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PARAMETER EVALUATION IN MICHAELIS-MENTEN KINETICS

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ABSTRACT

Parameter estimation reliability in enzyme kinetics depends upon the substrate range concentrations under assay. An inappropriate concentration set may lead to spurious values of Km and Vmax in the Michaelis-Menten approach. In this paper, the theoretical arguments for a practical criterium concerning the best work range of substrate concentrations are discussed on a velocity ratio basis (V1/Vn) as response to the pertinent substrate concentration ratio (S1/Sn).

INTRODUCTION

The Michaelis-Menten equation (I) has been subjected to a variety of comments on what may be referred to as textbook distortions, such as goodness-of-fit for estimates of error (1) and the comparison of reciprocal plots with microcomputer softwares (2).

$$V = \frac{Vmax \cdot S}{Km + S} \quad (*) \qquad (I)$$

In fact, inaccurate graphs of velocity vs concentration are particularly common in biochemical textbooks as has been pointed out recently by NAQUI (3), who alludes to the fact that in plots of V vs S the expression (II) applies

$$(dV/dS)_{S=0} = Vmax/Km \qquad (II)$$

As a suggestion we should like to recall a further helpful drawing aid: the slope (III)

$$(dV/dS)_{S=Km} = Vmax/4Km. \qquad (III)$$

At this point V = Vmax/2 as evidenced by the Michaelis-Menten equation (I) for S = Km.

The estimatives of parameters (Km and Vmax) by linearization (such as in Lineweaver-Burk representations, FIGURE 1) are at present somewhat obsolete having been superseded by computer techniques (4).

(*) The following symbols apply:

V: Inicial velocity of an enzyme-catalyzed reaction, obeying Michaelis-Menten kinetics; Vmax: Maximum conventional velocity of such a reaction; Km: Michaelis-Menten constant; Si (i = 1 ... n): Substrate concentration, S1 > ... > Sn; GC: Geometric Center. GC = $(S1 \times Sn)^{1/2}$

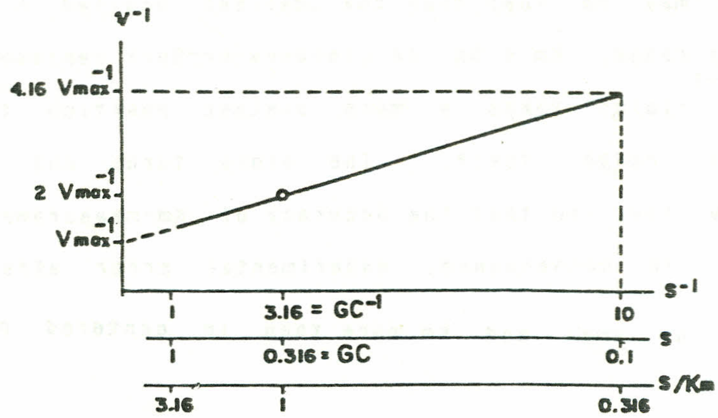

FIGURE 1. Lineweaver-Burk plot. Km lies within the experimental range Sn/S1 = 10; Km = 0.316.

This, however, does not exempt from searching for the most reliable range of S's in order to minimize the consequences of experimental error. As a premise, interpolations cause smaller distortions than extrapolations. In other words, a range of experimental S's encompassing Km is preferable to those not comprising it.

In enzyme kinetics the array of S's represents the independent variable and that of V's the dependent variable.

By geometric arguments it is well established that slopes near unit yield more dependable readings than those deviating considerably from it (5).

In Lineweaver-Burk plots the slope representing Michaelis-Menten kinetics is Km/Vmax. The intersection of the straight line with the ordinate registers $Vmax^{-1}$. So, 2 $Vmax^{-1}$ of the ordinate yields Km^{-1} on the abscissa (FIGURE 1). The value of Km in this figure may be labelled as "centered" (Sn < Km < S1) identifying itself with GC.

If the range of experimental S's does not encompass the hitherto unknown Km-value, there follows one of two possible alternatives:

a) Km may be less than the smallest applied S of the experimental range, Km < Sn. In Lineaweaver-Burk representations such a Km^{-1}-value takes a more distant position than the experimental range itself. The slope turns out to be precariously low, so that the accuracy of Km-measurements are endangered. In consequence, experimental error affects the computation of Vmax and Km more than in centered positions (FIGURE 2).

