Commentary

The Steady State Approximation in Enzyme Kinetics: Reflections in Its Centennial Year

Eric Barnsley[1]

1. Independent researcher

The now-called quasi- (or pseudo-) steady state (QSS) approximation was introduced by Briggs and Haldane in an analysis of an unusual kinetic scheme for an enzyme-catalysed reaction, it had no product binding. The approximation, though perhaps not the kinetic scheme used, was well founded, and led to a solution of the rate equation of an enzyme-substrate intermediate. That in turn allowed the derivation of the well-known equation describing the kinetics of many enzymes, the Henri-Michaelis-Menten equation. Unfortunately, in more realistic kinetic schemes with product binding, the application of the QSS approximation leads to an uncertainty in the nature of kinetic constants as described by rate constants. It is shown here that for reasonable special cases of two kinetic schemes, first analysed by Haldane, that during the pre-steady state the reverse reaction may cause the concentration of the enzyme-substrate intermediate to rise to give a QSS essentially that of its final equilibrium concentration. This leads to a revision of the structure of kinetic constants (as describe by rate constants), and is sufficient to show the Haldane Relationship between catalytic constants and the equilibrium constant of the reaction is not generally true.

Corresponding author: E. A. Barnsley, eabarnsley@t-online.de

Introduction

It is the centennial year of the publication by Briggs and Haldane [1] of an approximation that is useful in obtaining a solution for the concentration of an enzyme-substrate intermediate in enzyme-catalyzed reactions. The approximation applies when a substrate concentration is many orders of magnitude greater than that of the enzyme, as is usual in studies *in vitro*, and as a result of which the nett rate of

change of the enzyme concentration is negligible compared with that of the substrate. Thus, in the rate equation for an enzyme-substrate intermediate, that rate can then be sensibly equated to zero. Although this has been called a steady state, that expression was not used by Briggs and Haldane, and the descriptions pseudo-steady or quasi-steady state (QSS) are preferable. The analysis of Briggs and Haldane was complete for the unusual kinetic scheme they used, one with absolutely no binding of the product to the enzyme, but reactions are to some degree reversible and for some of these its application has provided ambiguous descriptions of the kinetic constants of the Henri-Michaelis-Menten (HMM) equation (equation 3).

The QSS approximation was used by Haldane [2][3] in analyses of two reversible kinetic models, schemes 1 and 2, below, and since then by many others in the analyses of numerous more complex two-substrate reaction schemes. The common procedure is to write the rate equation for all enzyme components, equate each of them to zero, and solve the resulting simultaneous equations to find the concentration of the intermediate that gives rise to product. A HMM equation can be obtained only for initial velocities (meaning those at the outset of the QSS), but whether the solution of the equations is done purely algebraically, or by using a matrix solution for simultaneous equations, or another algorithm such as that of King and Altman [4], or using Cleland's concept of partition analysis and net rate constants [5], the result always neglects the possible effect of the back reaction, during the pre-steady state, on the QSS concentration.

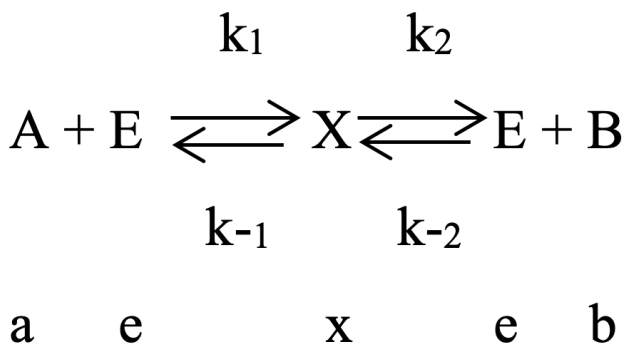
Results of analyses made around sixty years ago by Miller and Alberty [6] and Walter and Morales [7] showed that for scheme 1 at the beginning of the QSS, dx/dt may have an extremely low positive value with d^2x/dt_2 negative and both of these approaching zero, that is x is approaching its equilibrium value. This contrasts with the usual assumption that, if $dx/dt=0$, the value of x is a maximum and, as pointed out by Morales and co-workers [8][9], that d^2x/dt_2 is negative. The consequence that, at the outset of the QSS, x may essentially have its equilibrium concentration, leads to a significant difference between descriptions, written as rate constants, in the catalytic constants of the derived HMM equation (equation 1). This has have been entirely overlooked, even by the latter authors themselves. The QSS approximation may be useful for solving equations and leading to a HMM equation, but without further information the catalytic constants written in terms of rate constants are insecure.

