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THE CONSEQUENCES OF SYSTEMATIC ERROR IN ENZYME KINETICS

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SUMMARY

The consequences of systematic error in enzyme kinetics was considered by introducing a first order error term into the MICHAELIS–MENTEN–BRIGGS–HALDANE equation. The situations investigated were those involving over- or undercorrection of an enzyme or substrate blank reaction in the absence and presence of product inhibition and in the presence of a totally competitive, non-competitive or uncompetitive inhibitor. In addition, consideration was given to errors arising from a departure from the BEER-LAMBERT relationship and those proportional to the velocity or substrate concentration. Finally, attention has been called to the questionable validity of using weighting procedures to correct for random error in the presence of systematic error.

* Contribution No. 2560.

INTRODUCTION

The apparent initial rate of many enzyme catalyzed reactions is given by eqn. 1,*

$$v = \mathrm{d[P]}/\mathrm{d}t = -\mathrm{d[S]}/\mathrm{d}t = k[\mathrm{E}]\,[\mathrm{S}]/(K + [\mathrm{S}]) \qquad (1)$$

provided [E] and [S] are varied over a sufficiently limited range and all other reaction parameters are held constant. In this communication we shall be concerned initially with the consequences of a systematic error which will introduce an additional first order term into eqn. 1 to give eqn. 2.

$$v = k[\mathrm{E}]\,[\mathrm{S}]/(K + [\mathrm{S}]) + k_{\mathrm{A}}[\mathrm{A}] \qquad (2)$$

It is assumed in eqn. 2 that A will yield products which are indistinguishable from those arising from S.

When [E] is invarient, eqn. 1 is linear and eqn. 2 non-linear with respect to $1/v$ *vs.* $1/[\mathrm{S}]$, $[\mathrm{S}]/v$ *vs.* [S] or v *vs.* $v/[\mathrm{S}]$ (see ref. 1). Thus, it might be argued that the presence of a systematic error, of the type contemplated in eqn. 2, would necessarily be revealed by a non-linear relationship between the two members of any one of the above three pairs of parameters and that the only hazard would be one of confusing a situation described by eqn. 2 with one devoid of systematic error but characterized by a dependency upon [S] other than that associated with eqn. 1. However, this view ignores the fact that many enzymic studies are conducted over such a limited range of [S] that an existing non-linear relationship may not be revealed, with the result that k and K will be evaluated on the basis of an apparent linear relationship which in fact may vary with the relative magnitudes of [S] and K.

To illustrate the kinetic consequences of several of the more common systematic errors, eqn. 2 was evaluated using assumed but reasonable values for the various constants and independent variables and the results so obtained were compared with those arising in the absence of systematic error or errors. The first cases considered were those involving the so-called enzyme and substrate blanks.

It frequently is observed that products indistinguishable from those formed in a total system, otherwise described by eqn. 1, will arise from either or both E and S when examined separately. In practice the rates of these latter reactions may be individually determined and then subtracted from the rates observed for the total system**. Thus, the term k_{A} [A] in eqn. 2 may be specified as in Table I to give eqns. 2_a to 2_f***. These latter equations were evaluated for $K = 5.0 \cdot 10^{-2}\ M$, $k = 2.0 \cdot 10\ M/\mathrm{min}/M$, $\pm k_{\mathrm{A}_1} = \pm k_{\mathrm{A}_2} = \pm 2.0 \cdot 10^{-1}\ M/\mathrm{min}/M$, $\pm k_{\mathrm{A}_3} = \pm 2.0 \cdot 10^{-4}\ M/\mathrm{min}/M$, $[\mathrm{E}] = 5.0 \cdot 10^{-5}\ M$ and $[\mathrm{S}] = 5.0 \cdot 10^{-4}\ M$ to $5.0\ M$. The data so obtained are presented in Figs. 1a, 1b and 1c in the form of v *vs.* $v/[\mathrm{S}]$plots.

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* Where [E] and [S] are the initial enzyme and substrate concentrations and k and K are the two constants derivable from the dependence ot v upon [S] when [E] is invarient. For a discussion of the procedures involved see ref. 1.

