

Research Article

False Assumptions in Derivations of the Henri-Michaelis-Menten Equation When Using the Quasi-Steady-State Assumption

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Haldane used the quasi-steady state assumption in analyses of the kinetics of reversible enzyme-catalysed reactions, but to obtain an initial velocity used a false assumption which converted his analyses to ones for irreversible reactions. With the exception of a few special cases in which the steady state concentration of the species giving rise to the product has its equilibrium concentration, a correct analysis shows that the parameters of derived Henri-Michaelis-Menten equations for reversible reactions will in general have a term containing a product concentration. The determination of the value of an individual rate constant from an experimentally measured Michaelis constants requires a correct analysis of the kinetic scheme under consideration, and the arbitrary use of equations lacking a product concentration is invalid. These observations are discussed in terms of the teaching of enzyme kinetics and the use of numerical methods to obtain individual rate constants.

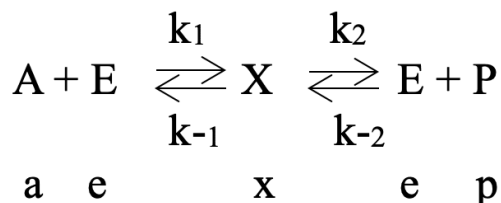
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1. Introduction

This work is an elementary review of early analyses of kinetic models for reaction that lead to a Henri-Michaelis-Menten (HMM) equation (equation 1), and of Haldane's analyses, made specifically for reversible reaction. Because of a faulty assumption he made, Haldane converted the analyses to ones for irreversible reactions. The work is intended primarily for teachers of enzyme kinetics and authors of text books containing such material, but it may also be noted that Haldane's error was incorporated into later research on more complex kinetic systems and into an algorithm designed to simplify the solution of rate equations. The pertinent references are in the following text. The HMM equation was first introduced theoretically ^[1], and then confirmed experimentally ^[2], in both cases after experimental studies on a reversible reaction, the hydrolysis of sucrose catalysed by invertase.

$$v_i = k_{cat} e_o a_o / (a_o + K_m) \quad (1)$$

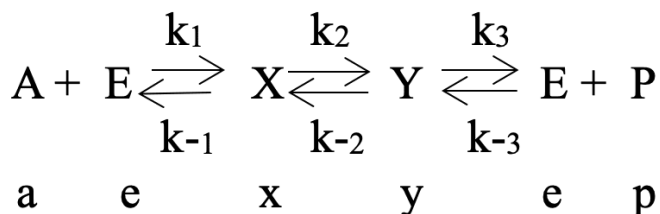
In equation 1, v_i is the initial reaction rate, a and e are the concentrations of reactant, A, and enzyme, E, respectively, and the subscripts o indicate initial concentrations. These initial conditions were used because it was known the reaction was inhibited by product, and v_i was measured in the experimental confirmation [2]. The kinetic model used in the derivation was scheme 1, where P is the product and the letters k are rate constants. Other small case letters are those used



Scheme 1.

for concentrations. In the first theoretical derivation of equation 1, however, no reference to the product concentration and the reverse reaction was required because of an assumption used in the derivation, that E and A come very rapidly to equilibrium and remain so.

Haldane [3][4] later made analyses of scheme 1 and scheme 2 using the now called quasi-



Scheme 2.

steady state (QSS) or pseudo-steady state assumption, which, together with Briggs [5], he had introduced in an analysis of a variant of scheme 1. In this variant of scheme 1, the authors used a completely irreversible reaction (all reference to a reverse binding of P with E was omitted, p played no role in the kinetics, and the velocity derived was that for the value of v at any concentration of A during the reaction.

