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Commentary

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

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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 in which the enzyme 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 the two kinetic schemes first analysed by Haldane, during the pre-steady state the reverse reaction affects the concentration of the enzyme-substrate intermediate so that it rises to give a QSS essentially that of its final equilibrium concentration. This leads to a revision of the structure of kinetic constants (as described by rate constants) and is sufficient to show that the Haldane Relationship between catalytic constants and the equilibrium constant of the reaction is not generally true.

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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-catalysed 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 net 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 reverse binding of the product to the enzyme, but reactions are to some degree reversible and for these the QSS assumption may lead to 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 those of

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King and Altman [4] and using Cleland [5], the result always neglects the role of the back reaction on the QSS concentration reached during the pre-steady state.

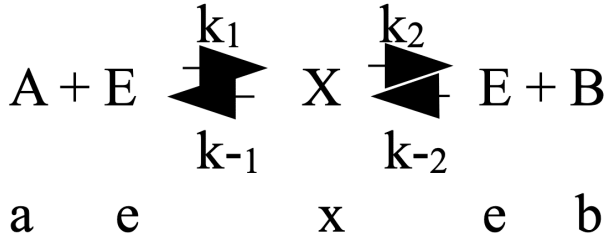
Results of analyses made around sixty years ago by Miller and Alberty [6] and Walter and Morales [7] showed for scheme 1 at the beginning of the QSS, that dx/dt may have an extremely low positive value with d^2x/dt_2 negative and both of these approaching zero. That is, x is approaching its equilibrium 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, of the catalytic constants of the derived HMM equation (equation 1). This has 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 $(k_{-1}/k_1 + k_2k_{-2})$ (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 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, the one 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 also useful in analyses of special cases of scheme 2. This scheme was introduced by Haldane [2][3] because he recognised that scheme 1 was inadequate to describe experimental 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 was reversible and subject to product inhibition, but Haldane also 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



Scheme 1.

doi.org/10.32388/1C11NK.2

In this scheme, A and B are reactants, E the enzyme and X an enzyme-reactant intermediate. The corresponding lower-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.

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

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, $v_i = k_2 x$, is given by equation 2, and in the HMM equation (generally written as equation 3), this identifies the catalytic constants as $k^A_{cat} = k_2$, and $K^A_m = (k_{-1} + k_2)/k_1$.

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

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

The approximation, $a_o = a + b$, neglects only the term $k_1 x(e_o - x)$ in comparison with $\{k_1 a_o + (k_{-2} - k_1)b\}e_o$, 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 $t = 0$).

$$(1/k_1 a_o e_o)dx = [1 - (x/e_o)\{(1 + (k_{-1} + k_2))/k_1 a_o\}]dt \quad (4)$$

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

Equation 5 describes the concentration x throughout the pre-steady state and the QSS. The substitution of Haldane's expression for K^A_m gives equation 6, and this describes a monotonic exponential curve.

$$x = a_o e_o\{1 - \exp[[-k_1(a_o + K^A_m)t\}/(a_o + K^A_m)] \quad (6)$$

For sensible values of a_o, e_o, K_m and k_1, the curve plateaus very rapidly at $x = a_o e_o/(a_o + K^A_m)$, 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 $a_o = K_m$, then for the reasonable values of $k_1 = 10^6 M^{-1} sec^{-1}$ and $K_m = 10^{-3} M, t = 3.5 \times 10^{-3}$ sec.

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

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 (which 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 $x = a_o e_o/\{a_o + (k_{-1}/k_1 + k_2/k_{-2})\}$. Consequently, although k_{cat} remains expressed as k_2, the K^A_m is $(k_{-1}/k_1 + k_2/k_{-2})$. In the reverse direction with $k_{-2} > k_1$, K^B_m and k^B_{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 $\{k_1 a_o + (k_{-2} - k_1)b + k_{-1} + k_2\}$ is abbreviated as N, dx/dt is substituted from equation 1, and dx^2/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.

$$d\mathrm{x}^2/d\mathrm{t}_2 = (\mathrm{k}_{-2} - \mathrm{k}_1)(d\mathrm{b}/d\mathrm{t})(\mathrm{e}_o - \mathrm{x}) - d\mathrm{x}/d\mathrm{t}\{\mathrm{k}_1\mathrm{a}_o + (\mathrm{k}_{-2} - \mathrm{k}_1)\mathrm{b} + (\mathrm{k}_{-1} + \mathrm{k}_2)\} \quad (8)$$

The term N contains b, which varies during the reaction, but N, (derived from $(\mathrm{k}_1\mathrm{a} + \mathrm{k}_{-2}\mathrm{b})$ in deriving equation 1), is always positive.