** For a discussion of the validity of this procedure see ref. 2.

*** Although eqns. 2_a and 2_b may appear to be equivalent to equations 2_b and 2_d it will be shown subsequently that the addition of $\pm k_{\mathrm{A}_1}[\mathrm{E_T}]$ to eqn. 1. leads to different rate equations than does the addition of $\pm k_{\mathrm{A}_2}[\mathrm{E_F}]$. This situation arises from the fact that in the former instance it is assumed that all of the enzyme present, $[\mathrm{E_T}]$, can give rise to a blank reaction whereas in the latter only the free enzyme, $[\mathrm{E_F}]$, can so react. While these pairs of rate equations are different they are kinetically indistinguishable so long as the conditions implicit in eqn. 1 are maintained.

It is evident from Figs. 1a, 1b and 1c that the systematic errors considered in Table I will lead to errors in the direction indicated in Table II. The information summarized in Table II may be interpreted in several ways. First, if a non-linear plot is observed it follows from Table II that the situations described by eqns. 2_a,

TABLE I

SYSTEMATIC ERRORS ASSOCIATED WITH ENZYME OR SUBSTRATE BLANKS

Eqn.	*Source of error*	k_A*	*[A]*	*Nature of error*
2_a	Enzyme blank	k_{A_1}	$[E_T]$**	Undercorr. for blank
2_b	Enzyme blank	k_{A_2}	$[E_F]$***	Undercorr. for blank
2_c	Enzyme blank	$-k_{A_1}$	$[E_T]$**	Overcorr. for blank
2_d	Enzyme blank	$-k_{A_2}$	$[E_F]$***	Overcorr. for blank
2_e	Substrate blank	k_{A_3}	[S]§	Undercorr. for blank
2_f	Substrate blank	$-k_{A_3}$	[S]§	Overcorr. for blank

* It is assumed that in every case the rate of reaction will be first order in [A].
** Total enzyme concentration, *i.e.*, $[E_T] = [E]$.
*** Free enzyme concentration, *i.e.*, $[E_F] = [E_T] - [ES]$.
§ Free substrate concentration. However, this will be equivalent to the initial or total substrate concentration, *i.e.*, $[S_T] \doteq [S]$, everywhere except in eqn. 3 where $[S_T] \doteq [S] + [P]$. The conditions are necessary for the validity of eqn. 1.

TABLE II

RELATION BETWEEN TRUE AND APPARENT VALUES OF K AND k

$k_A[A] =$	$k_{A_1}[E_T]$ (2_a)*	$k_{A_2}[E_F]$ (2_b)*	$-k_{A_1}[E_T]$ (2_c)	$-k_{A_2}[E_F]$ (2_d)	$k_{A_3}[S]$ (2_e)*	$-k_{A_3}[S]$ (2_f)**
K'***	$< K$	$< K$	$> K$	$> K$	$> K$	$< K$
k'§	$\lesseqgtr k$	$< k$	$\gtreqless k$	$> k$	$> k$	$< k$

* Simulates activation by excess substrate.
** Simulates inhibition by excess substrate.
*** Apparent value of K.
§ Apparent value of k.

2_b and 2_e will simulate activation by excess substrate, in the sense used by WOLF AND NIEMANN[3], and that of equation 2_f of inhibition by excess substrate*. Alternatively, if an apparent linear relationship is observed, over a narrow range of [S], constants evaluated on the basis of such a relationship will tend to be in error in the directions indicated in Table II. Finally, the data summarized in Table II are useful in identifying those errors which tend to be predominantly additive, *e.g.*, a combination of 2_c and 2_f, or of 2_d and 2_f. There is no combination involving both enzyme

* See ref. 1, p. 81 for the characteristics of inhibition by excess substrate. Cases 2_e and 2_f are of particular interest because of the requirement of relatively high values of [S] for the demonstration of either activation or inhibition by excess substrate. In these cases it is important that the substrate blank be evaluated with considerable precision because if it is overestimated inhibition by excess substrate will be simulated and if underestimated activation by excess substrate will be mimicked. In eqns. 2_a and 2_b the dependency of v upon [S] is the same as that encountered in the case of introduction of an endogenous substrate with the enzyme preparation[4]. However, in the latter instance the dependency of v upon [E] differs from that of equations 2_a and 2_b.

