

CLASSIC PAPER

Enzyme kinetics: the velocity of reactions

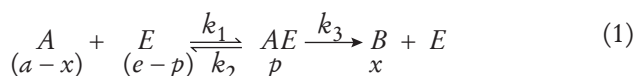
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In the post-genomic world, why are we interested in enzyme kinetics? Judging from the volume of papers published every year, the discipline is far from becoming obsolete. Rather, there is a growing interest spanning from theoretical aspects to practical applications, such as those of pharmacological relevance, with enzyme inhibition in pole position.

A classic paper is not necessarily one that has been, and continues to be, cited at a high rate. It can also be one that marked a starting point and stimulated the growth of an entire branch of science.

Enzymes at work

In 1925, in an article of fewer than two pages, George E. Briggs and John B.S. Haldane re-evaluated the equation of Michaelis and Menten “to examine its theoretical basis” and put forward an innovative hypothesis that called attention to a fundamental property of enzyme-catalysed reactions¹. They pointed out that the assumption of an equilibrium between substrate (A), enzyme (E) and their complex (AE), proposed by Michaelis and Menten², or the existence of an irreversible step in the formation of AE, proposed by van Slyke and Cullen³, were restrictive assumptions to describe the first step of reaction (see eqn 1). The symbols of the original paper are used here: a, the initial concentration of A; x, the concentration of product B at a time t; e, the total concentration of E; k₁, k₂ and k₃, rate constants (today’s customary k₁, k₋₁ and k₂ respectively); and p, the concentration of the AE complex.



Briggs and Haldane suggested that the rate of change of p, the concentration of the AE complex, is negligible compared with the rate of change of x and (a-x). There is no need to make assumptions about the rate constants since “data as to the course of the reaction can give no indication of the ratio of k₂ and k₃”¹. The rate equation for the mechanism shown in eqn 1, as written by Briggs and Haldane (eqn 2), was:

$$\frac{dx}{dt} = k_3 p = \frac{k_3 e (a - x)}{a - x + \frac{k_2 + k_3}{k_1}} \quad (2)$$

This becomes more familiar when more modern terminology is used, substituting: the reaction rate, v, for k₃p; k_{cat} for k₃; e₀ for the total enzyme concentration; s for the concentration of free substrate (a-x); and the Michaelis constant, K_m, for (k₂+k₃)/k₁, as shown in eqn 3:

$$v = \frac{k_{cat} e_0 s}{s + K_m} = \frac{V_s}{s + K_m} \quad (3)$$

A further substitution in eqn 3 (the limiting rate, V, for k_{cat}e₀) produces the traditional expression of the Michaelis–Menten equation, the fundamental equation of enzyme kinetics.

It is in its modern, widely used form (eqn 3), based on the treatment of Briggs and Haldane, not on the rapid equilibrium assumption used by Michaelis and Menten, that we recognize the importance of this theory. It represents a milestone in the history of enzyme kinetics, as the foundation of the steady-state treatment of enzyme-catalysed reactions.

The general message of Briggs and Haldane can be explained as follows: in the equilibrium treatment, the substrate dissociation constant (K_s=[E][S]/[ES]) tells us how much of the enzyme is present in the ES complex at thermodynamic equilibrium. K_m gives information about the proportion of enzyme present as ES as well, but for the reaction at steady-state, i.e. while the enzyme is actually ‘at work’, busy in turning over substrate. In the wake of these concepts, more intermediates, substrates and products can be added to eqn 1 and the catalytic step can be made reversible. Therefore eqn 3 gains broad validity and can describe mechanisms that are a great deal more complex than eqn 1. Briggs and Haldane, the veritable authors of the modern Michaelis–Menten equation, deserve an additional word of appreciation.

Dedicated to inhibitors

The effect of a competitive inhibitor on the rate of an enzyme-catalysed reaction can be appreciated using a modified form of the Michaelis–Menten equation, in which the K_m term in the denominator of eqn 3 is multiplied by the factor 1+(i/K_i), where i and K_i represent the concentration of inhibitor and its dissociation constant from the enzyme–inhibitor complex respectively. The resulting K_m(app) can then be used to calculate K_i, using appropriate plots at various inhibitor concentrations. A simple graphical method that simplifies this task and permits the direct calculation of K_i without the need for tedious secondary plots was published in 1953 by Malcom Dixon⁴ (yes, the same M. Dixon, co-author with M.C. Webb, of the best-seller *Enzymes*⁵). The Dixon plot for linear competitive inhibition is based on eqn 4.

$$\frac{1}{v} = \frac{K_m}{V_s} + \frac{1}{V} + \left(\frac{K_m}{V_s} \times \frac{i}{K_i} \right) \quad (4)$$

The plot of 1/v against i at constant s generates a straight line.

Measurements at a different substrate concentration give another straight line that intersects the previous one at a point whose abscissa co-ordinate equals $-K_m$. Even though this method cannot distinguish between competitive and mixed inhibition, and does not provide the value of the dissociation constant of the EIS complex for mixed and uncompetitive inhibitors, it is highly appreciated and widely used (3926 citations until September 2005, of which 262 were between 2000 and 2005). The Dixon plot was the first graphical method specifically dedicated to enzyme inhibition and its popularity must be related to its simplicity. One can also easily get rid of the above-mentioned deficiencies by the concomitant use of a similarly conceived graphical method⁶. This is one of several kinetic inventions published in the *Biochemical Journal* by Athel Cornish-Bowden, the inexhaustible enzyme kinetic volcano, well known for his *Fundamentals of Enzyme Kinetics*⁷. When used together, the two plots complement each other and permit identification of the mechanisms of inhibition and calculation the inhibition constants.

