the FEBS Journal

REVIEW ARTICLE

Validity of the Michaelis–Menten equation – steady-state or reactant stationary assumption: that is the question

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Keywords
enzyme kinetics, initial rate experiments, limiting rate, Michaelis–Menten constant, rapid-equilibrium assumption, reactant stationary assumption, steady-state assumption

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(Received 23 August 2013, revised 1 October 2013, accepted 8 October 2013)

doi:10.1111/febs.12564

The Michaelis–Menten equation is generally used to estimate the kinetic parameters, V and K_M, when the steady-state assumption is valid. Following a brief overview of the derivation of the Michaelis–Menten equation for the single-enzyme, single-substrate reaction, a critical review of the criteria for validity of the steady-state assumption is presented. The application of the steady-state assumption makes the implicit assumption that there is an initial transient during which the substrate concentration remains approximately constant, equal to the initial substrate concentration, while the enzyme–substrate complex concentration builds up. This implicit assumption is known as the reactant stationary assumption. This review presents evidence showing that the reactant stationary assumption is distinct from and independent of the steady-state assumption. Contrary to the widely believed notion that the Michaelis–Menten equation can always be applied under the steady-state assumption, the reactant stationary assumption is truly the necessary condition for validity of the Michaelis–Menten equation to estimate kinetic parameters. Therefore, the application of the Michaelis–Menten equation only leads to accurate estimation of kinetic parameters when it is used under experimental conditions meeting the reactant stationary assumption. The criterion for validity of the reactant stationary assumption does not require the restrictive condition of choosing a substrate concentration that is much higher than the enzyme concentration in initial rate experiments.

Introduction

The Michaelis–Menten equation is undoubtedly one of the most important mathematical expressions in biochemistry. It describes the initial rate of production formation (v_0) for a family of enzyme-catalysed reactions in terms of two parameters: the limiting rate (V) and the Michaelis–Menten constant (K_M) [1,2]. The initial velocity of the Michaelis–Menten equation is a rectangular hyperbolic function of the initial substrate concentration (s_0), which has the mathematical form:

$$v_0 = \frac{V s_0}{K_M + s_0}. \quad (1)$$

In the above expression, K_M gives s_0 at which v_0 is half V, so if $s_0 = K_M$ is substituted in Eqn (1), we obtain

Abbreviations
c, concentration of the enzyme–substrate complex; C, enzyme–substrate complex; e_0, initial enzyme concentration; E, enzyme; e, enzyme concentration; K_M, Michaelis–Menten constant; K_S, equilibrium dissociation constant of enzyme-substrate complex; K, van Slyke–Cullen constant; P, product; s_0, initial substrate concentration; S, substrate; s, substrate concentration; t_C, timescale of the enzyme–substrate complex; t_S, timescale of the substrate; v_0, initial rate of product formation; v_C, rate of change of the enzyme–substrate concentration; V, limiting rate; v_P, rate of change of the product concentration; v_S, rate of change of the substrate concentration.

$v_0 = \frac{1}{2}V$. v_0 is measured through initial rate experiments, which are performed by mixing the enzyme with a large excess of substrate. Under these conditions, the intermediate species builds up and achieves a pseudo-steady-state after an initial fast transient. After this point, v_0 changes slowly and is typically monitored through accumulation of product with time [2,3]. At low s_0, v_0 increases linearly with s_0. As s_0 increases, the linear relationship breaks down and v_0 increases less rapidly until it reaches the saturating value of V at high s_0 (see Fig. 1). Initial rate experiments are simple to perform and analyse. They are also relatively free from complications such as back reaction and enzyme degradation. As a consequence, they are the most commonly used experimental assay in enzyme kinetics.

The Michaelis–Menten equation was derived by Leonor Michaelis and Maud Menten in their seminal paper on enzyme kinetics which was published in the *Biochemische Zeitschrift* in 1913. In their paper, Michaelis and Menten measured the initial rates of the invertase reaction at different substrate concentrations. They showed that the Michaelis–Menten equation accurately describes the initial rates of the invertase reaction.