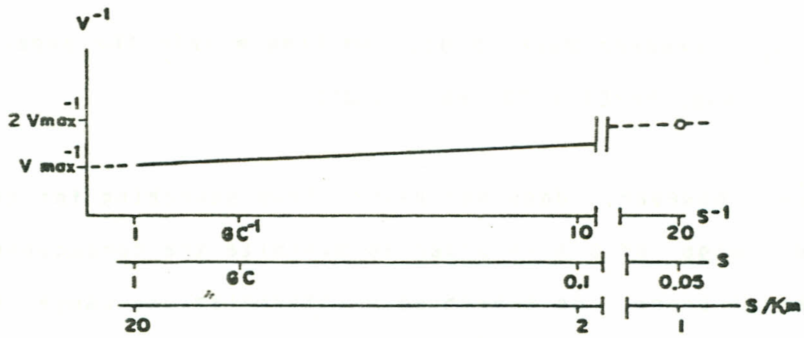

FIGURE 2. Lineweaver-Burk plot. Km is smaller than Sn, the smallest experimental value. S1/Sn = 10; Km = 0.05

b) Km may turn out to be greater than the largest applied S, Km > S1. As a consequence in Lineweaver-Burk plots Km^{-1} is located closer to the ordinate (FIGURE 3) which leads to unconveniently steep slopes, again imperiling accuracy of ordinate intersection readings (6, 7).

METHODOLOGY

An experimental set of data dealing with the beta-glucosidase of a fungus (Humicola sp) hydrolysing p-nitrophenyl beta-glucoside (8) may help make our point. The unabridged set of these observations runs from Sn = 0.05 to S1 = 2, expressed in empirical units. FIGURE 4 records the observed initial velocity as a function of the applied substrate concentration by way of

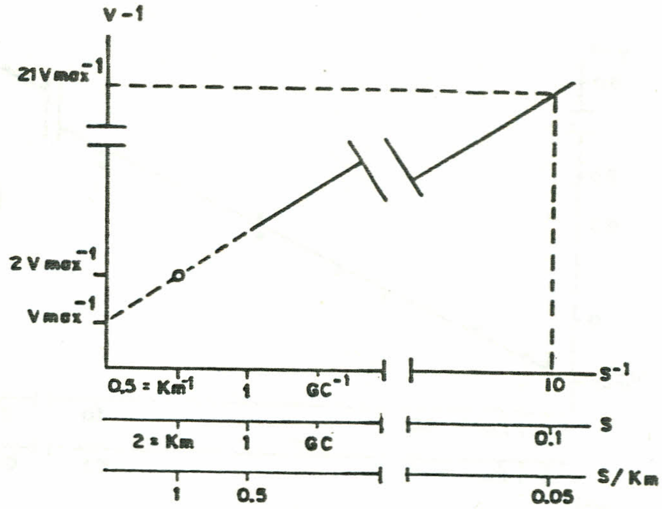

FIGURE 3. Lineweaver-Burk plot. Km is larger than S1, the largest experimental value S1/Sn = 10; Km = 2

the Lineweaver-Burk representation. Vmax and Km have been evaluated in the usual way, while the geometric center between Sn^{-1} and $S1^{-1}$ is located at $GC^{-1} = (S1 \times Sn)^{-1/2}$.

DISCUSSION

In a range, e. g., from $S1^{-1} = 1$ to $Sn^{-1} = 10$, the geometric center lies at $S^{-1} = 3.16$ (see also FIGURE 1). The goodness-of-centering can be expressed by the ratio V1/Vn where the subscripts correspond to those of their pertinent S's. This ratio depends not only on the centering but also upon the width of the substrate range, but not on the empirical scale. This can be taken as a reliable criterium for the centering of Km, stretching from unit (Km << Sn, FIGURE 2) to S1/Sn (for Km >> S1, FIGURE 3): ten in the present case.

The original equation of Michaelis-Menten (I) in its "reduced" form, where yi = Vi/Vmax and xi = Si/Km, takes the shape