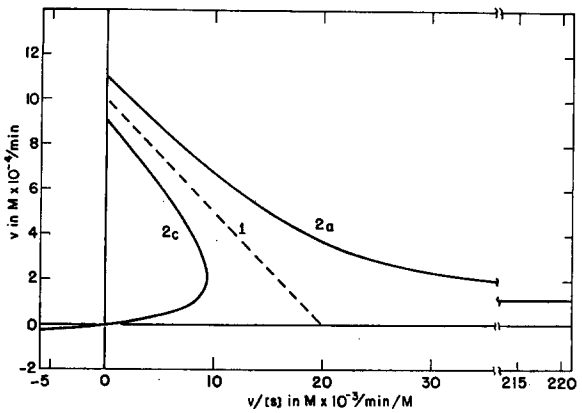

Fig. 1a. Systematic error effects on eqn. 1: eqns. 2_a and 2_c with $\pm k_{A_1} = \pm 2.0\ M/\text{min}/M$ to exaggerate the effects of the error term. Eqns 2_a and 2_c have asymptotes at $v = k_{A_1}[E_T]$ and $v = -k_{A_1}[E_T]$ and intercepts at $v = (k + k_{A_1})[E_T]$ and $v = (k - k_{A_1})[E_T]$ respectively.

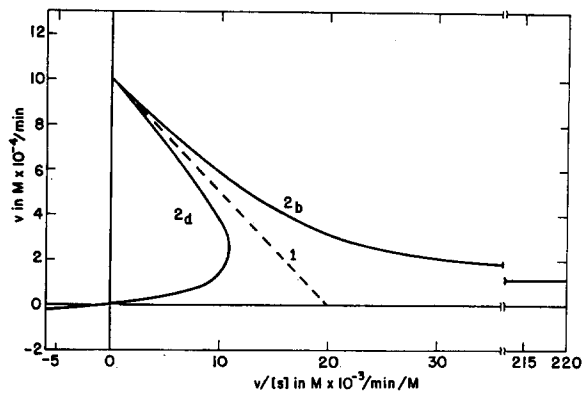

Fig. 1b. Systematic error effects on eqn. 1: eqns. 2_b and 2_d with $\pm k_{A_2} = \pm 2.0\ M/\text{min}/M$ to exaggerate the effects of the error term. Eqns. 2_b and 2_d have asymptotes at $v = k_{A_2}[E_T]$ and $v = -k_{A_2}[E_T]$ respectively and a common intercept at $v = k[E_T]$.

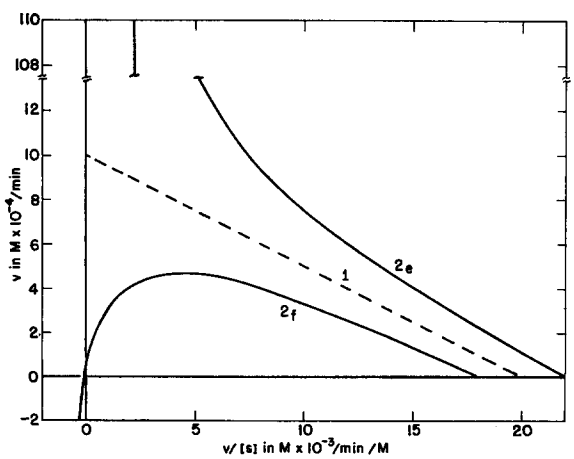

Fig. 1c. Systematic error effects on eqn. 1: eqns. 2_e and 2_f with $\pm k_{A_3} = \pm 2.0 \cdot 10^{-3}\ M/\text{min}/M$ to exaggerate the effects of the error term. Eqns. 2_e and 2_f have asymptotes at $v/[S] = k_{A_3}$ and $v/[S] = -k_{A_3}$ and intercepts at $v/[S] = k_{A_3} + k[E_T]/K$ and $v/[S] = -k_{A_3} + k[E_T]/K$ respectively.

and substrate errors that will be generally compensatory because errors arising from improper treatment of the enzyme blank will be dominant at low values of [S] and those associated with the substrate blank at high values of [S].

