular cases, like simultaneous linear fitting of a family of straight lines disclosing a single point convergence or focus (Junqueira & Mares-Guia, 1990) are well established. However, currently used methods are more commonly based on individual linear analysis. We hereupon propose an improved statistical procedure based on secondary linear plots following the primary LB representation, which estimates kinetic parameters and their related variances for any enzyme reaction, independently of its molecular inhibition mechanism.

The general kinetic model of an overall inhibited enzyme reaction is given by (Keleti, 1986):



where P is the product.

Formally,

$$v^{-1} = (1 + \alpha[I]K_I^{-1})V_{\max}^{-1} + (1 + [I]K_I^{-1})K_S V_{\max}^{-1}[S]^{-1}, \quad (1)$$

where $\alpha = K_S K_I^*^{-1} = K_I K_I^*^{-1}$; v and V_{\max} have their classical meaning, i.e., experimental and maximum reaction rates, respectively.

In short, this eqn. (1) may be represented by

$$v^{-1} = z + w[S]^{-1},$$

where

$$w = (1 + [I]K_i^{-1})K_s V_{\max}^{-1}, \quad (2)$$

and

$$z = (1 + \alpha[I]K_i^{-1})V_{\max}^{-1}. \quad (3)$$

A primary LB plot, viz V^{-1} versus $[S]^{-1}$, leads to a linear fitting for every individual $[I]$.

For a family of i straight lines each corresponding to a specific concentration of the inhibitor, $[I]$, linear regressions may allow the estimation of sets of i slopes (w) and i intercepts (z), as deduced from eqn. (1).

Taking into account secondary plots relating z 's and w 's versus $[I]$'s, the slopes (ψ_w and ψ_z),

$$\psi_w = K_s K_i^{-1} V_{\max}^{-1}, \quad (4)$$

and

$$\psi_z = \alpha K_i^{-1} V_{\max}^{-1}, \quad (5)$$

and intercepts (ξ_w and ξ_z),

$$\xi_w = K_s V_{\max}^{-1}, \quad (6)$$

and

$$\xi_z = V_{\max}^{-1}, \quad (7)$$

may be estimated.

The equations (4) through (7) lead to the parameter evaluations of eqn (1). The sequential linear regressions give rise to two kinds of statistical errors affecting the parameter values: (i) the standard deviation due to experimental dispersion and (ii) the systematic errors propagated through presumed estimation steps.

Data from a peroxidase reaction inhibited by quercetin is used to demonstrate the procedure.

The main purpose of this work is to establish a statistically-based criterion to select at least one out of a collection of possible molecular mechanisms

of enzyme inhibited reactions, which would permit the estimation of more reliable kinetic parameters.

MATERIAL AND METHODS

Enzyme reaction

Peroxidase (EC 1.11.1.7) catalyzes the reaction of guaiacol with H_2O_2 and its inhibition was studied by adding to the reaction medium a flavone, quercetin, extracted from the radish. The reaction was followed spectrometrically through its absorption at 470 nm in 1 cm light path cells. The assay medium contained also tris(hydroxymethyl) aminomethane (Tris)/HCl, pH 7.2, 10 mM, $CaCl_2$ 0.5 mM and H_2O_2 0.3 mM, and increasing amounts of guaiacol (10.0, 13.3, 20.0, 28.6 and 50.0 mM) and quercetin (0, 20, 50 and 65 μ M). The reaction was started by the addition of 50 μ L of a peroxidase (Merck) solution containing 10 mg of enzyme in 150 mL of Tris/HCl pH 7.2. The final assay volume was 3.0 mL and absorption readings were followed during 5 min, when the reaction rate was visibly slowing down. The temperature of the reaction cells was maintained between 22 and 23°C (Oliveira, 1994).