$$yi^{-1} = 1 + xi^{-1}. \qquad (IV)$$

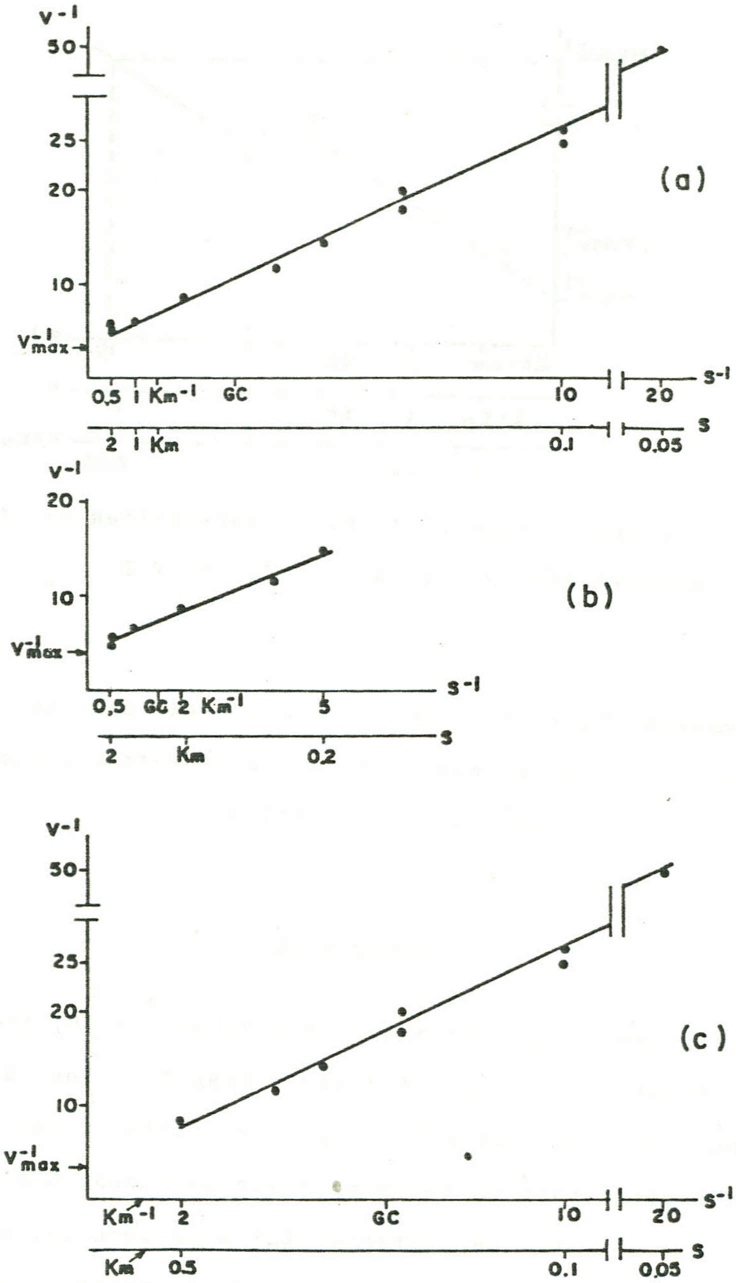

FIGURE 4. Lineweaver-Burk plot of a set of data dealing with the beta-glucosidase of a fungus (Humicola sp) hydrolysing p-nitrophenyl beta-glucoside (8). GC: Geometric center. (a) The whole set ranging from S1 = 2 Sn = 0.05; (b) A subset from S1 = 2 to Sn =0.2; (c) A subset from S1 = 0.5 to Sn = 0.05. Arbitrary units.

The velocity ratio R = V1/Vn may be expressed by

$$R = (1 + xn^{-1})/(1 + x1^{-1}), \quad (V)$$

since it has the same meaning as y1/yn, the pertinent velocity ratio (FIGURE 5).

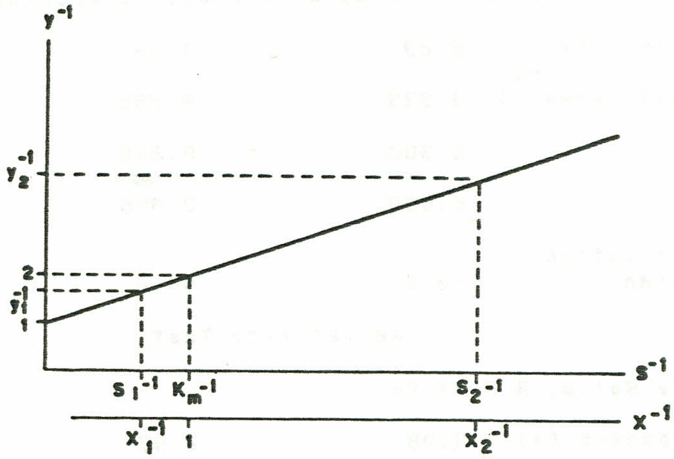

FIGURE 5. Michaelis-Menten equation in its reduced form, expressed through the Lineweaver-Burk plot. x^{-1} = Km/S; y^{-1} = Vmax/V.

For goodness-of-fit judgements arbitrary tolerance limits at 0.5 GC and 2 GC can be established, being equidistant from the GC in the geometric sense by 100%.

The Lineweaver-Burk data treatment to the above quoted <u>Humicola</u> set yields Vmax = 0.300 and Km = 0.689 (TABLE I).

