Research Article

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

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Haldaneused the quasi-steady state assumption in theoretical analyses of two kinetic models of enzyme-catalysed reversible isomerisation reactions, but these analyses gave incorrect predictions of the parameters of the kinetic constants of the Henri-Michaelis-Menten equation. The errors were due to a false assumption made to obtain the equation in terms of an initial velocity, and his results were parameters for irreversible reactions. Correct derivations are given for a minimally adequate kinetic model of an isomerization, an alternative derivation is introduced, and the results are discussed briefly in terms of the teaching of enzyme kinetics and of the role of numerical methods in determining individual rate constants. The observations made here may have consequences for analyses of more complex reactions in which the same false assumption has been used.

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1. Introduction

Haldane [1][2] used the quasi-steady state (QSS) assumption in theoretical analyses of schemes 1 and 2 which represent enzyme-catalysed reversible isomerization reactions, and obtained Henri-Michaelis-Menten (HMM) equations.

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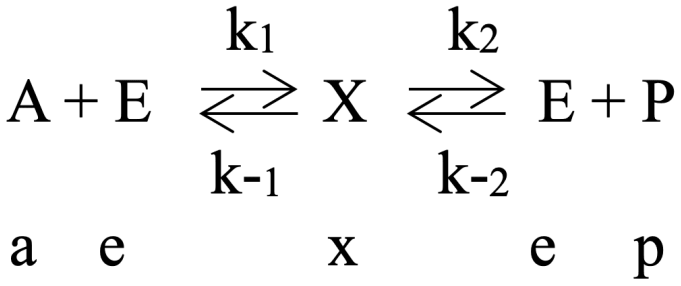
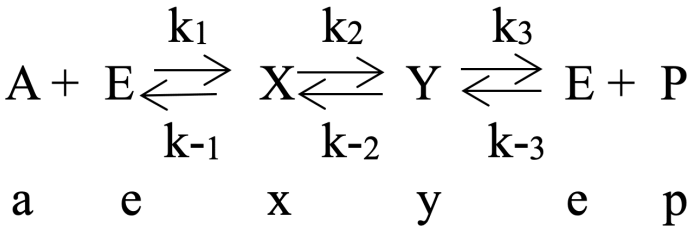


Scheme 1.



Scheme 2.

In these schemes the species A and P are interconverted, E represents free enzyme, and X and Y are enzyme-substrate intermediates. The small case letters k are rate constants and the others are for concentrations. Because the HMM equation involves only a reactant concentration, Haldane set the product concentration to zero in his equations, but this introduced an error that is still found in text books, and which has been copied in analyses of more complex reactions and into an algorithm [3] intended to aid the solution of rate equations. Because Scheme 1 is an inadequate minimal model, lacking at least one elementary step showing the interconversion of enzyme-bound reactant and product, the following discussion is continued largely with reference to Scheme 2. In a reversible reaction, the reverse reaction of P begins as soon as P is produced, and this affects the concentration of Y present at the outset of the QSS, whereas in an irreversible reaction, when both p and k_{-3} do not appear in the analysis, the concentration of Y is independent of the product concentration.

$$v_i = \frac{k_{\text{cat}} e_o a_o}{a_o + K_m} \tag{1}$$

It may be noted that the HMM equation (equation 1) was first introduced theoretically [4] and then confirmed experimentally [5] after experimental studies on a reversible reaction, the hydrolysis of sucrose catalysed by invertase. Scheme 1 was analysed, and the initial reaction rate (v_i in equation 1) was given in terms of the initial

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concentrations of A (a_o) and E (e_o) and (in modern form) two parameters, k_{cat} and K_m. These initial conditions were used because it was known the reaction was inhibited by product, and v_i was measured in the experimental confirmation [5], but in any case, the assumption, that A and E came very quickly into and remained in equilibrium, avoided in the analysis any consideration of a role for the product concentration.