Estimation of absolute kinetic parameters

Firstly, V^{-1} values, expressed as a dependent variable, are plotted as a function of $[S]^{-1}$ for every $[I]$. The LB plots are presented in Fig. 1 and linear parameters are presented in Table 1. The family of i lines may converge to a focus located, within the experimental errors, (i) on the abscissa axis (simple non-competitive inhibition, $\alpha = 1$); (ii) on the ordinate (competitive, $\alpha = 0$); (iii) on the second quadrant (mixed non-competitive, $\alpha < 1$); (iv) in the third quadrant (mixed non-competitive, $\alpha > 1$) or (v) may not converge at all (parallel lines; uncompetitive) (Engel, 1977), where the enzyme affinity toward the second ligand has somehow been changed, as the result of a previous engagement (ES or EI). The criterion to select one of the above alternatives requires a hypothesis test that is described in the next steps.

Secondly, the slopes w [eqn. (2)] of each straight line i are plotted against $[I]$, to give a new linear pattern (Fig. 2). The least square fitting of these new data allows an estimation of the slope, ψ_w [eqn. (4)] and the intercept, ξ_w [eqn. (6)].

Thirdly, the intercepts z_i [eqn. (2)] are plotted against $[I]$ and the new (Fig. 3) slope ψ_z [eqn. (5)] and intercept ξ_z [eqn. (7)] may now be estimated. All individual parameters are algebraically related and completely define eqn. (1).

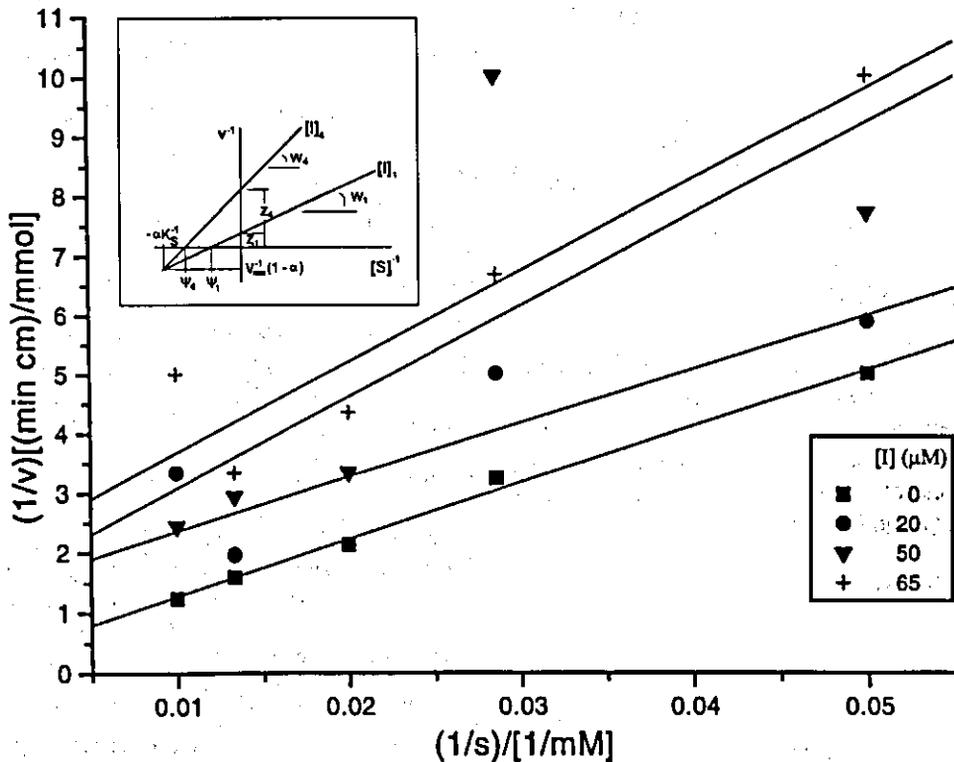


FIG. 1. Lineweaver-Burk representation of linear fittings, relating v^{-1} versus s^{-1} . Estimated regressions parameters are presented in Table 1. Inset: schematic graphical representation of used symbols.

TABLE 1. Linear parameters estimated from the Lineweaver-Burk Plot, according to eqn. (1).