Seven years before the republications of Haldane's original work on reversible reactions [4], Miller and Alberty [6] gave an analysis of scheme 1 that showed Haldane's analysis did not always provide the correct time

course of X in the reaction, and this was confirmed numerically by Walter and Morales [7], but this work appears to have been overlooked until recently [8][9]. Perhaps because of the importance of the ratio k_{-2}/k_1 that had been demonstrated with scheme 1 [6][7], I had assumed [8] that Haldane's derivations might be correct in one direction, but a re-analysis of his work shows that not only was that assumption incorrect, but that an additional assumption made by Haldane was wrong. The HMM equation for a reversible reaction requires an initial reactant concentration, and in his treatment of scheme 1 with a reaction starting with A, Haldane simply set p to zero in his equations. He did not why, but that always eliminates any role for k_{-2} in scheme 1. If used for scheme 2, setting p to zero eliminates a role k_{-3} , and for a reaction starting with P the role of k_1 . Consequently, his analyses were for completely irreversible reactions.

Haldane's assumption, that in reactions starting with A it is sufficient to write $p=0$, persists in text books, has become one of general use and is generally used in analyses of two-substrate reactions, and is even deliberately written into an algorithm [10] intended to simplify the analysis of kinetic schemes. The error is elementary and demonstrable with the simplest algebra, but I give a summary of Haldane's analysis of scheme 2 in section 2 because that makes the source of the error perfectly clear, and two of his equation are in fact useful in the correct analysis given in section 3. In this latter section, a valid approximation to obtain an initial velocity in the QSS is used, and it is concluded that derivations of the parameters of equation 1, obtained for reversible reactions and containing only rate constants, must, in general, be incorrect. In section 4, there is an alternative derivation of an HMM equation for reversible reactions, which does provide a little relief from the elementary but tedious algebra of section 3. In section 5, the current presentation of reversible kinetics in text books is discussed, and also draws attention to the added difficulty of using numerical methods for the determination of individual rate constants when these depend on analytically derived expressions for experimentally determined parameters of the HMM equation.

2. The problem with Haldane's analyses

Scheme 1 has always been an unsatisfactory model for an enzyme catalysed reaction because it contains steps relating only to the binding of A and P with E, and lacks an elementary step in which an interconversion of the enzyme-bound forms of A and P occurs. Haldane introduced scheme 2 because scheme 1 could not account for the observation, made during the hydrolysis of sucrose catalysed by invertase in the presence of methanol, that β -methyl glucoside accumulated. None the less, his analysis was for an isomerisation reaction, and I shall use the scheme as such.

Haldane [3][4] gave the rate equations for X and Y in scheme 2 as equations 2 and 3, equated them to zero, and solved them as simultaneous equations to give equations 4 and 5 for y and x respectively.

$$\frac{dx}{dt} = k_1 a(e_0 - x - y) + k_{-2} y - x(k_{-1} + k_2) \quad (2)$$

$$\frac{dy}{dt} = k_{-3}p(e_0 - x - y) + k_2x - y(k_{-2} + k_3) \quad (3)$$

$$y = \frac{\{k_1k_2a + k_{-3}(k_{-1} + k_2)p\}e_0}{k_1(k_{-2} + k_2 + k_3)a + k_{-3}p(k_{-1} + k_{-2} + k_2) + (k_{-1}k_{-2} + k_{-1}k_3 + k_2k_3)} \quad (4)$$

$$x = \frac{\{k_1a(k_{-2} + k_3) + k_{-2}k_{-3}p\}e_0}{k_1(k_{-2} + k_2 + k_3)a + k_{-3}p(k_{-1} + k_{-2} + k_2) + (k_{-1}k_{-2} + k_{-1}k_3 + k_2k_3)} \quad (5)$$

He was probably the first to note that in a QSS the rate of the reaction must very closely approximate the nett rate through each step in the reaction scheme, and he gave the reaction rate as $v=k_2x-k_{-2}y$. Using equations 4 and 5 he wrote the equation for v fully, but then immediately gave v with parameters written as K_m^A , K_m^P and $k_{cat}^Ae_0$ and $k_{cat}^Pe_0$ (the latter written as maximum velocities, V^A and V^P respectively). As an example, for a reaction starting with A, equations 6 and 7 were given, and they do not contain a term in p and k_{-3} (and for a reaction starting with P his parameters do not contain a and k_1).