An alternative procedure for interpreting the kinetic consequences of systematic errors of the above kind, which is also useful for assessing the significance level of the individual errors, is based upon transposition of equations 2_a to 2_f to equations $2'_a$ to $2'_f$ inclusive.

$$v\,[S] + vK = (k + k_{A_1})\,[E_T]\,[S] + k_{A_1}\,[E_T]\,K \qquad (2'_a)$$

$$= k\,[E_T]\,[S] + k_{A_2}\,[E_T]\,K \qquad (2'_b)$$

$$= (k - k_{A_1})\,[E_T]\,[S] - k_{A_1}\,[E_T]\,K \qquad (2'_c)$$

$$= k\,[E_T]\,[S] - k_{A_2}\,[E_T]K \qquad (2'_d)$$

$$= (k\,[E_T] + k_{A_3}\,K)\,[S] + k_{A_3}\,[S]^2 \qquad (2'_e)$$

$$= (k\,[E_T] - k_{A_3}\,K)\,[S] - k_{A_3}\,[S]^2 \qquad (2'_f)$$

Examination of these latter equations readily discloses how the various systematic errors interact with k and K. Furthermore, division of both members of equations $2'_a$ to $2'_f$ by $(K + [S])$ reveals that systematic errors of the kind under consideration can be ignored only if $v \gg k_{A_1}\,[E_T]$ or $k_{A_1} \ll k\,([S]/(K + [S]))$ for cases 2_a and 2_c, $v \gg k_{A_2}\,[E_T]\;(K/(K + [S]))$ or $k_{A_2} \ll k\,([S]/K)$ for cases 2_b and 2_d and $v \gg k_{A_3}\,[S]$ or $k_{A_3} \ll k\,[E_T]/(K + [S])$ for cases 2_e and 2_f.

It has been suggested upon several occasions that evaluations based upon eqn. 1 should be weighted to compensate for the altered error function arising from transformation of a hyperbolic to a linear function[5]. While this practice may be justified for data containing only random error the question arises as to its suitability for data containing both random and systematic errors. Values of k and K were obtained from equations 2_a to 2_f for several tenfold ranges in [S] using both weighted and unweighted linear least-squares fits of lines based upon $1/v$ *vs.* $1/[S]$ plots. It was found that either procedure could lead to values of k and K which were closer to the true values depending upon the particular range of [S] investigated. However, when equations 2_e and 2_f were employed the unweighted fits generally but not invariably led to more accurate values of k and K than did the weighted fits. Thus, it is clear that the practice of weighting to accommodate random error, when both random and systematic errors are present, is no panacea and if used must be used with discretion to avoid causing greater errors in the values of the kinetic constants than would obtain were it not used.

Eqn. 2 may be extended to accommodate situations involving reversible inhibition by reaction products or added inhibitor[1,6]. In the former instance eqn. 2 may be modified to give eqn. 3.

$$v = \{k\,(K_1/(K_1 - K))\,[E]\,[S]\}/\{K((K_1 + [S_T])/(K_1 - K)) + [S]\} + k_A\,[A] \qquad (3)$$

Evaluation of eqn. 3, with $k_A\,[A]$ defined as in Table I, $K_1 = 2K$, K and $0.5\,K$, $[S_T] = K$ and with the values of k, K, $\pm k_{A_1}$, $\pm k_{A_2}$, $\pm k_{A_3}$ and [E] used previously, was based upon the treatment of FOSTER AND NIEMANN[7-12]. In principle, the integral $\int_0^t$ (eqn. 3) dt was evaluated for $k_A\,[A] = 0$, to obtain values of t for arbitrarily chosen extents of reaction of 20 and 40 %. The integral $\int_0^t$ (eqn. 3) dt was then evaluated