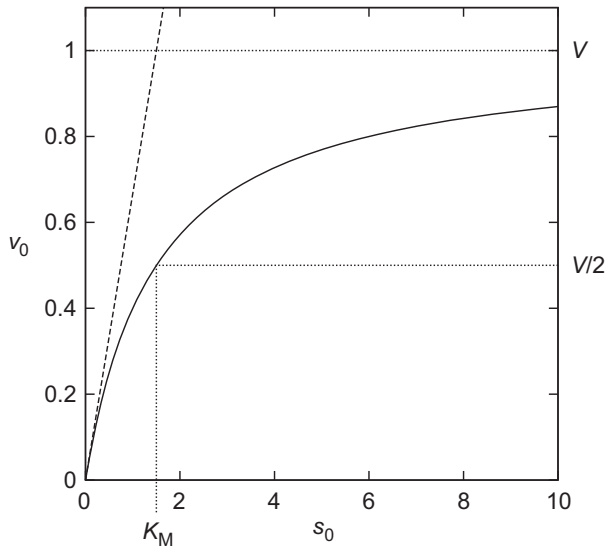

Fig. 1. Initial velocity v_0 plotted against the initial substrate concentration s_0 for the reaction mechanism (2) obeying the Michaelis–Menten equation (Eqn 1). The dependence of v_0 on s_0 follows a rectangular hyperbola with an asymptote on the v_0 axis at V. The s_0 for which $v_0 = \frac{1}{2}V$ is equal to K_M. At very small values of s_0, v_0 follows a linear relationship given by Vs_0/K_M, as shown in the figure. The kinetic parameters, V and K_M, are estimated by fitting v_0 for various s_0 using the Michaelis–Menten equation (Eqn 1). Parameters values used for this figure are: $V = 1$ mOD/min and $K_M = 1.5$ μM.

Michaelis and Menten are considered the founders of modern enzymology, because their initial rate experiments have served as a standard for most of the enzyme kinetics experiments over the last century [2,4].

The derivation of the Michaelis–Menten equation (Eqn 1) requires making some assumptions about the experimental conditions of the enzyme-catalysed reactions [5]. In most biochemistry textbooks, the Michaelis–Menten equation is derived using the steady-state assumption [4,6]. If the Michaelis–Menten equation is to be used to estimate K_M and V, it is essential to know whether or not the steady-state assumption is valid in any given experimental assay for an enzyme-catalysed reaction.

In this work, I review the foundations of the Michaelis–Menten equation, examine the literature investigating the validity of the steady-state assumption and provide general principles to derive criteria for the validity of the Michaelis–Menten equation to estimate the kinetic parameters in initial rate experiments. I argue that the strongest criterion to use the Michaelis–Menten equation for estimating K_M and V in test tube experimental assays is not the steady-state assumption, but the reactant stationary assumption, which assumes that the substrate concentration does not change significantly during the initial transient of the enzyme-catalysed reaction. I also show that the laboratory practice for initial rate experiments of choosing a substrate concentration that is much higher than the enzyme concentration is unnecessarily restrictive for the validity of the reactant stationary assumption.

Derivation of the Michaelis–Menten equation

In 1902, Henri [7,8] proposed the following reversible reaction mechanism between a substrate S and an enzyme E, giving the enzyme–substrate complex C, which irreversibly yields the product P:

$$S + E \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} C \xrightarrow{k_2} P + E, \qquad (2)$$

where k_1, k_{-1} and k_2 are rate constants of the reaction. Henri derived an equation for the rate of product formation to explain the enzyme action, but he did not propose an experimental assay to study enzyme-catalysed reactions nor a protocol to estimate the rate constants of the reaction [4]. In addition, reaction mechanism (2) is a simplification, which assumes that the overall enzyme-catalysed reaction is irreversible and has only one intermediate complex. Despite these imperfections, modern textbooks present reaction

mechanism (2) as the starting point for introducing and interpreting enzyme kinetics [2,3,9].

The difficulty of investigating the behaviour of the enzymatic reaction was largely resolved when Michaelis and Menten [10] showed that enzymes can be studied by measuring v_0 using Eqn (1) [4,5,11] under what it is currently known as the rapid-equilibrium assumption [3,12,13]. For this reason, Eqn (1) and reaction mechanism (2) are known, respectively, as the Michaelis–Menten equation and reaction mechanism, although these authors clearly recognised Henri as the originator of both.