$$K_m^A = \frac{k_{-1}k_{-2} + k_{-1}k_3 + k_2k_3}{k_1(k_{-2} + k_2 + k_3)} \quad (6)$$

$$k_{cat}^A = \frac{k_2k_3}{k_{-2} + k_2 + k_3} \quad (7)$$

One may assume that, once again when starting with A and as with scheme 1, he set p to zero in one of the equations. That could have been done equally in either of equations 2, 3, 4, 5 or in $v=k_2x-k_{-2}y$. This always leads to the results he gave, but the shortest algebra, to obtain an equation for y in a reaction starting with A, is to set p to zero in equation 3, solve it together with equations 2 for y , and write $v_1=k_3y$. But writing $p=0$ always eliminates k_{-3} , and a glance at scheme 2 with p and k_{-3} omitted shows the reaction is now an irreversible one. In one important respect, Haldane's analysis is then similar to that of Briggs and Haldane [5] in their introduction of the QSS approximation. It is that of an absolutely irreversible reaction in which the concentration of product plays no role in determining the concentrations of X and Y reached at the outset of the QSS. Haldane's equations 6 and 7 are for the parameters of an irreversible reaction and not, as he supposed, for a reversible reaction. This situation arises in analyses of the reaction in both the forward and reverse directions.

3. A corrected analysis of scheme 2.

It seems reasonable to conjecture that Haldane set p to zero somewhere in his equations because, at the outset of the QSS when $p \ll a$, the concentration a could simply be replaced with a_0 . In reversible reactions, however, the production of P occurs as soon as Y has formed, and although its concentration at the outset of the QSS might be negligible compared with a_0 , its formation during the pre-steady state will affect the concentration of Y present at the outset of the QSS. This was first observed with Scheme 1 [6][7] for a reaction starting with A when, if $k_{-2} \geq k_1$, x rises in the pre-steady state to its final equilibrium concentration. It might also be noted that Morales and co-workers [11][12] pointed out that the condition $dx/dt=0$ indicates a maximum value of x only if d^2x/dt^2 is negative,

and that this maximum value of x (when dx/dt is zero and then becomes negative during the QSS) occurs only when $k_1 > k_{-2}$.

The rate equation for scheme 2 is also given by equation 8, and at the outset of the QSS p will be present.

$$v = k_3 y - k_{-3} p (e_0 - x - y) \quad (8)$$

The conservation equation for A is $a_0 = a + x + y + p$, and it approximates to $a_0 \approx a + p$ when a_0 is several orders of magnitude greater than e_0 . This approximation is at the basis of the QSS assumption, and not an additional independent assumption. Substituting $a = a_0 - p$ in equations 4 and 5 gives equations 9 and 10. A reasonable assumption is that at the outset of the QSS, the initial reverse reaction rate is negligible compared with the forward rate, and $k_{-3} p (e_0 - x - y)$ might be neglected in equation 8, reducing it to $v_i = k_3 y$.

$$x = \frac{[k_1 a_0 (k_{-2} + k_3) + p \{k_{-2} k_{-3} - k_1 (k_{-2} + k_3)\}] e_0}{k_1 a_0 (k_{-2} + k_2 + k_3) + p \{k_{-3} (k_{-2} + k_2 + k_{-1}) - k_1 (k_{-2} + k_2 + k_3)\} + (k_{-1} k_2 + k_{-1} k_3 + k_2 k_3)} \quad (9)$$

$$y = \frac{[k_1 k_2 a_0 + p \{k_{-3} (k_{-1} + k_2) - k_1 k_2\}] e_0}{k_1 a_0 (k_{-2} + k_2 + k_3) + p \{k_{-3} (k_{-2} + k_2 + k_{-1}) - k_1 (k_{-2} + k_2 + k_3)\} + (k_{-1} k_{-2} + k_{-1} k_3 + k_2 k_3)} \quad (10)$$