For model 1, when k_1 is greater than k_{-2}, the K_m for reactant A is that given by Haldane, $(k_{-1}+k_2)/k_1$, but when $k_{-2}>k_1$ the concentration of the intermediate X is essentially its equilibrium concentration and its

K_m is ($k_{-1}/k_1+k_2/k_{-2}$) (see below). Without information about the relative values of the binding constants k_1 and k_{-2}, an experimentally derived K_m cannot be interpreted in terms of rate constants. Although the kinetic scheme 1, used by both Miller and Alberty [6] and Walter and Morales [7] must be viewed as inadequate (see references 2 and 3), I show below that analyses of reasonable special cases of scheme 2 lead to similar consequences to those deduced for scheme 1. A full analysis of scheme 2 is not possible, but computer-aided numerical computations, starting with appropriate sets of rate constants for each fixed equilibrium constant, could survey this matter. A further consequence of these observations is that the Haldane Relationship is not generally valid.

The analysis of reversible reactions

A thorough analysis of scheme 1 has already been given [6]. The scheme itself is inadequate, however, because X gives rise directly to A and B and the scheme probably lacks an elementary step. It is, however, that used by Henri [10] and Michaelis and Menten [11] in deriving the HMM equation, and it remains the standard starting point in teaching enzyme kinetics. I shall outline here a simplified version of the analysis of Miller and Alberty [6], because it is useful in analyses of special cases of scheme 2. This scheme was introduced by Haldane [2][3] because he recognized that scheme 1 was inadequate to describe experimental



Scheme 1.

results in the hydrolysis of sucrose, catalysed by invertase, which was the reaction for which Henri [10] and Michaelis and Menten [11] produced their kinetic analyses. These authors knew the reaction

doi.org/10.32388/1C11NK

was reversible and subject to product inhibition, but Haldane pointed out that scheme 1 could not explain the formation of methyl glucoside when the reaction was carried out in the presence of methanol.

Scheme 1

In this scheme, A and B are reactants, E the enzyme and X an enzyme-reactant intermediate. The corresponding small case letters represent their concentrations, and the letters k are rate constants. The conservation equation for E is given by $e_o = e+x$, the approximate conservation equation for A by $a_o=a+b$, and in both of these the subscript o refers to the total concentration. Using these, the rate equation for X is given by equation 1 for which there is no analytical solution.

$$dx/dt=\{k_1a_o+(k_{-2}-k_1)b\}e_o- x\{k_1a_o+(k_{-2}-k_1)b+k_{-1}+k_2\} \quad 1.$$

In the usual analysis [2][3], when b is negligible compared with a_o, terms in b are neglected, so that at the beginning of the QSS the initial velocity, $v_i= k_2x$, is given by equation 2, and this

$$v_i = k_2e_oa_o/\{a_o+(k_{-1}+k_2)/k_1\} \quad 2.$$

$$v_i = k_{cat}e_oa_o/(a_o+K_m) \quad 3.$$

identifies in the HMM equation (generally written as equation 3) the catalytic constants as $k_{cat}^A=k_2$, and $K_m^A=(k_{-1}+k_2)/k_1$. The approximation, $a_o=a+b$, neglects only the term $k_1x(e_o-x)$ in comparison with $\{k_1a_o+(k_{-2}-k_1)\, b\}\, e_o$, and is well justified under the usual conditions *in vitro* when $a_o >> e_o$.