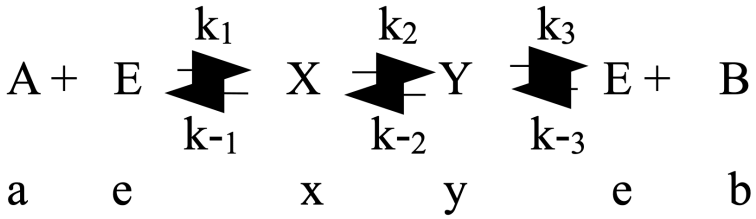
$$\begin{aligned} \mathrm{x}/\mathrm{e}_o = \; & [(\mathrm{k}_{-2} - \mathrm{k}_1)(d\mathrm{b}/d\mathrm{t}) - \mathrm{N}\{\mathrm{k}_1\mathrm{a}_o + (\mathrm{k}_{-2} - \mathrm{k}_1)\mathrm{b}\}]/ \\ & [(\mathrm{k}_{-2} - \mathrm{k}_1)(d\mathrm{b}/d\mathrm{t}) - \mathrm{N}\{\mathrm{k}_1\mathrm{a}_o + (\mathrm{k}_{-2} - \mathrm{k}_1)\mathrm{b}\} - \mathrm{N}(\mathrm{k}_{-1} + \mathrm{k}_2)] \end{aligned} \quad (9)$$

Thus, in equation 9, the numerator is greater than the denominator, implying the impossible solution $\mathrm{x}/\mathrm{e}_o > 1$. Consequently, when $d\mathrm{b}/d\mathrm{t}$ and $d\mathrm{x}/d\mathrm{t}$ are both always positive, $d\mathrm{x}^2/d\mathrm{t}_2$ cannot be zero, and $\mathrm{x}_{\mathrm{equ}}$ is the concentration in the QSS. (If the time-course of x passes through a QSS before it rises to $\mathrm{x}_{\mathrm{equ}}$, there would be an inflection (a change in the sign of the curvature) at which $d\mathrm{x}^2/d\mathrm{t}_2 = 0$).

The values of the kinetic constants are matters for experimental measurement, but a comparison of values to be expected from the 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 the scheme when $\mathrm{k}_{-2}/\mathrm{k}_1$, $\mathrm{K}_{\mathrm{m}}^{\mathrm{A}} = (\mathrm{k}_{-1}/\mathrm{k}_1 + \mathrm{k}_2/\mathrm{k}_{-2})$ and that of Haldane is $\mathrm{K}_{\mathrm{m}}^{\mathrm{HA}} = (\mathrm{k}_{-1} + \mathrm{k}_2)/\mathrm{k}_1$. The ratio $\mathrm{K}_{\mathrm{M}}^{\mathrm{HA}}/\mathrm{K}_{\mathrm{m}}^{\mathrm{A}}$ simplifies to $(1 + \mathrm{k}_{-2}\mathrm{K}_{\mathrm{equ}}/\mathrm{k}_1)/(1 + \mathrm{K}_{\mathrm{equ}})$, and consequently, for a given reaction (constant $\mathrm{K}_{\mathrm{equ}}$), the ratio $\mathrm{K}_{\mathrm{M}}^{\mathrm{HA}}/\mathrm{K}_{\mathrm{m}}^{\mathrm{A}}$ is directly proportional to $\mathrm{k}_{-2}/\mathrm{k}_1$, is 1 when $\mathrm{k}_{-2} = \mathrm{k}_1$, and has a slope of $\mathrm{K}_{\mathrm{equ}}/(1 + \mathrm{K}_{\mathrm{equ}})$. For different reactions at a given $\mathrm{k}_{-2}/\mathrm{k}_1$, $\mathrm{K}_{\mathrm{M}}^{\mathrm{HA}}/\mathrm{K}_{\mathrm{m}}^{\mathrm{A}}$ decreases with an increase in $\mathrm{K}_{\mathrm{equ}}$, and the slope (obtained by differentiation) is given by $(\mathrm{k}_{-2}/\mathrm{k}_1 - 1)/(1 + \mathrm{K}_{\mathrm{equ}})^2$.