But p remains in the equation for y given by equation 10. Expanding $k_3 y$ using equation 10, and rewriting the coefficients as $k_1 k_2 = \alpha$, $k_{-3} (k_{-1} + k_2) = \beta$, $k_1 (k_{-2} + k_2 + k_3) = \gamma$, $k_{-3} (k_{-2} + k_2 + k_{-1}) = \delta$, and $(k_{-1} k_{-2} + k_{-1} k_3 + k_2 k_3) = \varepsilon$, this gives equation 11 where p^* is the concentration of p present at the outset of the QSS.

$$\frac{v_i}{e_0} = \frac{k_3 \alpha a_0 + p^* k_3 (\beta - \alpha)}{a_0 \gamma + p^* (\delta - \gamma) + \varepsilon} \quad (11)$$

$$v_i = \frac{\frac{e_0 k_2 k_3 a_0}{k_{-2} + k_2 + k_3} \left\{ 1 + \frac{p^* (\beta - \alpha)}{k_1 k_2 a_0} \right\}}{a_0 + \frac{p^* (\delta - \gamma) / \gamma + \varepsilon}{k_1 (k_{-2} + k_2 + k_3)}} \quad (12)$$

Rearrangement of equation 11 leads to equation 12, an HMM equation of which the parameters are given in equations 13 and 14.

$$k_{\text{cat}}^A = \frac{e_0 k_2 k_3}{k_{-2} + k_2 + k_3} \left\{ 1 + \frac{p^* (\beta - \alpha)}{k_1 k_2 a_0} \right\} \quad (13)$$

$$K_m^A = \frac{p^* (\delta - \gamma) / \gamma + (k_{-1} k_{-2} + k_{-1} k_3 + k_2 k_3)}{k_1 (k_{-2} + k_2 + k_3)} \quad (14)$$

If equation 8 is used in full as a measure of v_i , equation 15 results, and p^* is always present in the parameters.

$$v_i = \frac{\frac{e_0 k_2 k_3 a_0}{k_{-2} + k_2 + k_3} \left\{ 1 + \frac{p^* [k_3 (\beta - \alpha) - k_{-3} \varepsilon]}{k_1 k_2 k_3 a_0} \right\}}{a_0 + \frac{p^* (\delta - \gamma) / \gamma + (k_{-1} k_{-2} + k_{-1} k_3 + k_2 k_3)}{k_1 (k_{-2} + k_2 + k_3)}} \quad (15)$$

These become those given by Haldane if p^* is equated to zero, a condition that is possible only if the reaction is absolutely irreversible.

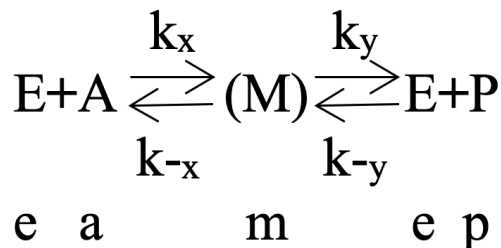
There are special cases when p disappears from the parameters. If $\beta=\alpha$, p disappears from k_{cat} in equation 13, and this requires $k_{-3}/k_1 = (k_{-1}+k_2)/k_2$. (If the full equation 8 is considered, leading to equation 15, p^* does not disappear). From the K_m in equation 14 and 15, p^* will disappear if $\delta=\gamma$, and this requires the $k_{-3}=k_1$ and $k_3=k_{-1}$ which reduces the equilibrium constant to one determined completely by the ratio k_2/k_{-2} . In scheme 1, there is a special case ($k_1=k_{-2}$) in which terms in p disappear from both numerator and denominator of the equation for dx/dt and allow an integration of the rate equation ^[6], but for scheme 2 there is not such a case for dy/dt .