Derivation of v_0 by Brings and Haldane

Currently, the mathematical protocol developed by Briggs and Haldane in 1925 [14] is considered the standard approach to derive the Michaelis–Menten equation for reaction mechanism (2) using the steady-state assumption [2,4]. Briggs and Haldane [14] applied the law of mass action to determine the rate of c as follows:

$$v_C = k_1 es - (k_{-1} + k_2)c. \tag{3}$$

They pointed out that C need not be in equilibrium with E and S, but within a very short time after starting the reaction, the rate of formation of C will almost balance its rate of destruction. Hence, C builds up to a pseudo-steady-state level, where its concentration is nearly constant. Thus, by making the steady-state assumption

$$v_C \approx 0, \tag{4}$$

reaction mechanism (2) has an enzyme conservation law

$$e_0 = e + c. \tag{5}$$

Substituting e from the enzyme conservation law (Eqn 5) into Eqn (3), and applying the steady-state assumption (Eqn 4), we can solve c in terms of s, thus

$$c = \frac{e_0 s}{K_M + s}, \tag{6}$$

where K_M is the Michaelis–Menten constant,

$$K_M = \frac{k_{-1} + k_2}{k_1}. \tag{7}$$

In the second step of reaction mechanism (2), the enzyme catalysis takes place with a first-order rate constant, k_2, known as the turnover number. The turnover number represents the maximum number of substrate molecules converted to product per active site per unit time, or the number of times the enzyme is 'turned over' per unit time. In the second step, the rate of product concentration is defined by the law of mass action as

$$v_P = k_2 c. \tag{8}$$

From the enzyme conservation law (Eqn 5), $c \leq e_0$, and so, provided that the experimental condition is valid,

$$\frac{e_0}{s_0} \ll 1, \tag{9}$$

then $s_0 \gg c$ during the build-up of c. Therefore, during the initial transient of the reaction, the free substrate concentration can be approximated by

$$s \approx s_0. \tag{10}$$

Substituting Eqn (6) with Eqn (10) into v_P as defined in Eqn (8), leads to:

$$v_0 = \frac{V s_0}{K_M + s_0}, \tag{11}$$

with the limiting rate defined as $V = k_2 e_0$. In fact, Eqn (11) is known as the Michaelis–Menten equation for the single-enzyme, single substrate catalysed reaction mechanism (2), even though it was derived using the Briggs and Haldane treatment.

This recapitulation of the derivation of v_0 using the Briggs and Haldane treatment provokes an important question: under what experimental conditions and range of rate constants is the steady-state assumption valid? To address this question, I present a historical review of literature investigating the validity of the steady-state assumption for the Michaelis–Menten reaction mechanism.

A historical review of the validity of the steady-state assumption

The validity of the steady-state assumption for the Michaelis–Menten reaction mechanism (2) was formally discussed for the first time in 1955 by Laidler [15] who suggested that a large ratio of s_0 to e_0 (equivalent to Eqn 9) is the main prerequisite for the validity of the assumption through a mathematical analysis. Ten years later, Hommes [16], Walter and Morales

[17] and Walter [18] mapped the range of validity of the steady-state assumption for both the irreversible and reversible Michaelis–Menten reaction mechanisms using early analog computer simulations. They found notable shortcomings with the validity of the steady-state assumption for cases with large reversible constants of the enzyme–substrate intermediates, such as k_{-1}.

In 1965, Wong [19] made an attempt to develop a continuous description of the initial transient and the steady-state phases of reaction mechanism (2), and concluded that the initial transient must be brief for the steady-state assumption to be applicable, which is achieved by increasing the s_0/e_0 ratio. In 1979, Stayton and Fromm [20] found the steady-state assumption to generally hold true for $s_0/e_0 > 100$ by exploring a wide range of rate constant values and initial reaction conditions using computer simulations.