The correlation coeficient (r^2 = 99.6 %) discloses a highly reliable set: On the other hand the Km value 0.689 lies far apart from the geometric center (GC = 0.316) of the whole set; its position lies above the proposed tolerance limits 0.158 (= 0.316 x 0.5) and 0.632 (= 0.316 x 2). So the assayed range of S's turns out to be rather low for Km evaluation notwithstanding the fact that Km still lies within the applied S's. To emphasize the

TABLE I - Parameter analysis and reliability test of a set of data dealing with the beta-glucosidase of a fungus (*Humicola* sp) hydrolysing p-nitrophenyl beta-glucoside (8).

	Range of S's (Sn to S1)		
	Whole Set	Bisected Sets	
	0.05 to 2	0.2 to 2	0.05 to 0.5
Lineweaver-Burk Parameter Searching			
Slope (Km/Vmax)	2.29	1.94	2.33
Intercept ($Vmax^{-1}$)	3.333	4.255	2.786
Vmax	0.300	0.235	0.359
Km	0.689	0.456	0.838
r^2 %-Correlation coefficient	99.6	98.8	99.7
Reliability Test			
Velocity Ratio, R = V1/Vn			
Expected (*)	10.98	2.67	6.63
Observed (**)	9.40	2.69	5.91
R-Tolerance limits (***)			
Lower	3.86	2.23	2.23
Upper	10.37	4.49	4.49
Km^{-1}	1.451	2.193	1.193
GC^{-1}	3.16	1.58	6.32
$(Km.GC)^{-1}$	4.590	3.467	7.547
u = ln [1/(Km.GC)]	1.524	1.243	2.021
y'^{-1} at u	1.702	1.439	2.420

(*) From the fitted Lineweaver-Burk straight line.
(**) From experimental data.
(***) Replacing Km either by 0.5 x GC (lower limit) or by 2 x GC (upper limit) on equation (V).

importance of the proper choice of S's, we bisected the whole range into one portion of low and another one of high S's, each fragment comprising a ratio of S1/Sn = 10. Details of fractioning and results can be followed in TABLE I as well as in FIGURES 4 and 6.

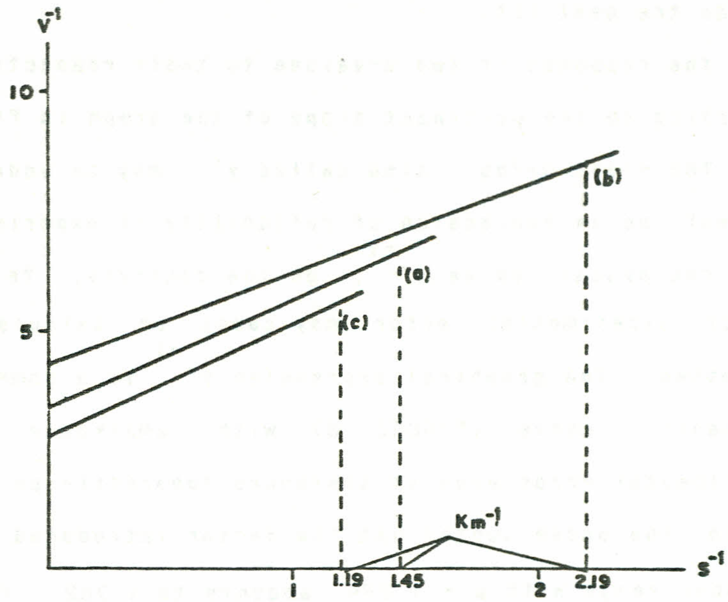

FIGURE 6. Set of data used in FIGURE 4, demonstrating the interference of range-width and position on Km and Vmax estimations. (å) The whole set ranging from S1 = 2 Sn = 0.05; (b) A subset from S1 = 2 to Sn =0.2; (c) A subset from S1 = 0.5 to Sn = 0.05.

In search for the best position of a range of S's it seems advisable to introduce a logarithmic scale (5, 9) whereby unified S1/Sn ratios acquire equal lengths. So, e. g., a range chosen as S1/Sn = e, i.e.,

$$\ln S1 - \ln Sn = 1, \qquad (VI)$$

has only one definite length, wherever the range may lie.

Introducing $x = e^{u}$, the equation (IV) assumes the form

$$y^{-1} = 1 + e^{-u} \qquad (VII)$$

A plot of y vs u produces the graph in FIGURE 7. In this figure one may verify the assumption that a range in the neighbourhood of u = 0 exhibits the highest slope. As a consequence the experimental error has the smallest influence on the results in this region. Thus, a range in such a position yields the best fit.

