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

The Henri-Michaelis-Menten (HMM) equation (equation 1) was first introduced theoretically

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

[1] and then confirmed experimentally [2], in both cases after experimental studies on a reversible reaction, the hydrolysis of sucrose catalysed by invertase. 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. The kinetic model used in the derivation was scheme 1, where P is the product and the letters k are rate constants. Other small

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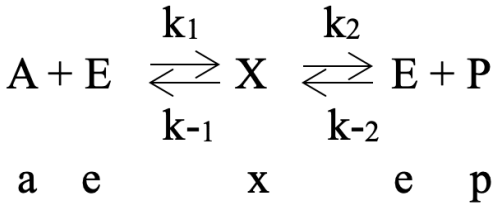
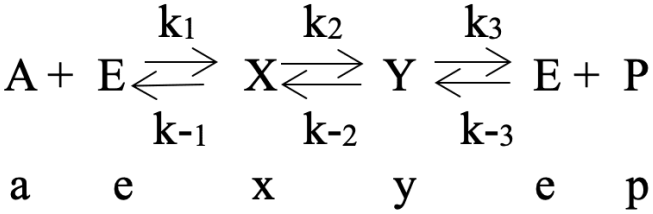


Scheme 1.

case letters are those used for concentrations. In the theoretical derivation of equation 1, 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-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 and p played no role in the kinetics), and the velocity derived was that for any value of the concentration of A during the reaction.



Scheme 2.

Seven years before the republications of Haldane's original work [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 was wrong. The HMM equation involves an initial reactant concentration, and somewhere in his analyses Haldane set terms in p to zero

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in his equations. That always excluded any role for k_{-2} in scheme 1 and for k_{-3} in scheme 2, and for a reaction starting with P the role of k_1, and the analyses were for completely irreversible reactions.

Because Haldane's erroneous assumption persists in text books, is generally used in analyses of two-substrate reactions (with the exception of the two-substrate reaction with random order of addition), and is deliberately written into an algorithm [10] intended to simplify the analysis of kinetic schemes, a summary of Haldane's analysis of scheme 2 is given in section 2. It is elementary, but the source of the error needs to be made perfectly clear. In section 3, there is an analysis of scheme 2 that uses the QSS assumption and a valid approximation to obtain an initial velocity in the QSS, and it is concluded that a description of the parameters of equation 1 in terms of rate constants alone 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

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

$$\frac{dy}{dt} = k_{-3} p(e_0 - x - y) + k_2 x - y(k_{-2} + k_3) \tag{3}$$

them to zero, and solved them as simultaneous equations to give equations 4 and 5 for y andx

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

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

respectively. 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 $k_{cat}^A e_o$ and $k_{cat}^P e_o$ (the latter written as maximum velocities, V^A and V^P respectively). How he actually obtained the parameters (for example, equations 6 and 7 for a reaction starting with A), is not stated, but they do not contain a term in p and k_{-3} (and for a reaction starting with P do not contain a and k_1). Setting p to zero in any of equations 2, 3, 4,5 and $v=k_2x-k_{-2}y$, always leads to Haldane's results. 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 $v_i=k_3y$. But p=0 always eliminates k_{-3}, and a glance at scheme 2 with k_{-3} omitted shows the reaction is now an irreversible one. In one important respect, Haldane's analysis is similar to that used by 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 the 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.

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

$$k_{\text{cat}}^A = \frac{k_2k_3}{k_{-2} + k_2 + k_3} \tag{7}$$

3. A corrected analysis of scheme 2.

It can be only a matter conjecture why, in his analyses, Haldane set p to zero somewhere in his equations, but perhaps he believed that, at the outset of the QSS when p<<a, the concentration a could simply be replaced with a_o. 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_o, the 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 given by equation 8, and at the outset of the QSS p will be

$$v = k_3y - k_{-3}p(e_0 - x - y) \tag{8}$$

present. The conservation equation for A is $a_o = a+x+y+p$ and it approximates to $a_o \approx a+p$ when a_o is several orders of magnitude greater than e_o. This approximation is at the basis of the QSS assumption, and not an additional independent assumption. Substituting $a=a_o-p$ in equations 4 and 5 gives equations 9 and 10.

$$x = \frac{[k_1a_0(k_{-2} + k_3) + p\{k_{-2}k_{-3} - k_1(k_{-2} + k_3)\}]e_0}{k_1a_0(k_{-2} + k_2 + k_3) + p\{k_{-3}(k_{-2} + k_2 + k_{-1}) - k_1(k_{-2} + k_2 + k_3)\} + (k_{-1}k_2 + k_{-1}k_3 + k_2k_3)} \tag{9}$$

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

A reasonable assumption is that at the outset of the QSS, the initial reverse reaction rate is negligible compared with the forward rate, and the term in p in equation 8 may neglected, reducing it to $v_i=k_3y$. But that does not remove p from equation 10, that for y. Expanding k_3y using equation 10, and rewriting the coefficients as $k_1k_2 = \alpha$, $k_{-3}(k_{-1}+k_2) = \beta$, $k_1(k_{-2}+k_2+k_3) = \gamma$, $k_{-3}(k_{-2}+k_2+k_{-1}) = \delta$, and $(k_{-1}k_{-2}+k_{-1}k_3+k_2k_3) = \varepsilon$, this gives equation 11 where p* represents the concentration of p present at the outset of the QSS.