Seven years before the republications of Haldane's original work on reversible reactions [2], 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]. My own assumption [8], that Haldane's derivations might be correct in one direction, was wrong. The HMM equation for a reversible reaction requires an initial reactant concentration, and in whichever direction the reaction is considered, setting the product concentrations to zero in analyses of schemes 1 and Scheme 2 converts the analyses to ones for irreversible reactions.

In the following, section 2 contains a brief review of Haldane's analysis of Scheme 2, given here in order to fully clarify the consequence of setting the product concentration to zero, but also to provide two of Haldane's equations (numbers 4 and 5, below) useful in the correct analysis that follows in section 3. In section 4, an alternative route to the HMM equation is given, and in section 5 these results are reviewed in terms of changes needed in text books on the subject and in teaching. The consequent increase in difficulty in using numerical methods for the identification of individual rate constants is also briefly discussed. The consequences of the use of Haldane's wrong assumption in analyses of more complex reactions is too broad a subject to be covered here. This later work may well have led to an HMM equation, but the present work should prompt caution about any identification of its parameters.

2. The problem with Haldane's analyses

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 in the interconversion of X and Y the scheme becomes a minimal one for such a reaction.

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

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

He gave [1][2] 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.

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

$$x = \frac{\{k_1 a(k_{-2} + k_3) + k_{-2}k_{-3}p\}\, e_o}{k_1(k_{-2} + k_2 + k_3)a + k_{-3}p(k_{-1} + k_{-2} + k_2) + (k_{-1}k_{-2} + k_{-1}k_3 + k_2k_3)} \tag{5}$$

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 in shorthand form 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).

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

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

As examples, for a reaction starting with A, equations 6 and 7 were given for the parameters of equation 1, 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). For Scheme 1 when starting with A, he stated explicitly that he had set p to zero, and for Scheme 2 that could have been done at any stage in equation 3, 4, 5 or in $v=k_2x-k_{-2}y$. The shortest algebra to obtain his results for an equation for y (in a reaction starting with A) is to set p to zero in equation 3, together with equations 2 solve it for y, and write $v_i=k_3y$. But a glance at Scheme 2 with p omitted (and consequently also k_{-3}) shows the reaction is now an irreversible one. In one important respect, the analysis is then similar to that of Briggs and Haldane [10] in their introduction of the QSS approximation with a version of Scheme 1. Both were analyses for reactions that are irreversible in the last step, and for 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. The error arises in analyses of the reaction in both directions.

3. A correct analysis of Scheme 2

The HMM equation generally requires the initial reactant concentration, a_o, and only in irreversible reactions [for example, 1, 2,10,11], when a velocity in the HMM equation is given for any prevailing concentration of reactant, is this avoided. It seems reasonable to conjecture that Haldane set p to zero somewhere in his equations because, at the outset of the QSS, when p<<a, the concentration of A could be approximated with a_o. In reversible reactions, however, the production of P occurs as soon as Y has formed, and although the concentration p at the outset of the QSS may well be negligible compared with a, 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 for Scheme 1, that the condition $dx/dt=0$ occurs at 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 $k_1 > k_{-2}$.

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

The rate equation for Scheme 2 is also given by equation 8, and at the outset of the QSS p will be 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 is not an additional independent assumption. Substituting $a=a_o-p$ in equations 4 and 5 gives equations 9 and 10.

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

$$y = \frac{[k_1 k_2 a_o + p\{k_{-3}(k_{-1}+k_2) - k_1 k_2\}]\, e_o}{k_1 a_o(k_{-2}+k_2+k_3) + p\{k_{-3}(k_{-2}+k_2+k_{-1}) - k_1(k_{-2}+k_2+k_3)\} + (k_{-1}k_{-2} + k_{-1}k_3 + k_2k_3)} \tag{10}$$

A reasonable assumption is that at the outset of the QSS, the initial reverse reaction rate is negligible compared with the forward rate, and $k_{-3}p(e_o-x-y)$ might be neglected in equation 8, reducing it to $v_i=k_3y$. But p remains in the equation for y, given by equation 10. 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* is the concentration of p present at the outset of the QSS. Rearrangement of equation 11 leads to equation 12, an HMM equation of which the parameters are given in equations 13 and 14.