The sign of the slope of dy/dt in the QSS is of interest. It is not always negative, and can be determined by differentiation of equation 10. After differentiation, dp/dt is positive, the terms in p disappear in the numerator, the denominator is positive, and the sign of the slope is that of $(k_1k_{-3}a_0+\beta-\alpha)$. On expansion, this has a positive sign if $k_{-3}>k_1k_2/(k_1a_0+k_{-1}+k_2)$, which agrees with the difference between the maximum value of y and the equilibrium concentration given previously ^[8]. The sign of the slope of dy/dt also reveals one other special case. This may be zero, and y will be constant during the whole QSS, but this is a very special case. For a reaction with a given enzyme the rate constants are fixed quantities and so the relationship is valid at only one single concentration of a_0 at which k_1a_0 must be sufficiently large to provide the very small period of a pre-steady state.

It should be pointed out that p^* is itself not a constant. It was simply introduced as the concentration of p present at the outset of the QSS, but for a fixed set of rate constants it will then depend on a_0 . Thus, although terms in p^* contributes significantly to the K_m , the changes in K_m obtained with different a_0 may be too small to be detected given the available precision of measurements, and the important factor is the combination of p^* with rate constants. (One may also point out that “the outset of the QSS” is ill defined except that it occurs, in the words of Briggs and Haldane ^[5], in the “first instant” (after an instantaneous mixing of all reactants)).

4. An alternative approach to the HMM equation

There is an alternative approach to obtaining a theoretical HMM equation, and that is to assume a reaction comes rapidly to a QSS in which all substrate-enzyme intermediates are contained in an assembly that is written as (M) in scheme 3.



Scheme 3.

Here, k_x is the rate constant for the reaction of E with A to give a component X in (M), and k_{-x} the constant for that reaction in the reverse direction. The rate constant for the reaction of a component of (M), Y, that gives rise to P is k_y , and k_{-y} that for the reverse reaction. (There is no implication that X and Y are consecutive steps in the reaction, and other components of (M) may also be present). The conservation equations are $e_0 = e + m$ and $a_0 = a + p + m = a + p$. In the QSS, $dm/dt = \{k_x a_0 + p(k_{-y} - k_x)\}(e_0 - m) - k_{-x} f_x m - k_y f_y m \approx 0$, where f_x and f_y are x/m and y/m respectively. The terms f_x and f_y are not strictly constants, but do change extremely slowly during the QSS as do x , y and m themselves. When p^* is the product concentration at the outset of the QSS, then $m = e_0 \{k_x a_0 + p^*(k_{-y} - k_x)\} / \{k_x a_0 + p^*(k_{-y} - k_x) + k_{-x} f_x + k_y f_y\}$. If it is assumed that initially in the QSS the reverse reaction is negligible, then v_i is given by $v_i = k_y f_y m$, and expanded this leads to $v_i = k_y f_y a_0 e_0 \{1 + p^*(k_{-y} - k_x)/k_x a_0\} / \{a_0 + [p^*(k_{-y} - k_x) + k_{-x} f_x + k_y f_y]/k_x\}$. This is an HMM equation, it depends only on the QSS assumption and one further reasonable assumption, that initially in the QSS the rate of the reverse reaction may be neglected. It is obtained independently of the details of the kinetic scheme within (M), and also displays the proper role of the concentration of P present at the outset of the QSS in contributing to the concentration of Y. Perhaps more interestingly, this approach can be expanded to accommodate reactions in which E is a mixture of isomeric forms, and also reactions with multiple substrates, but a closer identification of f_x and f_y requires a return to the algebra of section 3.

5. Discussion

Equating the product concentration to zero, as did Haldane in order to find initial velocity for the rate equation of a reversible reaction, has now been used as an approximation for more than ninety years, an astonishingly long time for the persistence of an elementary error. In the present analysis of scheme 2, an isomerization reaction, it is shown that the parameters of an HMM equation can be written in terms of rate constants alone under two circumstances. These occur if a reaction involves no rebinding of a product after its release from an enzyme intermediate (the reaction is in fact completely irreversible) or if in the steady state the intermediate giving rise to the product has its equilibrium concentration, in which case $K_m^A = (k_{-1}k_{-2}(1 + K_{\text{equ}}))/k_1(k_{-2} + k_2)$. Reactions with a

very high equilibrium constant may be described as essentially irreversible, but this is usually ill defined, and might even occur with scheme 2 if $k_{-2} \approx 0$. Generally, the situation is unknown, and when starting a reaction with A, the QSS concentration of Y will in general lie between the extremes of its equilibrium value and a maximum for an irreversible reaction. The parameters of the HMM equation will then contain a term in p^* , the concentration of product at the outset of the QSS.