In 1980, Seshadri and Fritzsch [21,22] investigated the steady-state assumption for the Michaelis–Menten reaction mechanism (2) with reversible P formation using a scaling and simplification mathematical technique known as singular perturbation analysis. To apply singular perturbation analysis, it is necessary to estimate the timescale of the initial transient and the steady-state period of the enzyme-catalysed reaction. In 1967, Heineken *et al*. [23] used singular perturbation analysis to implement the steady-state assumption in reaction mechanism (2) for the first time. Based on the findings of Laidler [15], Wong [19] and Stayton and Fromm [20], Heineken *et al*. [23] assumed that the ratio of e_0 to s_0 needed to be small ($e_0/s_0 \ll 1$) to apply the steady-state assumption. They also showed that the relative magnitude of k_2 does not guarantee the validity of the steady-state assumption. In contrast, Seshadri and Fritzsch [21,22] used a different criterion:

$$\frac{e_0}{K_M} \ll 1. \tag{12}$$

Seshadri and Fritzsch [21] cited Reich and Sel'kov [24] as the source of their choice of criterion, but the latter authors provided no motivation for their choice. In addition, none of the above authors provide a biophysical rationale for selection of the timescale used to implement the singular perturbation analysis. Klonowski [25] provides a general discussion of timescales. However, the timescales selected to apply the steady-state assumption discussed by Klonowski were also introduced without motivation. It is worth noting that Klonowski found in the Russian literature that the steady-state assumption is valid when $e_0 \ll s_0$, or when

$$s_0 \ll K_S \text{ and } s_0 \ll K. \tag{13}$$

In the above conditions, $K_S = k_{-1}/k_1$ is defined as the equilibrium dissociation constant of C, and $K = k_2/k_1$ is the Van Slyke–Cullen constant [26]. Note that K_M can be written as $K_M = K_S + K$.

Using linear approximations and a modal analysis technique, Palsson and Lightfoot [27] and Palsson [28] derived Eqn (12) as the criterion for the validity of the steady-state assumption in 1984. In 1996, de la Selva *et al*. [29] obtained the same criterion by studying the slope of the rate of P formation versus S depletion at equilibrium using mathematical asymptotic analysis.

One of the most rigorous analyses of the validity of the steady-state assumption for reaction mechanism (2) was performed by Schauer and Heinrich [30] in 1979. They investigated the numerical errors resulting from applying the steady-state assumption by studying the time-dependent change in s and c. They proposed three criteria to minimize the errors in the implementation of the steady-state assumption. The first criterion is that the depletion of s must be small during the initial transient of the enzyme-catalysed reaction. The second criterion is that the reaction timescale of C must be faster than the reaction timescale of S. The third criterion is that the instantaneous P formation rate must always be smaller than the limiting rate V under the steady-state assumption.

Segel [6] and Slemrod [31] mathematically formalised the analysis of Schauer and Heinrich using a mathematical scaling and simplification technique, and obtained simpler formulae for the three criteria described above. They derived the following condition:

$$\frac{e_0}{K_M + s_0} \ll \left(1 + \frac{K_S}{K}\right)\left(1 + \frac{s_0}{K_M}\right), \tag{14}$$

for the validity of the steady-state assumption, i.e. $v_C \approx 0$. Interestingly, condition (14) is an extension of the conditions (9), (12) and (13). The domain for which the steady-state assumption is valid was extended by Borghans *et al*. [32] based on Segel's formulae and using a mathematical change of variables to study the total substrate concentration (the sum of s and c) rather than s. They proposed the following condition for the validity of the steady-state assumption:

$$\frac{Ke_0}{(K_M + e_0 + s_0)^2} \ll 1. \quad (15)$$

Since the total substrate concentration cannot be depleted by the formation of c during the initial transient, it is unfair to draw direct comparisons between condition (14) and the newly derived condition (15) for the total substrate concentration. In addition, the formulation using the total substrate concentration has a limited practical utility in estimating kinetic parameters, because the total substrate concentration cannot be measured experimentally in initial rate experiments. However, using this change of variable, analytical approximations have been derived to both estimate kinetic parameters using progress curves analysis [33,34] and investigate the dynamics of complex enzyme-catalysed reactions [35,36].