$$\frac{v_i}{e_o} = \frac{k_3\alpha a_o + p{*}k_3(\beta-\alpha)}{a_o\gamma + p{*}(\delta-\gamma) + \varepsilon} \tag{11}$$

(These correct an error made in an earlier preprint (doi.org/10.32388/LRF3TR)).

$$v_i = \frac{\frac{e_o k_2 k_3 a_o}{k_{-2}+k_2+k_3}\left\{1 + \frac{p{*}(\beta-\alpha)}{\alpha a_o}\right\}}{a_o + \frac{p{*}(\delta-\gamma)+\varepsilon}{\gamma}} \tag{12}$$

$$k_{\text{cat}}^A = \frac{k_2k_3}{k_{-2}+k_2+k_3}\left\{1 + \frac{p{*}(\beta-\alpha)}{\alpha a_o}\right\} \tag{13}$$

$$K_m^A = \frac{p{*}(\delta-\gamma) + (k_{-1}k_{-2} + k_{-1}k_3 + k_2k_3)}{k_1(k_{-2}+k_2+k_3)} \tag{14}$$

If equation 8 is used in full as a measure of v_i, p* is also always present in the parameters. The parameters become those given by Haldane if p* is equated to zero, a condition that is possible only if the final step of the reaction is 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 $k_{-3}/k_1 = k_2/(k_{-1}+k_2)$. (If the full equation 8 is considered, p* does not disappear). From the K_m in equation 14, p* will disappear if $\delta=\gamma$, which requires the $k_{-3}=k_1$ and $k_3=k_{-1}$. This reduces the equilibrium constant to one determined completely by the ratio k_2/k_{-2}. For Scheme 1, there is a special case ($k_1=k_{-2}$) in which the terms

in p disappear from both numerator and denominator of the equation for *d*x/*d*t, and this allows the integration of the rate equation for x [6]. For Scheme 2 there is not such a case for *d*y/*d*t.

The sign of the slope of *d*y/*d*t in the QSS is also of interest. The general form of the equation for y (from equation 10) is equation 15.

$$\frac{y}{e_o} = \frac{\alpha a_o + p(\beta - \alpha)}{a_o\gamma + p(\delta - \gamma) + \varepsilon} \tag{15}$$

$$\frac{1}{e_o}\frac{dy}{dt} = \frac{\frac{dp}{dt}\left[a_o(\beta\gamma - \alpha\delta) + (\beta - \alpha)\varepsilon\right]}{D^2} \tag{16}$$

Differentiation leads to equation 16 in which D is the denominator of equation 15 and is positive (see the denominators in equations 4 and 5). Because *d*p/*d*t is positive, the sign of the slope of *d*y/*d*t is that of [a_o(βγ-αδ) + (β-α)ε], a constant equivalent to [$k_1k_{-3}a_o$+ $k_{-3}(k_{-1}+k_2)$-k_1k_2]. From the beginning of the QSS until *d*y/*d*t becomes zero at equilibrium, the sign of *d*y/*d*t does not change and is positive if $k_{-3}>k_1k_2/(k_1a_o+k_{-1}+k_2)$. This agree with the earlier comparison of y with its equilibrium value [8]. When *d*y/*d*t is always positive beyond the outset of the QSS, there is a possibility that, at or near the end of the QSS, it might increase before becoming zero at equilibrium. The progress curve of y would then pass through an inflection point at which d^2y/dt_2 would be zero, and this possibility can be tested by the differentiation of equation 16 which leads to equation 17.