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 methods of numerical integration, and this would require that, for each of a range of equilibrium constants, reasonable sets of rate constants be examined.



Scheme 2.

Each set would be consistent with the chosen equilibrium constant, $\mathrm{K}_{\mathrm{equ}} = \mathrm{k}_1\mathrm{k}_2\mathrm{k}_3/\mathrm{k}_{-1}\mathrm{k}_{-2}\mathrm{k}_{-3}$. 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 starting a reaction with A, when $\mathrm{k}_{-3} > \mathrm{k}_1$, 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 $\mathrm{k}_{\mathrm{cat}}$.

One special case occurs if X and Y are always in equilibrium [12], and a second special case is that when $\mathrm{k}_{-1} = \mathrm{k}_3$. The conservation equation for A is $\mathrm{a}_o = \mathrm{a} + \mathrm{b} + \mathrm{x} + \mathrm{y}$, and this gives the following exact differentials: $d\mathrm{a} + d\mathrm{b} = -d\mathrm{x} - d\mathrm{y}$. The rate equations for $d\mathrm{a}/d\mathrm{t}$ and $d\mathrm{b}/d\mathrm{t}$ are equations 10 and 11, and rearrangement of their sum and substitution of $\mathrm{a} = (\mathrm{a}_o - \mathrm{b})$ and $\mathrm{k}_{-1} = \mathrm{k}_3$ leads to equation 12. If $\mathrm{k}_{-3} = \mathrm{k}_1$, the terms in b disappear in equation 13, and the variables in $(\mathrm{x} + \mathrm{y})$ and t can be separated and integrated as shown for x in Scheme 1.

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

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

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

The QSS is that when $(x + y)$ closely approaches $(x_{equ} + y_{equ})$, 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 $k^A_{cat} = k_2k_3/(k_{-2} + k_2)$ and $K^A_m = k_3(k_{-2}k_{-3} + k_1k_2)/k_1k_{-3}(k_{-2} + k_2)$.

Discussion

The analysis presented in this communication for scheme 1 recapitulates that in the publications of Miller and Alberty [6], but for simplicity employs an approximation of the conservation equation for the reactant, and also shows 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 provide expressions for K_m and k_{cat} is not adequate. Although the QSS approximation itself may be an excellent numerical one, the description of catalytic constants in terms of rate constants is ambiguous. 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]... $k^A_{cat}K^B_m/k^B_{cat}K^A_m = K_{equ}$. 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 in the special cases of scheme 2 when $k_1 = k_{-3}$, 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 rebinding of product to the enzyme: it is not scheme 1. The long failure (also by this author) to recognise the insufficiency of the usual application of the QSS approximation is a puzzle. The failure persists in textbooks of biochemistry and enzyme kinetics, and the literature is replete with uncertain descriptions of catalytic constants written in terms of rate constants.

Modern methods of evaluating experimental kinetic measurements are made by computer-aided numerical integration. This applies to the direct determination of catalytic constants 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 a numerical analysis has been published [18], but its scope was limited and not designed to reveal in general the relevance of k_1/k_{-3}. For more complex ones, it is clear that, in the first place, K_{equ} for the reaction should be defined, and for that K_{equ} reasonable sets of rate constants consistent with it 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 enzyme 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 may be helpful if all textbooks 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. 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.

Statements and Declarations

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Potential Competing Interests

No potential competing interests to declare.

Data Availability

This is a theoretical study and did not generate new data. All sources analysed are cited in the References section.

Author Contributions

E.A.B. was the sole author and is responsible for the conception, analysis, and writing of the manuscript.

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Declarations

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