for values of t corresponding to those above to obtain apparent values of [S'] that differed from those of [S] because of contributions arising from the term k_A [A]. The slopes of $([S_o] - [S_t])/t$ *vs.* $(\ln ([S_o]/[S_t]))/t$ and $([S_o] - [S'_t])/t$ *vs.* $(\ln ([S_o]/[S'_t]))/t$ plots[7-12], which were but slightly curved in the latter instance, were then used to arrive at an estimate of the apparent values of K'_1 relative to those of K_1, for which k_A [A] = 0. It was found that for equations 3a, 3b, and 3e $K'_1 > K_1$ and for 3c, 3d and 3f $K'_1 < K_1$. In other words, systematic error arising from no or under-correction of the enzyme or substrate blank reactions could lead one to over-estimate the magnitude of an apparent enzyme-product dissociation constant whereas over-correction of either of the two blank reactions could lead to the opposite result.

For an added inhibitor, eqn. 2 may be transformed into eqns. 4, 5 and 6, which may be associated with "totally competitive", "totally non-competitive"

$$v = \{k\,[E]\,[S]\}/\{K\,(1 + [I]/K_2) + [S]\} + k_A\,[A] \quad (4)$$

$$v = \{k\,[E]\,[S]/(1 + [I]/K_2)\}/\{K + [S]\} + k_A\,[A] \quad (5)$$

$$v = \{k\,[E]\,[S]\}/\{K + [S]\,(1 + [I]/K_2)\} + k_A\,[A] \quad (6)$$

and "totally uncompetitive" inhibition respectively[1]. Eqns. 4, 5 and 6 were evaluated for the values of k, K, $\pm k_{A_1}$, $\pm k_{A_2}$, $\pm k_{A_3}$, [E] and [S] used previously but with $(1 + [I]/K_2) = 1.0$, 1.1, 2.0 and 10.0 respectively. The data so obtained were presented in $1/v$ *vs.* $1/[S]$ plots to produce six sets of curves, each set consisting of a family of four curves. When examined over a sufficiently wide range of [S] the non-linearity of each curve was readily apparent. However, in contrast to the case involving only [S] as the independent variable, *vide ante*, it did not appear to be worth while to attempt to identify any of the above curves with those arising in the absence of a systematic error from a dependency upon [S] and [I] other than that assumed in eqns. 4, 5 and 6 principally because situations involving a simultaneous dependency upon $[S]^n$ and $[I]^m$ are largely unexplored. Instead, attention was directed to the more immediate question as to the possibility of confusing one type of inhibition with another by examining a system over a sufficiently narrow range of [S] as to be led

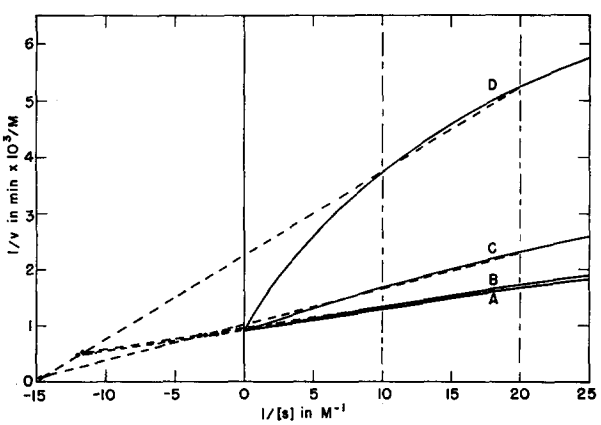

Fig. 2. Simulation of mixed inhibition by a "totally competitive" inhibitor: solid lines, eqn. 4a with $k_{A_1} = 2\ M/\text{min}/M$ to exaggerate the effects of the error term and with $(1 + [I]/K_2) = 1$, 1.1, 2 and 10 for curves A, B, C and D respectively; broken lines, extrapolated cords of eqn. 4a between $1/[S] = 20$ and $10\ M^{-1}$.

to the conclusion that the dependence of v upon [S] and [I] was linear rather than non-linear. For each family of four curves two or more ranges of [S], each entailing a two-fold variation in [S], were selected as to encompass as large a portion of each curve without leading to negative values of v. Each of the four chords of the line segments associated with a particular range in [S] was extrapolated to its intersection with the other three chords as illustrated in Fig. 2. These points of intersection then were examined to determine their ability to simulate a single point of intersection and to determine their proximity to the $1/v$ and $1/[S]$ axes. This procedure was repeated for all other ranges of [S] and for the other five sets of curves. This information, interpreted in terms of type of inhibition simulated is given in Table III.