Conditions for the validity of the derivation of the Michaelis–Menten equation

I have shown how the Michaelis–Menten equation can be derived by applying the steady-state assumption (the Briggs and Haldane derivation). This derivation implicitly assumes that $s \approx s_0$ while c builds up during the initial transient of the reaction. In 2008, this implicit condition was named the reactant stationary assumption by Hanson and Schnell [37], although it was originally defined computationally by Côme [38] in 1979. Over the last century, the majority of enzymologists did not consider the reactant stationary assumption to be an independent assumption from the steady-state assumption.

If the steady-state assumption implicitly assumes $s \approx s_0$ during the initial transient of the reaction, is the reactant stationary assumption part of the steady-state assumption? The answer is no. In 2008, Hanson and Schnell [37] showed that the steady-state assumption can be valid without ensuring $s \approx s_0$ during the initial transient for reaction mechanism (2). Therefore, the steady-state assumption can be valid when the reactant stationary assumption is invalid. Hanson and Schnell also showed that the reactant stationary assumption can be valid when the steady-state assumption is invalid for reaction mechanism (2) in the presence of endogenous substrate. This can also occur for enzymes that catalyse reversible reactions [39].

The result of Hanson and Schnell's work has important implications for the validity of the Michaelis–Menten equation. Deriving the Michaelis–Menten equation requires the adoption of two distinct and independent assumptions: the steady-state assumption and the reactant stationary assumption. However, it is unclear under what assumptions (steady-state assumption, reactant stationary assumption or both) it is appropriate to use the Michaelis–Menten equation to estimate kinetics parameters. Below, I introduce the simpler formulae introduced by Segel [6] and Segel and Slemrod [31] to determine the regions of validity of the steady-state assumption and the reactant stationary assumption for reaction mechanism (2) and illustrate Hanson and Schnell's findings. Here, I focus on the steady-state assumption, because it is based on the Briggs and Haldane treatment, which is considered the standard approach to derive the Michaelis–Menten equation. After deriving the conditions for validity of both the steady-state assumption and the reactant stationary assumption, I will discuss the appropriate conditions for the application of the Michaelis–Menten equation.

Conditions for the validity of the steady-state assumption

In reaction mechanism (2), the steady-state assumption makes the rate of formation of C almost in balance with its rate of destruction after an initial fast transient, which means that we can take $v_C \approx 0$. This implies that c remains approximately constant during the steady-state regime of the reaction. From the biophysical point of view, this occurs when the time of c build-up (t_C) is much smaller compared to the time (t_S) during which the s changes appreciably. Therefore, the condition for the validity of the steady-state assumption is expressed in mathematical terms as:

$$t_C \ll t_S. \quad (16)$$

To solve the above condition, it is necessary to estimate the timescales t_C and t_S. To estimate t_C, Segel [6] assumes that the s does not change appreciably during t_C. By making $s = s_0$ and substituting it into Eqn (3), he transforms v_c into a linear differential equation with the solution:

$$c(t) = \frac{e_0 s_0}{K_M + s_0}\left[1 - \exp\left(-\frac{t}{t_c}\right)\right], \quad (17)$$

where

$$t_C = \frac{1}{k_1(K_M + s_0)}. \quad (18)$$

To estimate t_S, Segel [6] calculates how long it will take for a significant change to occur in the rate of

change of s using an expression that divides the total amount of s (given by s_0) by the maximum rate of s ($|v_S|_{\max}$):

$$t_S = \frac{s_0}{|v_S|_{\max}}. \tag{19}$$

If the steady-state assumption is valid, $|v_S|_{\max} = v_0$, because there is a symmetrical relationship between the substrate depletion and product formation rates (see for example, [40]). Substituting v_0 from Eqn (11), t_S is equal to

$$t_S = \frac{K_M + s_0}{V}. \tag{20}$$

Now we are in the position to calculate the criterion for the validity of the steady-state assumption by substituting Eqns (18) and (20) into Eqn (16), and rearranging the equation after some algebraic calculations (see Appendix S1)

$$\frac{e_0}{K_M + s_0} \ll \left(1 + \frac{K_S}{K}\right)\left(1 + \frac{s_0}{K_M}\right). \tag{21}$$

The above condition is identical to Eqn (14). According to condition (21), the steady-state assumption can be valid for situations where $e_0/s_0 \approx 1$, as long as $K_M \gg 1$, $K_S/K \gg 1$, or $s_0/K_M \gg 1$. This implies that the steady-state assumption is valid in a less restrictive parameter range than stated in most of the literature presented in the previous section, in which it was stated that the steady-state assumption is only valid when $e_0/s_0 \ll 1$.