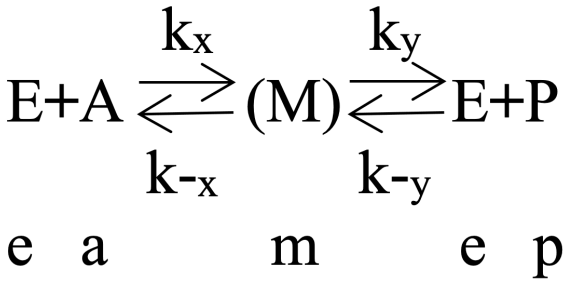
$$\frac{\frac{d^2y}{dt_2}}{\{e_o\left[a_o(\beta\gamma - \alpha\delta) + (\beta - \alpha)\varepsilon\right]\}} = \frac{\left(\frac{d^2p}{dt_2}\right)D^2 - \left(\frac{dp}{dt}\right)^2 2D(\delta - \gamma)}{D^4} \tag{17}$$

The term (d^2p/dt_2) D^2 is negative and the sign of (dp/dt)^{2}2D(δ-γ)]/D^4 is that of (δ-γ)]. Thus, when *d*y/*d*t is positive and d^2p/dt_2 is negative throughout the QSS, then d^2y/dt_2cannot be zero if $k_{-3}(k_{-2}+k_2+k_{-1}) > k_1(k_{-2}+k_2+k_3)$ and there is not an inflection. (d^2y/dt_2 is then zero only at equilibrium when *d*p/*d*t and d^2p/dt^2 are both zero). This has an interesting implication. If y is rising extremely slowly in the QSS and continues without an upward inflection in this state until equilibrium, then at the outset of the QSS y must be below but very close to its equilibrium concentration. The QSS value of y might then be approximated by its equilibrium value.

It should be pointed out that p* is itself not a constant as are α, β, γ, δ and ε, but will depend on a_o. It may also be noted that “the outset of the QSS” is ill defined except that it occurs, in the words of Briggs and Haldane [10], in the “first instant” of the reaction (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.

There, 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 Y in (M) 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 $e_o=e+x+y+n$, where n is the sum of the concentration of components other than X and Y in (M), and $a_o=a+p+x+y+n\approx a+p$. In the QSS, $dm/dt\approx 0$ so that $dm/dt=\{k_x a_o+p(k_{-y}-k_x)\}(e_o-m)-k_{-x}f_x m-k_y f_y m \approx 0$, 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 $m=e_o\{k_x a_o+p^*(k_{-y}-k_x)\}/\{k_x a_o+p^*(k_{-y}-k_x)+k_{-x}f_x+k_y f_y\}$. 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 $v_i=k_y f_y a_o e_o\{1+p^*(k_{-y}-k_x)/k_x a_o\}/\{a_o+[p^*(k_{-y}-k_x)+k_{-x}f_x+k_y f_y]/k_x\}$. 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. 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

In a reanalysis of Scheme 2 (an isomerization reaction) given in section 3, it is now shown that the parameters of an HMM equation can be written in terms of rate constants alone under two circumstances. These occur when a reaction involves no rebinding of a product after its release from an enzyme intermediate (the reaction is not reversible) and when in the QSS the intermediate giving rise to the product has its equilibrium concentration and $K_m^A=\{k_{-1}k_{-2}(1+K_{equ})/k_1(k_{-2}+k_2)\}$. A close approximation to these states may be possible. The first occurs if a

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reaction is essentially irreversible (with a very high equilibrium constant, but this might occur with Scheme 2 if $k_{-2} \approx 0$), and the second if dy/dt is positive and close to and slowly approaching zero at equilibrium. In general, the situation is unknown, and so when starting a reaction with A, the QSS concentration of Y will most probably lie between its equilibrium value and that of an irreversible reaction. Between these extremes, and whatever the numerical difference between their values of y might be, the parameters of the HMM equation will contain a term in the concentration of product at the outset of the QSS (p*). Because the two extremes lead to quite different expressions for the parameters of the HMM equation, any further approximation, using only one of them, requires evidence about which of the two is appropriate, if at all.