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

Rearrangement of equation 11 leads to equation 12, an HMM equation of which the parameters are given in equations 13 and14.

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

If equation 8 is used in full as a measure of v_i, equation 15 results, and p* is always present in the parameters. These become those given by Haldane if p* is equated to zero, a condition that, as pointed out above, is possible only if the reaction is absolutely irreversible.

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

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

$$v_i = \frac{\frac{e_0 k_2 k_3 a_0}{k_{-2}+k_2+k_3}\left\{1 + \frac{p^*[k_3(\beta-\alpha) - k_{-3}\varepsilon]}{k_1 k_2 k_3 a_0}\right\}}{a_0 + \frac{p^*(\delta-\gamma)/\gamma + (k_{-1}k_{-2}+k_{-1}k_3+k_2k_3)}{k_1(k_{-2}+k_2+k_3)}} \quad (15)$$

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 *d*x/*d*t and allow an integration of the rate equation, but for scheme 2 there is not one for *d*y/*d*t.

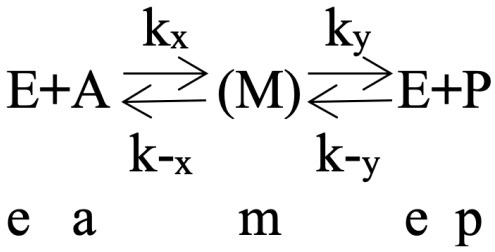
The sign of the slope of *d*y/*d*t in the QSS is of interest. It is not always negative, and can be determined by differentiation of equation 10. After differentiation, *d*p/*d*t is positive, the terms in p disappear in the numerator, the denominator is positive, and the sign of the slope is that of $(k_1k_{-3}a_0+\beta-\alpha)$. 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 *d*y/*d*t 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_o at which k_1a_o 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_o. Thus, although terms in p* contributes significantly to the K_m, the changes in K_m obtained with different a_o 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. (This is not unexpected: when using the QSS, the assumption is $dy/dt \approx 0$ and not $dy/dt=0$. (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. 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.



Scheme 3.

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 $e_o=e+m$ and $a_o=a+p+m \approx a+p$. In the QSS, $dm/dt=\{k_xa_o+p(k_{-y}-k_x)\}(e_o-m)-k_{-x}f_xm-k_yf_ym \approx 0$, 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* the is the product concentration at the outset of the QSS, then $m=e_o\{k_xa_o+p^*(k_{-y}-k_x)\}/\{k_xa_o+p^*(k_{-y}-k_x)+k_{-x}f_x+k_yf_y\}$. If it is assumed that initially in the QSS the reverse reaction is negligible, then v_i is given by $v_i=k_yf_ym$, and expanded this

leads to $v_i=k_yf_ya_oe_o\{1+p^*(k_{-y}-k_x)/k_xa_o\}/\{a_o+[p^*(k_{-y}-k_x)+k_{-x}f_x+k_yf_y]/k_x\}$. This is an HMM equation, it depends only on the QSS assumption and one further reasonable assumption, that initially in the QSS the 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

The faulty approximation made by Haldane when analysing reversible reactions using the QSS assumption, and the wrong parameters the HMM equations he derived, have had an extraordinary long life and appear to this day in textbooks. Furthermore, his faulty approximation has been used in analyses of two substrate reactions as well as in an algorithm intended to aid the solution of rate equations. Section 2 shows that his analyses were for irreversible reactions and not, as he supposed, for reversible ones. Section 3 does not use Haldane's faulty approximation, that of equating p to zero to obtain initial velocities, but gives a correct analysis of scheme 2 in which, at the outset of the QSS, the rate of the reverse reaction is assumed negligible compared with that of the forward reaction. This shows that the parameters of the HMM equation generally contain a term in p. This is also the result if the full equation 8 with p=p* is used. But special cases were noted when the steady state concentration of y is its equilibrium concentration, and these lead to an expression for K_m lacking a term in p*: $K_m=(k_{-1}k_{-2}(1+K_{equ})/k_1(k_{-2}+k_2)$. In general, the term in p* cannot simply be neglected. When it is, the reaction becomes an irreversible. When it is not, an equilibrium state for y might be present in the QSS, but we do not know, and so we may not assume it simply for the sake of obtaining a K_m formulated only by rate constants.

Text book derivations of rate equations derived using Haldane's "p=0" assumption need revision. The equations in section 3 look complex, and the derivations when given line by line may be too burdensome for students lacking a well-practised background in algebra, they are algebraically elementary. An alternative approach to an HMM equation is given in section 4, and may be helpful, but that will require a choice by teachers 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. That derived from Michaelis and Menten's work [2] may be 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 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 avoiding 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 effectively 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, they provides 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 the K_m, numerical methods may be used to obtain individual rate constants of reactions from 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.

That basic data would be the experimental results obtained for an 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 for the forward and reverse reactions, and perhaps in fortunate cases an association constant obtained from pre-steady state studies. The kinetic model should have a sound basis and be correctly analysed. The expressions for the K_m deduced by Haldane for A with scheme 2 (using it a one for an isomerisation) contain altogether only six rate constants, but the correct analytical K_m expressions contains all six rate constants and in addition term in p* and rate constants together. 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_o 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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