Condition for the validity of the reactant stationary assumption

I now focus on the validity condition of the reactant stationary assumption for reaction mechanism (2). For the reactant stationary assumption to be valid, there must be a negligible decrease in s during the initial transient, t_C. This decrease, which we denote by Δs, is certainly less than the product of t_C and the maximal rate of s at the start of the reaction: $|v_S|_{t=0}$. This implies that

$$\left|\frac{\Delta s}{s_0}\right| = \frac{t_C |v_S|_{t=0}}{s_0} \ll 1. \tag{22}$$

Applying the law of mass action to the first elementary step ($E + S \rightarrow C$) of reaction mechanism (2) leads to $|v_S|_{t=0} = |k_1 e_0 s_0|$. Using the definition of t_C (Eqn 18), we can expand Eqn (22) to

$$\frac{e_0}{K_M + s_0} \ll 1, \tag{23}$$

which is the criterion for validity of the reactant stationary assumption. It is easy to see that, when Eqn (23) is valid, Eqn (21) must also be valid. According to condition (23), the reactant stationary assumption can be valid for situations when $e_0/s_0 \approx 1$ as long as $K_M \gg 1$. Dividing the numerator and denominator of the left-hand side of Eqn (23) by K_M, and rearranging the condition to

$$\frac{e_0}{K_M} \ll \left(1 + \frac{s_0}{K_M}\right), \tag{24}$$

it can be seen that the reactant stationary assumption is also valid when $e_0/s_0 \approx 1$ as long as $e_0 \ll K_M$. Therefore, the reactant stationary assumption is a stronger condition than that required for the steady-state assumption, and is sufficient for the validity of the steady-state assumption.

Is the Michaelis–Menten equation valid under the steady-state assumption or the reactant stationary assumption?

Following the simpler formulae introduced by Segel [6] and Segel and Slemrod [31], it was found that condition (23) ensures $s \approx s_0$ during the initial transient, but it also ensures $v_C \approx 0$ during the steady-state period. The regions of validity of the steady-state assumption and reactant stationary assumption are illustrated graphically in Fig. 2 by plotting conditions (21) and (23). In order to graphically represent the conditions, the threshold for 'much smaller than unity' was arbitrarily set as equal to 0.1. Figure 2 shows the boundaries of the regions of validity of the steady-state assumption and the reactant stationary assumption in the e_0/K_M and s_0/K_M plane. The plane is divided into three regions: the upper region where the reactant stationary assumption and the steady-state assumption are both invalid, the middle region where the steady-state assumption is valid but the reactant stationary assumption is invalid, and the bottom region where both the reactant stationary assumption and the steady-state assumption are valid. As e_0 is increased or s_0 is decreased, the reactant stationary assumption becomes invalid first, then the steady-state assumption also becomes invalid.

Hanson and Schnell [37] investigated what would happen to the estimation of kinetic parameters when the Michaelis–Menten equation, derived using the Briggs–Haldane treatment (Eqn 11), is used in the