The equations in section 3 look complex, and the full derivations may be considered too burdensome for students lacking a well-practised background in algebra. An alternative approach to an HMM equation is given in section 4, and may be a little easier, but its use will require teachers to make a choice appropriate to circumstances. If later a more detailed analysis that reveals the nature of f_x and f_y is required, then the algebra in section 3 (and more) is required.

The earliest derivations of an HMM equation [4][5][10][13] should not be discarded in teaching. Although they involve inadequate kinetic models, they have historical interest, are algebraically short, and together provide a good cautionary tale. The derivation of Michaelis and Menten [5] is the simplest, but 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 [1] to avoid the experimentally observed problems of product inhibition, 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 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 [10] was for a completely theoretical and absolutely irreversible reaction. These early analyses, based on variants of Scheme 1, provide a warning about the care needed in the choice of kinetic schemes and their analysis, and in the consequent interpretation of measured parameters. But because reversible reactions are probably not the least numerous in intermediary metabolism, and the QSS assumption is a good general one *in vitro* when $a_o >> e_o$, a continued misidentification of the parameters of the HMM equation is indefensible. Consequently, text books need revision to avoid the additional but wrong assumption that Haldane made [1][2] in his application QSS assumption.

It may well be noted that an HMM equation is fully consistent with the QSS assumption leading to it. Briggs and Haldane [10] had suggested that on average $(dx/dt)/(da/dt)$ would be less than $-e_o/a$, and used some then current information to justify this. (The validity of the latter is not entirely clear because one equation they gave for dx/dt,

without its origin, cannot be correct because it is dimensionally inconsistent. None the less, it is a good argument that, in a reaction in which $a_o >> e_o$ and E is reformed, the nett change in e must always be less than that in a, otherwise the reaction would stop). The equation for the intermediate giving rise to the product has the form, for example with Scheme 2, of $y=e_o a_o/(a_o+K_m)$, and this itself shows the dependence of y on a_o. The differentiation of the equation with respect to a, and considering a reaction when $a_o = K_m$, leads to $dy/da = (dy/dt)/(da/dt) = (+/-)\, e_o/4a_o$. The uncertainty of the sign is because that of dy/dt may be positive, zero or negative. Briggs and Haldane also clearly recognised that writing $dx/dt=0$ was an approximation, and they did not use the expression "steady state". This came into use to describe their assumption, but was later replaced by the more precise terms quasi- or pseudo-steady state.

The observations made here also have implications for the use of computer-aided numerical methods to obtain individual rate constants. Numerical methods can now be used to determine directly from primary experimental data the numerical values of the parameters k_{cat} (or V) and K_m of an HMM equation, and also their statistical significance [14]. These methods are 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 to the rate constants. The basic experimental data usually available would be the equilibrium constant of the reaction, K_{eq}, 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, of course, be as simple as reasonable, and be analysed correctly. The expression for the K_m deduced by Haldane for A with Scheme 2 (an isomerisation) contains only five rate constants, but the correct analytical expression contains these same five rate constants and in addition a term with p* in combination with all six rate constants. If Scheme 2 is extended to the hydrolysis of sucrose, (and a constant water concentration is used with 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,* for the time course of single reactions do not appear to have been made with a scheme more complex than Scheme 1. Consequently, the usefulness of numerical methods to obtain unambiguous values of single rate constants remains to be determined.

This paper has described the source of an error made by Haldane almost a Century ago, and it was a quite elementary one, but one that has been propagated for a long time. Kinetics was the first method that allowed some understanding of the operation of enzymes, and is still practically valuable, but it is most probable that advances

in fully understanding the mechanisms of enzymic catalysis will depend entirely on modern physico-chemical methods that, for example in Scheme 2, reveal directly possible events occurring between X and Y and also provide their rate constants. There is a great deal of published work into kinetic schemes more complex than Scheme 2 that has carried Haldane's original error with it, and it would require a great amount of work to tabulate all of this and to evaluate possible consequences for conclusions drawn about (and then from) the parameters of any HMM equation deduced. When earlier work is considered, that should be done with caution.

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