Miller and Alberty [6] noted, however, that when $k_1=k_{-2}$, the terms in b in equation 1 disappear, and the variables in the equation can be separated to give equation 4. Integration gives equation 5 (the constant of integration is given by the condition that x=0 when t=0).

$$1/k_1a_oe_o)dx=[1-(x/e_o)\{(1+(k_{-1}+k_2)/k_1a_o\}]dt \quad 4.$$

$$x=a_oe_o\{1-\exp-(k_1a_o+k_{-1}+k_2)t\}/\{a_o+(k_{-1}+k_2)/k_1\} \quad 5.$$

Equation 5 describes the concentration x throughout the pre-stead state and the QSS. The substitution of Haldane's expression for K_m^A gives equation 6, and this and equation 6

$$x=a_oe_o\{1-\exp[[-k_1(a_o+K_m^A)t\}/(a_o+K_m^A)] \quad 6.$$

describe monotonic exponential curves. For sensible values of a_o, e_o K_m and k_1, the curve plateaus very rapidly at $x=a_oe_o/ (a_o+K_m^A)$ which is the usual solution for x. Equation 6 rearranges to give equation 7, and if for example, x/x_{equ} is given the value 0.999 and if $a_o=K_m$,

$t = -\log_e(1-x/x_{equ})/k_1(a_o+K_m)$ 7.

then for the reasonable values of $k_1=10^6 M^{-1}sec^{-1}$ and $K_m = 10^{-3}M$, t = 3.5 $x10^{-3}$ sec. The QSS is reached after an extremely short pre-steady state; in the words of Briggs and Haldane [1] "in the first instant."

Miller and Alberty [6] then used approximation methods to examine the result with different relative values of k_1 and k_{-2}. For the case when $k_1>k_{-2}$, x rose to a maximum before declining (that is the usual description of events at the beginning of the QSS), and for the condition $k_{-2}>k_1$, they stated their approximations were not reproducible although some did show x rising to a plateau. This situation was later clarified by Walter and Morales [7] who used computer aided calculations to show that, when $k_{-2}>k_1$, x always plateaued at the equilibrium value, so that x is given by $x=a_oe_o/\{a_o+(k_{-1}/k_1+k_2/k_{-2})\}$. Consequently, although k_{cat} remains express as k_2, the K_m^A is $(k_{-1}/k_1+k_2/k_{-2})$. In the reverse with direction with $k_{-2}>k_1$, K_m^B and k_{cat} would be those given by Haldane [2][3]. Substitution of these results in the Haldane Relationship does not lead to the equilibrium constant of the reaction.

The results calculated by Walter and Morales [7] may not be expected intuitively, but the result is a necessary one when $k_{-2}>k_1$. The differentiation of equation 1 leads to equation 8. If for convenience in equation 8, the term $\{k_1a_o+(k_{-2}-k_1)\,b+k_{-1}+k_2\}$ is abbreviated as N,

$$dx^2/dt_2=(k_{-2}-k_1)(db/dt)(e_o-x)-dx/dt\{k_1a_o+(k_{-2}-k_1)b+(k_{-1}+k_2)\} \quad 8.$$

dx/dt is substituted from equation 1, and d^2x/dt_2 is equated to zero, then separation of the terms in e_o and x leads to the value of x/e_o given by equation 9. The term N contains b which

$$x/e_o=[(k_{-2}-k_1)(db/dt)-N\{k_1a_o+(k_{-2}-k_1)b\}]/[(k_{-2}-k_1)(db/dt)-N\{k_1a_o+(k_{-2}-k_1)b\}-N(k_{-1}+k_2)] \quad 9.$$