Simpleness without simplicity, and no maths required

What follows is a good remedy to allay the fears of mathematicophobic users. With the fast evolution of information technology, modern enzyme kinetics is becoming a computation-intensive discipline. This is fine but does not mean that graphical analysis of kinetic data is outmoded. If you are not convinced of this statement, you should read *The direct linear plot: a new graphical procedure for estimating enzyme kinetic parameters* by Robert Eisenthal and Athel Cornish-Bowden⁸. If you read just the title you might grumble, “Okay, okay, just another graphical representation of the Michaelis–Menten equation”. In this case, you are encouraged to read the whole article, which will immediately get your attention (this suggestion may be superfluous, as most readers probably already know that the direct linear plot is a smash hit in biochemistry). The article was flanked by an accompanying paper by the same authors (in inverted order) featuring a thorough analysis of the statistical implications of the new method⁹. Evidently the description of the graphical procedure in the first paper⁸ is convincing enough that most readers trust in it without verifying its mathematical fundamentals in the second paper⁹, at least judging from the citations (1475 for the first and 291 for the second paper).

When compared with three popular linear transformations of the Michaelis–Menten equation, namely the double reciprocal plot (Lineweaver–Burk), the s/v against s plot (Hanes–Wolf), and the v against v/s plot, often called the Eadie–Hofstee plot, the direct linear plot offers several practical and statistical advantages. Its mathematical expression is obtained by rearranging eqn 3 thus:

$$V = v + \frac{v}{s} \times K_m \quad (5)$$

So how does it work? Initial velocity data, v , collected at various substrate concentrations, s , are put in a graphic by placing s on the negative side of the abscissa, which represents K_m , and v on the ordinate, which represents the limiting rate, V . A line is then drawn through the two points, and the final plot consists of a set of straight lines. These intersect at a common point only in the absence of experimental error, a situation that never occurs in

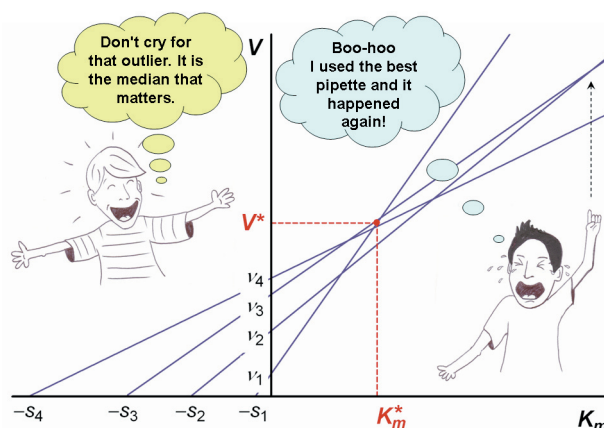


Figure 1. Direct linear plot. Determination of the best estimates of V and K_m (V^* and K_m^*) from the median of all intersection points (red dot). Robust parameter estimation makes the plot very tolerant to the presence of outliers.

practice, so that a pattern like that shown in Figure 1 is obtained.

What may strike first-time users of the direct linear plot is the treatment of K_m and V as variables and of the measured values s and v as constants, a practice that “may seem perverse”, as stated by Cornish-Bowden himself⁷. There is, however, nothing perverse in this method. Indeed, the mathematical background of a graphical procedure has never been so minutely explored. The group of statistics to which the direct linear plot belongs is known as distribution-free or non-parametric statistics. The plot provides an estimation of non-parametric confidence limits for V and K_m and the best values of these parameters are directly read from the plot as the coordinates of the median of all intersection points (Figure 1). Distribution-free statistics do not make particular assumptions about errors and, unlike parametric statistics, which take the sample mean as the best value of a given parameter, they use the median as the best estimator. The median, which is the middle value of a series of numbers, is much less sensitive to the presence of outliers than the mean of the same series, and its determination does not require calculation, only simple counting of the intersection points, e.g. from left to right, as shown in Figure 1; don’t cry any more about outliers.

Other classic enzyme kinetics papers

Although only three papers are acknowledged in this article, the actual inventory of excellent contributions is very long. Looking over the century’s output in the *Biochemical Journal* reveals innovative enzyme kinetic theories and methods that significantly contributed to its maturation towards a quantitative science. Such contributions extend over a number of branches of enzyme kinetics, including statistics^{10–14}, the burst reaction of chymotrypsin¹⁵, two- and three-substrate reactions^{16,17}, tightly bound substrates and inhibitors^{18,19}, methods for the derivation of steady-state equations^{20–23}, and integrated rate equations^{24,25}. To this partial and arbitrary list many others could be added.

Several articles (not necessarily classics), particularly those published in the first three decades of the past century, are appealing for their historical content and the copious details, including personal notes, that cannot be found elsewhere. So

if you have a few hours to spare, browse the early volumes in the archive of the *Biochemical Journal* (www.biochemj.org) and read articles such as that by H.P. Barendrecht²⁶, which fascinates with its lively criticism of results from the early days of enzyme kinetics: “the researches of the last few years on the kinetics of enzyme action have brought more confusion than clearness in this field”. He also comments that, “enzyme action spreads like radiation from an enzyme particle as centre... what kind of radiation this may be cannot yet be decided”. There is a touch of romanticism in this statement, is there not?

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