The equations in section 3 look complex, and the derivations when given line by line may be considered too burdensome for students lacking a well-practised background in algebra, even though they are algebraically elementary. An alternative approach to an HMM equation is given in section 4, and may be helpful, but that will require teacher to make a choice appropriate to circumstances, and in any case when a more detailed analysis that reveals the nature of f_x and f_y is required, the algebra in section 3 is required.

The earliest derivations of an HMM equation ^{[1][2]} have both historical interest and educational value. The derivation of Michaelis and Menten ^[2] is direct and easier algebraically, but should be properly evaluated. The assumptions of a rapid initial and continued equilibrium between substrate and enzyme is quite arbitrary, and also removes from the analysis any need to consider the reverse reaction. (The use of initial velocities was originally proposed to avoid the experimentally observed problems of product inhibition ^[1], and the lack of terms in p in an equation for the initial reaction velocity was incidental). The later work of Van Slyke and Cullen ^[13] on the hydrolysis of urea catalysed by urease usually receives only passing mention in textbooks, but their assumption, that the process was a succession of two irreversible reactions, was at least well argued, and the result was a newly formulated K_m . (The analysis also used a novel route to a rate equation for the reaction, but may have contained the unstated assumption of a steady state). The QSS approximation introduced by Briggs and Haldane ^[5] was for a completely theoretical and absolutely irreversible reaction, one that is possible only in the extreme when product concentrations can play no role. Taken together, these early analyses provide a good warning about the care needed in the development of kinetic schemes and the consequent interpretation of measured parameters.

The observations made here also have implications for the use of computer-aided numerical methods. These can now be used to determine directly from primary experimental data both the numerical values of the parameters k_{cat} (or V) and K_m for an HMM equation and also their statistical significance ^[14]. These methods may be superior to the graphical ones used earlier, but the methods provide no formulation of parameters. Johnson ^{[15][16]} has shown how, given a measured value of a K_m , numerical methods may be used to obtain individual rate constants of reactions with an analytically predicted K_m , but he has also pointed out ^[15] that, when there are too many degrees of freedom in the theoretical scheme, limited data may not allow the assignment of unique values for rate constants. The basic experimental data usually available would be the equilibrium constant, K_{equ} , (with which subsequently calculated sets of rate constants must always agree, for example in scheme 2, $K_{equ} = k_1k_2k_3/k_{-1}k_{-2}k_{-3}$), experimentally measured parameters of the HMM equation for the forward and reverse reactions, and perhaps

in fortunate cases an association constant obtained from pre-steady state studies. The kinetic model should be, of course, as simple as reasonable, and be correctly analysed. The expression for the K_m deduced by Haldane for A with scheme 2 (using it as one for an isomerisation) contains only five rate constants, but the correct analytical K_m expressions contains these same five rate constants and in addition a term with p^* in combination with all rate constants. If scheme 2 is extended to the hydrolysis of sucrose, (and a constant water concentration is incorporated into k_1) there will potentially be an additional six rate constants related to the release and rebinding of the products, glucose and fructose (the number will depend on the mechanism of product release). Computations of this complexity are still awaited. Calculation, *ab initio*, from the time course of single reactions with different a_0 do not appear to have been made with a scheme more complex than scheme 1. Consequently, the true scope of numerical methods to obtain an unambiguous determination of single rate constants remains to be determined, and it is quite possible that advances will depend entirely on modern physico-chemical methods that reveal directly events occurring between X and Y and their associated rate constants.

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