[I]	Slope (w)	Intercept (z)	r^2
0	94.92	0.32	0.99
20	90.74	1.45	0.83
50	153.43	1.54	0.73
65	152.99	2.14	0.94

Finally, four hypotheses are hereby tested, using the t-distribution as a statistical criterion (Table 2): (i) if the set of slopes of the linear regressions relating w 's, from eqn. (2), versus $[I]$ is statistically zero (Fig. 2), the straight lines in the primary plot (Fig. 1) may be assumed as being parallel (uncompetitive inhibition); (ii) if the set of slopes

from the linear regression relating the intercepts z 's, from eqn. (3), versus $[I]$ [eqn. (5)] is statistically zero (Fig. 3), the focus may be located on the ordinate (competitive inhibition); a set of ϕ 's at $[S]^{-1} = -K_s^{-1}$ are calculated from eqn. (1), by making $v^{-1} = 0$; (iii) if the slope of the linear regression relating ϕ versus $[I]$ is statistically zero (Fig. 4), there is a focus located on the abscissa at $-K_s^{-1}$ ($\alpha = 1$, non-competitive); and (iv) if the previous three hypotheses are rejected, α being neither one nor zero, the inhibition may be mixed (partially competitive), provided that the straight lines in the primary plot converge to a point on the second or third quadrant having an ancillary coordinate at $[S]^{-1} = -\alpha K_s^{-1}$. Values of v^{-1} (namely, ϕ 's) at this abscissa point are plotted against $[I]$ (Fig. 5), and the statistical test for zero slope value indicates whether or not the mixed inhibition model may be accepted.

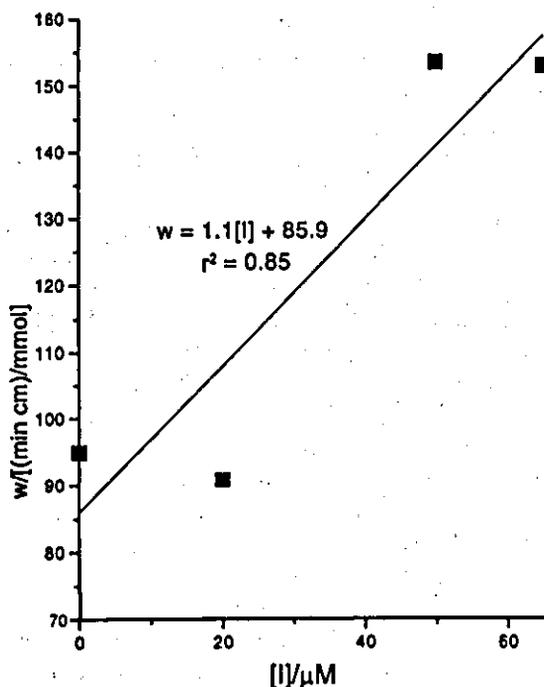


FIG. 2. Linear regression of slopes (w) estimated from fittings shown in Fig. 1 and Table 1 versus inhibitor concentrations, $[I]$. The zero slope hypothesis (uncompetitive mechanism) is rejected within a level of error of 5% probability.

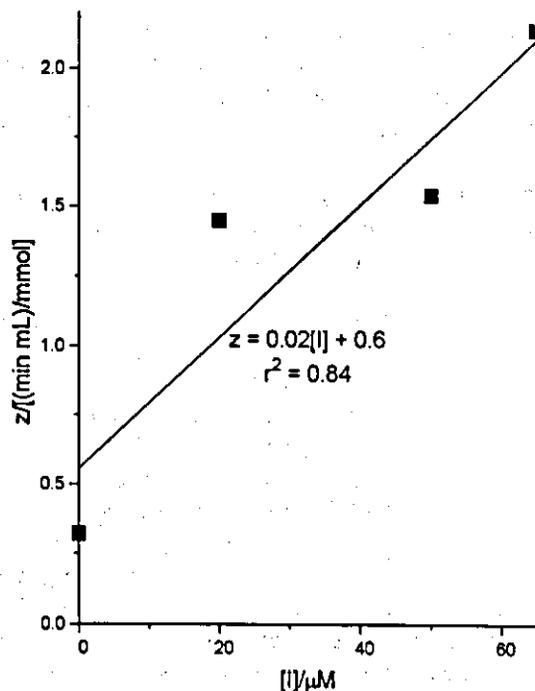


FIG. 3. Linear regression of intercepts (z) estimated from fittings shown in Fig. 1 and Table 1 versus inhibitor concentrations, $[I]$. The zero slope hypothesis (competitive mechanism) is rejected within a level of error of 5% probability.