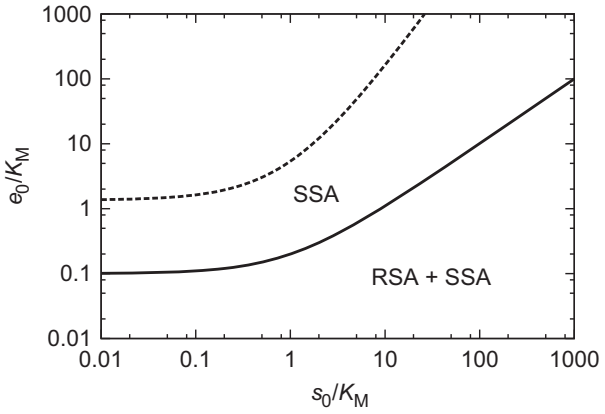

Fig. 2. Limits of the validity of the steady-state assumption and reactant stationary assumption for the irreversible single-enzyme, single-substrate enzyme reaction mechanism (2). In the area labelled 'RSA + SSA', the reactant stationary assumption (RSA) and the steady-state assumption (SSA) are both valid, and the Michaelis–Menten equation (Eqn 11) can be used to estimate the kinetic parameters K_M and V. In the area labelled 'SSA', the steady-state assumption is valid, but the reactant stationary assumption does not hold. Note that the criterion for the validity of the reactant stationary assumption is a sufficient condition for the validity of the steady-state assumption. However, if the reactant stationary assumption is not valid, the use of the Michaelis–Menten equation leads to inaccuracies of 10–1000-fold in the estimation of the kinetic parameters K_M and V. In the top area, neither the steady-state assumption nor the reactant stationary assumption are valid. Parameters used for this figure are: $K_M = 1$ μM and $K_S/K = 12.5$.

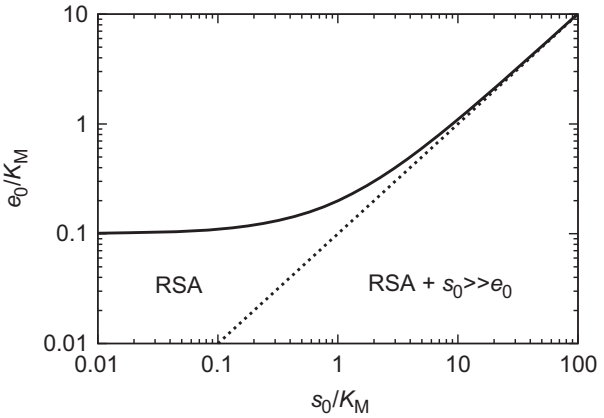

Fig. 3. The condition $s_0 \gg e_0$ is unnecessarily restrictive in initial rate experiments for the irreversible single-enzyme, single-substrate enzyme reaction mechanism (2). The Michaelis–Menten equation (Eqn 11) can be used to accurately estimate the kinetic parameters K_M and V when the reactant stationary assumption (RSA) is valid. Note that in the area labelled 'RSA + $s_0 \gg e_0$', both assumptions are valid, but the reactant stationary assumption is also valid in the region labelled 'RSA'. When $s_0 \approx e_0$, the reactant stationary assumption is valid as long as $K_M \gg 1$ or $e_0 \ll K_M$. In the top area, neither the condition $s_0 \gg e_0$ nor the reactant stationary assumption are valid. The value of the Michaelis–Menten constant used in this figure is 1 μM.

region where the steady-state assumption is valid, but the reactant stationary assumption is not. They found that the v_0 for the single-enzyme, single-substrate reaction mechanism (2) may lead to widely inflated estimates of K_M and V. The values can over-estimate the real K_M and V values by as much as 10–1000-fold. This clearly indicates that the Michaelis–Menten equation can only be used to accurately estimate kinetic parameters when the reactant stationary assumption is valid, i.e. when condition (23) is satisfied.

It may be argued that the identification of the region of validity of the reactant stationary assumption has a limited significance for the practice of initial rate experiments, because these enzyme kinetics experiments are generally arranged such that $s_0 \gg e_0$. Although the reactant stationary assumption is valid when $s_0 \gg e_0$, this condition is unnecessarily restrictive, as shown in Fig. 3. As illustrated in the figure, and explained in the analysis in the previous sub-section, the Michaelis–Menten equation can be used even when $s_0 \approx e_0$, as long as $e_0 \ll K_M$.

If the reactant stationary assumption is not valid but the steady-state assumption holds, it is necessary to make some corrections to rate equations and experimental assays. When considering the Briggs and Haldane treatment for reaction mechanism (2), if we do not adopt the reactant stationary assumption, we have to ignore the assumption that $s \approx s_0$ during the initial transient. Substituting Eqn (6) into $v_P = k_2 c$, as defined above (Eqn 8), leads to a new equation for the rate of change of product concentration

$$v_P = \frac{Vs}{K_M + s}. \tag{25}$$

To obtain accurate estimates of K_M and V using Eqn (25), it is necessary to measure both v_P and s simultaneously during the pseudo-steady-state period under experimental conditions when the steady-state assumption holds [39]. This complicates the kinetic experiment and could potentially require both s and e, because both S and E can be depleted with the formation of C under experimental conditions where s cannot be considered to be approximately equal to s_0 [2].