The response of two u-values to their respective V's varies according to the pertinent slope of the graph in FIGURE 7.

The slope dy/du, also called y', may be understood in this context as an expression of reliability of experimental values. Its reciprocal value y'^{-1}, on the contrary, tells about the effect experimental error may cause on calculated parameter estimates. The graphical expression y'^{-1} vs u comes out to be an exponential curve (FIGURE 8) with remarkable influences of experimental error even at distances apparently as small as $u = \pm 2$. In the above quoted set the factor introduced by a somewhat improper range with u = 1.524 amounts to 1.702. The correct Km-value of the considered Humicola experiment thus may be found through the expression ln Km $\pm$ ln (y'^{-1}). In consequence, taking into account the whole set, labelled by Km = 0.689 and y'^{-1} = 1.702 (TABLE I), the most plausible value for Km lies between 1.173 (= 0.689 x 1.702) and 0.405 (= 0.689/1.702). This span for the site of Km refers to what may be called a "systematic" error. It contributes to the error obtained through inevitable

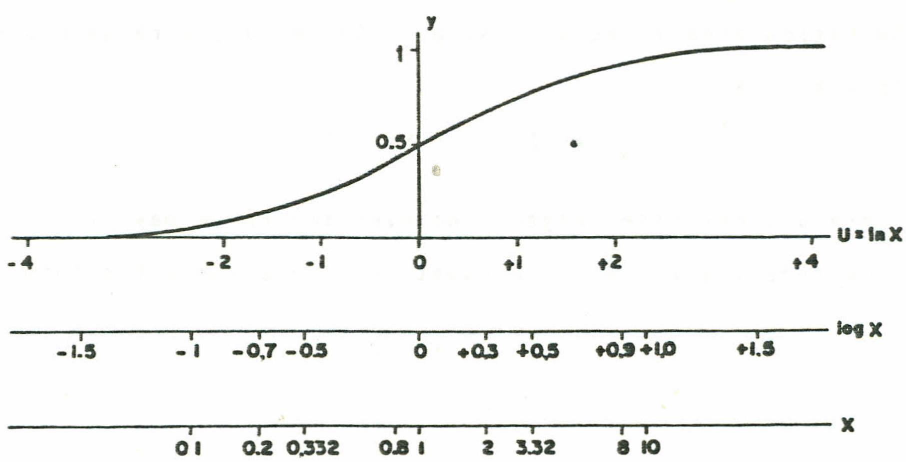

FIGURE 7. Reduced Michaelis-Menten equation $y^{-1} = 1 + e^{-u}$. Substrate concentrations x are recorded as e^{u}.

measurement shortcomings expressed by its standard deviation. TABLE I also makes clear that the range from 0.2 to 2 offers a more dependable reading for Km than the improperly centered though broader range from 0.05 to 2. An analogous result can be drawn of course for Vmax-evaluations.

In conclusion we suggest the following procedure for a reasonably dependable estimate of Km and Vmax: In a preliminary trial, one may choose two S's with the ratio $S1/Sn = 10$. If the observed V's turn out to result in a velocity ratio $R < 2.2$, measurements with lower S's should be tried. On the other hand, if $R > 4.5$, higher S's should be tested. These two arbitrary R-limits are then a practical prior criterium of reliability in enzyme kinetics studies.

Such recommendations should not lead one to underestimate the otherwise useful information on low and high S's. So, cooperativity, positive as well as negative, shows a peculiar profile at low S/Km, while substrate-excess inhibition itself is more strongly characterized the higher S/Km is.

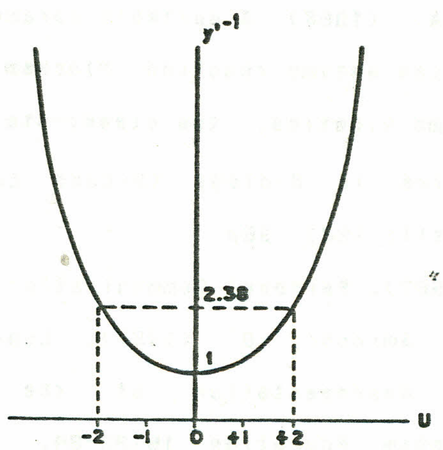

FIGURE 8. Reduced and derived Michaelis-Menten equation. Effect of experimental errors $y'^{-1} = e^{u} + 2 + e^{-u}$ as function of u. The y'^{-1}-values were still normalized by dividing them by 4, yielding $y'^{-1} = 1$ when $u = 0$. See text for further details.

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