TABLE III

SIMULATION OF ALTERNATIVE TYPES OF INHIBITION

As the significance of the error term $k_A[A]$ diminishes, simulation of alternative types of inhibition become less probable. However, even when this element of confusion becomes unimportant errors in the magnitudes if k, K and K_2 may persist.

Eqn.	$k_A[A]$	$k_{A_1}[E_T]$	$k_{A_2}[E_F]$	$-k_{A_1}[E_T]$	$-k_{A_2}[E_F]$	$k_{A_3}[S]$	$-k_{A_3}[S]$
4	"C"*	M**, (N)***	M**	M**	M**	M**	M**, (N)***
5	"N"§	M**	N§§	M**, (C)***, (N)***	N§§	M**, N§§	M**, N§§
6	"U"§§§	X*	X*	M**	M**	M**, (N)***	X*

* "Totally competitive" with systematic error.
** Inhibition of mixed types, *i.e.*, combination of partially competitive and partially non-competitive, etc.
*** Fortuitous simulation for a singular range of [S], or more accurately $[S]/K$.
§ "Totally non-competitive" with systematic error.
§§ No change in type but only in magnitude of k, K and K_2.
§§§ "Totally un-competitive" with systematic error.
* No currently recognized type.

It is evident from these data that situations interpreted as involving "totally non-competitive" inhibition require careful scrutiny in order to be certain that the type of inhibition and/or the magnitude of the inhibition constants are not a consequence of a systematic error arising from improper treatment of the enzyme or substrate blank reactions of a system which in fact may be "totally competitive" or "totally non-competitive" in basic character.

In the preceding discussion particular attention has been paid to systematic errors arising from enzyme or substrate blank reactions because such reactions are a common feature of many enzyme catalyzed reactions. In particular situations other systematic errors may arise. For example, if the specific substrate or reversible inhibitor is in facile equilibrium with a form, *e.g.*, a micelle, which interacts with the enzyme to a lesser degree than the monomer, K or K_2 will be over-evaluated since the observed values of v will exhibit the same dependencies upon [S] and [I] as in eqns. 1 and 4 with k_A [A] = 0. Alternatively, if a systematic error is present which is proportional to v, *i.e.*, eqn. 2 with k_A [A] = k_{A4} v, then only the value of k will be influenced and the plots will remain linear. An example of this situation would be a systematic error in the time scale. Similarly, a systematic error in the determination of [S] which is proportional to [S] will be reflected only in the value of K. Finally,

when a reaction is followed spectrophotometrically by measuring $-\mathrm{d}[S]/\mathrm{d}t$ or $\mathrm{d}[P]/\mathrm{d}t$, most departures from the BEER-LAMBERT relationship are representable by an equation of the form $[C]_{obs.} = [C]_{act.} \pm k_B [C]^n$ which can be differentiated to $\mathrm{d}[C]obs./\mathrm{d}t = \mathrm{d}[C]act./\mathrm{d}t \pm k_B\, n\, [C]I^{-1}\, \mathrm{d}[C]/\mathrm{d}t$. When $n = 1$, the systematic error will be proportional to v and, hence, reflected only in the value of k. Determining $\mathrm{d}[P]/\mathrm{d}t$ when $n \neq 1$ will introduce no error since $[P] = 0$ at zero time. However, determining $-\mathrm{d}[S]/\mathrm{d}t$ when $n = 2$ will give the rate expression the form of eqn. 2_e or 2_f, while other values of n, except $n = 0$, will give somewhat different curves when plotted in the usual manner.

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