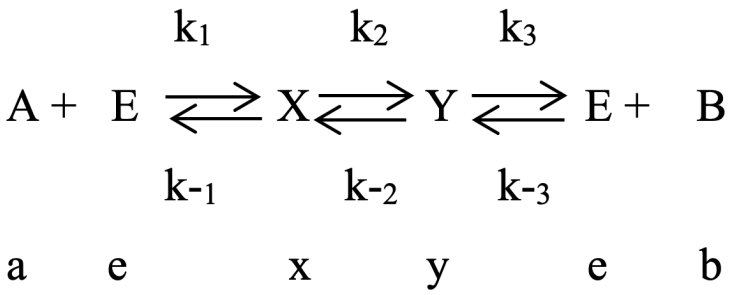
varies during the reaction, but N (derived from $(k_1a+k_{-2}b)$ in deriving equation 1) is always positive. Thus, in equation 9, the numerator is greater than the denominator, implying the impossible solution $x/e_o>1$. Consequently, when db/dt and dx/dt are both positive, dx^2/dt_2 cannot be zero. It becomes zero only at equilibrium, and so consequently there is not an inflection (change in the sign of the curvature) in the time course of x as it rises to x_{equ}. ($d^2x/dt_2 = 0$ is required at an inflection point).

The values of the kinetic constants are matters for experimental measurement, but a comparison, of values to be expected from analyses obtained here with those of Haldane, shows just how large an error in Haldane's results would exist if individual rate constants were known. Thus, for scheme when $k_{-2}>k_1$, $K_m^A=(k_{-1}/k_1+k_2/k_{-2})$ and that of Haldane is $K_m^{HA}=(k_{-1}+k_2)/k_1$. The ratio K_M^{HA}/K_m^A simplifies to (1+k-

$_2K_{equ}/k_1)/(1+K_{equ})$, and consequently, for a given reaction (constant K_{equ}), the ratio K_M^{HA}/K_m^A is directly proportional to k_{-2}/k_1, is 1 when $k_{-2}=k_1$, and has a slope of $K_{equ}/(1+K_{equ})$. For different reactions at a given k_{-2}/k_1, K_M^{HA}/K_m^A decreases with increase in K_{equ}, and the slope (obtained by differentiation) is given by $(k_{-2}/k_1-1)/(1+K_{equ})^2$.

Scheme 2

Haldane [2][3] also applied the QSS approximation in his analyses to the more realistic kinetic scheme 2 in which an elementary step with the formation of an enzyme-product species (Y) has been added. A complete analysis of scheme 2 could be made by modern



Scheme 2.

methods of numerical integration, and this would require that, for each of a range of equilibrium constants, reasonable sets of rate constants be examined. Each set would be consistent with the chosen equilibrium constant, $K_{equ}= k_1k_2k_3/k_{-1}k_{-2}k_{-3}$. Reasonable special cases of scheme 2, however, reduce the analysis to that given for scheme 1, and are sufficient to show that, in these special cases, and staring a reaction with A, when $k_{-3}>k_1$, y has its equilibrium value in the QSS. This again leads to expressions for K_m different from that given by Haldane, and also a different one for k_{cat}.

One special case occurs if X and Y are always in equilibrium [12], and a second special case is that when $k_{-1}=k_3$. The conservation equation for A is $a_o=a+b+x+y$, and this gives the following exact differentials: $da+db = -dx-dy$. The rate equations for da/dt and db/dt are equations 10 and 11, and rearrangement of their sum and substitution of $a=(a_o-b)$ and $k_{-1}=k_3$, leads to equation 12. If $k_{-3}=k_1$, the terms in b disappear in equation 13, and the

$da/dt = k_{-1}x - k_1 a(e_o - x - y)$ 10.

$db/dt = k_3 y - k_{-3} b(e_o - x - y)$ 11.

$d(x+y)/dt = = e_o\{k_1 a_o + (k_{-3} - k_1)b\} - (x+y)\{k_1 a_o + (k_{-3} - k_1)b + k_{-1}\}$ 12.

variables in (x+y) and t can be separated and integrated as shown for x in Scheme 1. The QSS is that when (x+y) closely approach ($x_{equ}+y_{equ}$), and the QSS concentration of Y is y_{equ}. For the condition $k_{-3}>k_1$, (x+y) in equation 12 can be dealt with as was x in scheme 1 to show that the QSS is also given by ($x_{equ}+y_{equ}$). The results are not in agreement with those of Haldane (for the condition $k_{-3}=k_1$), and are $k_{cat}^{A}=k_2k_3/(k_{-2}+k_2)$ and $K_m^{A}= k_3(k_{-2}k_{-3}+k_1k_2)/k_1k_{-3}(k_{-2}+k_2)$.