Conclusion

In enzyme kinetics, the Michaelis–Menten equation is widely believed to be valid under the steady-state

assumption. In standard biochemistry textbooks, the application of the steady-state assumption implicitly assumes that there is an initial transient during which s remains approximately constant (i.e. $s \approx s_0$), while c builds up and achieves pseudo-steady-state (i.e. $v_C \approx 0$). The implicit assumption that $s \approx s_0$ during the initial transient is known as the reactant stationary assumption. However, in this review, I have presented evidence showing that the reactant stationary assumption is not an implicit part of the steady-state assumption, but rather a separate and distinct assumption.

Analogous expressions to the Michaelis–Menten equation (Eqn 1), v_0, has been derived for a number of enzyme-catalysed reactions: the Van Slyke and Cullen urease reaction, reactions for adsorption of gases onto solids, linear competitive and uncompetitive enzymatic reactions, and some allosteric reactions [1,2]. For the derivation of the Michaelis-Menten equation, it is essential to apply both the steady-state assumption and the reactant stationary assumption, contrary to the widespread belief that the Michaelis–Menten equation is valid under the criterion for validity of the steady-state assumption only. In fact, rate equations in the form of the Michaelis–Menten equation are often said to be 'steady-state kinetic' equations.

During the last 25 years, it has been shown that the criterion for validity of the reactant stationary assumption is sufficient for validity of the steady-state assumption for the irreversible single-enzyme, single-substrate reaction [6], irreversible linear competitive [41], uncompetitive and mixed enzymatic reactions [42], and irreversible enzyme-catalysed reactions with alternative substrates [43]. Recently, Hanson and Schnell [37] demonstrated that the reactant stationary assumption is a necessary condition for the validity of the Michaelis–Menten equation to estimate kinetic parameters. They showed that the estimation of kinetic parameters using the Michaelis–Menten equation can lead to widely inflated values of K_M and V if experiments are performed under conditions where the steady-state assumption is valid but the reactant stationary assumption does not hold. Therefore, experiments must be performed under conditions that guarantee the validity of the reactant stationary assumption.

Surprisingly, initial rate experiments in enzyme kinetics are generally performed so that $s_0 \gg e_0$. From the biophysical point of view, this condition ensures that the enzyme is saturated with the substrate, causing the enzyme–substrate complex to build up and to remain in pseudo-steady-state for a long time. As shown in this review, $s_0 \gg e_0$ is one of the conditions for the steady-state assumption to be valid, but more importantly, it is a condition for the validity of the reactant stationary assumption. However, the condition $s_0 \gg e_0$ is unnecessarily restrictive. The analysis presented here shows that the Michaelis–Menten equation can be used even when $s_0 \approx e_0$ as long as $K_M \gg 1$ or $e_0 \ll K_M$ (see Fig. 3). As a consequence, biochemists can relax the condition $s_0 \gg e_0$ for the initial rate experiments of enzyme-catalysed reactions if the order of magnitude of K_M is known *a priori*.

This review also draws attention to the fact that the Michaelis–Menten equation, although widely believed to be valid under 'steady-state kinetics', is in reality truly valid under 'reactant stationary kinetics'.

Acknowledgements

I am indebted to Marc R. Roussel (Department of Chemistry and Biochemistry, University of Lethbridge, Alberta, Canada) for calling my attention to the problem of the reactant stationary approximation in enzyme kinetics a few years ago. I would also like to thank Mark Whidden, Allison Ho and Caroline Adams for carefully reading the manuscript. This work has been partially supported by the James S. McDonnell Foundation (Grant No. 220020223) under the 21st Century Science Initiative Studying Complex Systems Program.

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Supporting information

Additional supporting information may be found in the online version of this article at the publisher's web site:

Appendix S1. Derivation of conditions for validity of the steady-state assumption.