TABLE 2. Hypothesis-tests concerning the inhibition mechanism by using t-distribution.

Inhibition mechanism	Slope	r^2	t	Probability	Decision
Uncompetitive	1.099	0.85	3.35	0.05	Rejected
Competitive	0.024	0.85	3.26	0.05	Rejected
Non-competitive	0.000	0.30	0.92	0.43	Not rejected
Mixed	0.000	0.01	0.01	0.99	Not rejected

RESULTS AND DISCUSSION

It was not possible to unambiguously discern any trend for a non-MM kinetics, by inspecting Fig. 1 only. In addition, the trends expressed in Figs. 2, 3 and 4 suggest non-validity of competitive, uncompetitive and non-competitive inhibitions. This interpretation is corroborated by the individual trajectories on the third quadrant, as shown in Fig. 5. The points are evenly scattered around the horizon-

tal line representing the linear regression. Furthermore, it gives no clue for a non-linear fitting (Orsi & Tipton, 1979; Johnson & Frasier, 1985). On the basis of these arguments, it is plausible to consider the hypothesis that the kinetic mechanism follows the partial or the mixed inhibition model (Keleti, 1986). According to the t-distribution analysis (Table 2) the uncompetitive and competitive inhibitions are statistically rejected within <0.05 probability. The other two mechanisms, non-competitive and

mixed, are rejected with a much higher error level, viz 0.43 and 0.99, respectively. This means that one of them could be accepted, but further experimental and statistical refinements would be required. The highly scattered experimental data, as shown by the primary LB plot (Fig. 1) is the main cause of such a lack of discrimination. The experimental error weighting and higher number of measured points in the primary linear regression would be a way to improve statistical confidence, but independent biochemical information would be ultimately necessary for a consistent decision. Because partial inhibition exhibits a focus on a plane, not on a coordinate axis, the focus location reflects the change in enzyme affinity to one ligand by a former association with another one. Similar observations in all branches of chemistry sustain this seemingly general phenomenon. For instance, the first reaction of an organic acid with an alcohol virtually always interferes with further sequential steps of the esterification. In the case of the enzyme reaction, a focus on the ordinate

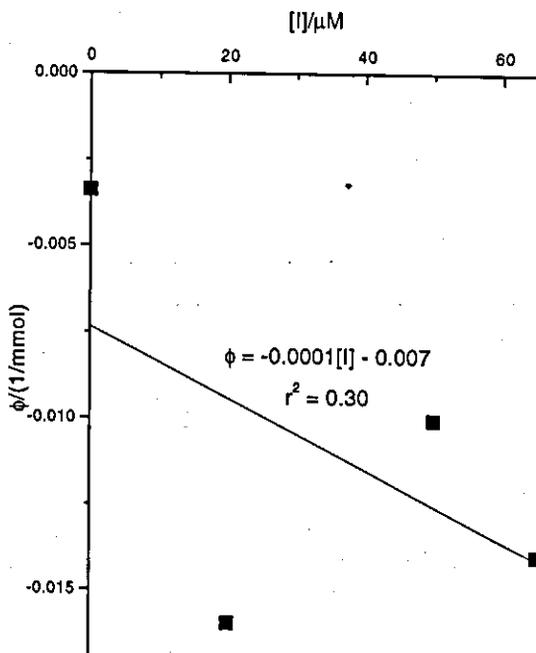


FIG. 4. Linear regression of $\phi = [S]^{-1}$ for $v^{-1} = 0$ versus $[I]$ at $[S]^{-1} = -\alpha K_s^{-1}$. The zero slope hypothesis (non-competitive mechanism) is rejected within a level of error of 43% probability.

or on the abscissa, as well as a plot of slopes in response to a second ligand is confined to particular cases and to those where a statistically tenable decision cannot be made.