Discussion

The analysis presented in this communication for scheme 1 recapitulate that in the publications of Miller and Alberty [6], but for simplicity employ an approximation of the conservation equation for the reactant, and also show why the computations of Walter and Morales [7] for scheme 1 are to be expected. For reasonable special cases of scheme 2, a similar analysis is outlined, and these analyses show that use of the QSS assumption as introduced by Haldane [2][3] to provides expressions for K_m and k_{cat} is not adequate. Although the QSS approximation itself may be an excellent numerical one, it may lead to an ambiguity in the description of catalytic constants in term of rate constants. Haldane also wrote (2,3, p.82), “Perhaps, however, the most important result of the above investigation is ... [an equation for the velocity of the reaction which ... when equilibrium is reached ... [leads to the equation]... $k_{cat}^{A}K_m^{B}/k_{cat}^{B}K_m^{A}=K_{equ}$. So this quantity is equal to the equilibrium constant, [K_{equ}] which depends on the free energies of A and B, and is independent of the catalyst.” (Paraphrased, with added words in brackets [], by this author). As recently as 2004, Alberty [13] stated that his earlier work with Bock [14], using results obtained with the enzyme fumarase, had confirmed the validity of this Haldane Relationship. It has been shown by Rose [15], however, that there are several possible kinetic pathways within each of the four sub-units of fumarase, and so the actual mechanism cannot be described by either scheme 1 or 2. The Haldane Relationship is correct when $k_1=k_{-2}$ in scheme 1, and when $k_1=k_{-3}$ in scheme 2, but otherwise it is not generally correct.

Haldane’s analyses [2][3] were followed by their application to complex models of two-substrate reactions in which the concentration of one reactant was kept constant (fixed), allowing the derivation of HMM

equations with “apparent” kinetic constants, each pair for one fixed concentration, and all of these analyses will have the same uncertainties outlined for Haldane’s work. It should be noted that, for the kinetic scheme they used, the results deduced by Briggs and Haldane [1] are irreproachable, but that scheme was exceptional, lacking any product enzyme binding: it was not scheme 1. The long failure (also by this author) to recognize the insufficiency of the usual application of the QSS approximation is a puzzle.

Modern methods of evaluating experimental kinetic measurements are made by computer aided numerical integration. This applies to the direct determination of catalytic constant for the HMM equation directly from primary experimental data [16], and to determining actual rate constants for a given model based on the expressions for k_{cat} and K_m determined by the QSS approximation [17]. For scheme 2 and more complex ones, it is clear that, in the first place, K_{equ} for the reaction should be known, and for that, reasonable sets of rate constants consistent with K_{equ} should be examined. Such extensive computations may demonstrate if for scheme 2 the relative values of the binding constants of reactants and products are the only determining factor that leads to the steady state in one direction being the final equilibrium concentration of enzymic intermediates. For more complex schemes for which computations are made starting with a derived analytical equation based on the QSS approximation, the reliability of that equation must be checked. It will probably be helpful if all text books describing the use of the QSS assumption are updated.

In contemplating the historical developments in studies of enzyme kinetics it may be questioned if the QSS assumption as applied was superior to the equilibrium one of Henri [10] and Michaelis and Menten [11]. The former is based on a very good approximation which is generally true when $a_o >> e_o$, and was thought to give a more general interpretation of the kinetic constants in the HMM equation whereas the equilibrium assumption was arbitrary and identified a K_m as a dissociation constant. But as applied the QSS assumption did not reveal all possible constants correctly, and furthermore, it does not always provide a solution, such as in the analysis of a two-substrate reaction with random order of substrate binding. Both the equilibrium and the QSS assumption may lead with relative simplicity to an HMM equation, but for both further information about individual rate constants is required to determine the structure of catalytic constants.

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Declarations

Funding: No specific funding was received for this work.

Potential competing interests: No potential competing interests to declare.