It is interesting to compare estimations of kinetic parameters obtained from the present LB (v^{-1} vs s^{-1}) linearization with those from other techniques, namely Hanes (sv^{-1} vs s), and direct non-linear fitting using Michaelis-Menten (v vs s) model. Results are presented in Table 3. Fig. 6 represents LB plots based on the set of kinetic parameters estimated according to the present procedure. Unlike the primary LB plots in Fig. 1, the straight lines in Fig. 6 converge to a common point, as expected from the mixed non-competitive inhibition model. The Eadie-Hofstee (vs^{-1} vs v) procedure gave inconsistent results and is not presented. Notice that in the Hanes & Eadie-Hofstee linearization strategies, the two variables, v and s , are not completely separated, as required for linear fitting. On the other hand, non-linear fittings are strongly influenced by experimental data in the asymptotic region of the v - s curve. In the present case, results are highly discrepant from linear methods as a consistent value for V_{max} could not be found, and estimations of the other kinetic parameters are consequently affected. Results of the non-linear fitting in Table 3 were obtained from si-

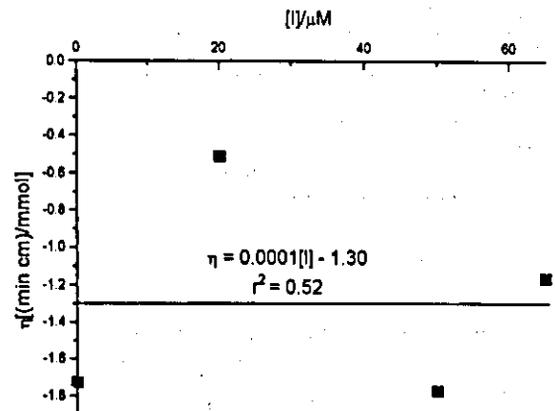


FIG. 5. Linear regression of $\eta = [S]^{-1}$ versus $[I]$ at $[S]^{-1} = -\alpha K_s^{-1}$. The zero slope hypothesis (mixed mechanism) is rejected within a level of error of 99% probability.

TABLE 3. Estimated kinetic parameters from linear and non-linear fittings.

Kinetic parameter	Non-linear v vs s	Lineweaver-Burk v ⁻¹ vs s ⁻¹	Hanes vs ⁻¹ vs s
$\hat{V}_{\max} / \Delta A_{470} \text{ min}^{-1} \text{ cm}^{-1}$	1.7	1.79	1.64
\hat{K}_S / mM	124.7	153.85	135.95
$\hat{K}_I / \mu\text{M}$	75.5	78.19	86.67
$\hat{\alpha}$	3.0	3.32	3.74

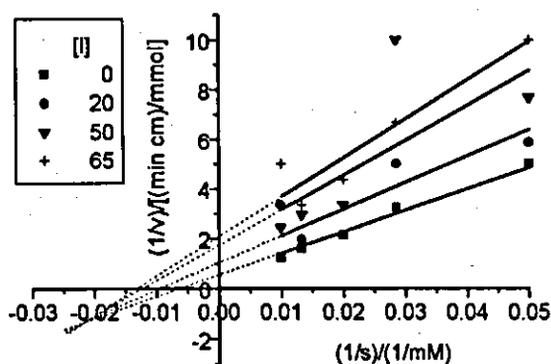


FIG. 6. Lineweaver-Burk plot and estimated regression lines from estimated kinetic parameters of Table 3.

multaneous least square convergence, by pooling sets of v-s data from all four inhibition concentrations into one input set. A Fortran program was written based on the iterative Marquardt (1959, 1963) algorithm, to perform least square minimization.

CONCLUSIONS

1. The present statistical procedure is a simple, general and clear way to carry out kinetic analysis of enzymatic reactions under inhibition action, based only on consecutive linear regressions.

2. It puts in evidence that the experimental errors can play an essential role on the final decision about the inhibition mechanism.

3. It enables one to show how close to the Michaelis-Menten mechanism the kinetic model actually is, as all proposed inhibition mechanisms can theoretically be rejected.

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