# Reactant Stationary Approximation in Enzyme Kinetics

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# Reactant Stationary Approximation in Enzyme Kinetics

**Sonya M. Hanson**[†] **and Santiago Schnell***[,‡]

*Department of Physics and Astronomy, University of Southern California, Los Angeles, California 90089-0484, and Indiana University School of Informatics and Biocomplexity Institute, 901 East Tenth Street, Bloomington, Indiana 47408-3912*

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In the application of the quasi-steady-state approximation, it is generally assumed that there is an initial transient during which the substrate concentration remains approximately constant while the complex concentration builds up. In this paper, we address the assumption that the substrate concentration does not change significantly during this initial transient and name it the reactant stationary approximation. For the single enzyme, single substrate reaction, the reactant stationary approximation is generally considered a sufficient condition to apply the quasi-steady-state approximation. Studying the dynamic behavior of this reaction with endogenous substrate, we show that the quasi-steady-state approximation and reactant stationary approximation are two separate approximations. We discuss the consequence of this result for the determination of reaction parameters in enzyme catalyzed reactions.

## 1. Introduction

The quasi-steady-state approximation (QSSA), known also as the steady-state approximation or the pseudo-steady-state hypothesis, is the most common simplification method in biochemical kinetics. It has been systematically employed in the field for more than 80 years.[6,15]

In the single enzyme (E), single substrate (S) reaction known as the Michaelis−Menten (MM) mechanism of enzyme action,[11] the enzyme combines with substrate to reversibly form enzyme−substrate complex (C). The complex then irreversibly yields the product (P) and releases the free enzyme:

$$\mathrm{S} + \mathrm{E} \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} \mathrm{C} \xrightarrow{k_2} \mathrm{P} + \mathrm{E} \tag{1}$$

By applying the law of mass action, the time course of this reaction is described by a nonlinear system of ordinary differential equations (ODEs). The QSSA simplifies this system by assuming one of the dependent variables, usually the concentration of the enzyme−substrate complex ($c$) to be approximately constant after a fast initial transient.

With this simplification, the QSSA helps determine the kinetics parameters in enzyme kinetics experiments. Initial rate experiments are usually used to estimate these kinetics parameters.[4,5] They are simple to perform and analyze, being relatively free of complications such as back-reaction and enzyme degradation. These experiments are performed during the reaction's longest kinetic timescale, the quasi-steady-state (QSS) period. When an enzyme is mixed with a large excess of substrate, the complex builds up in a fast initial transient. The reaction then achieves a QSS period in which the enzyme−substrate concentration ($c$) remains approximately constant. This behavior during the QSS period justifies the application of the mathematical approximation of the QSSA. In initial rate experiments, the initial rate or velocity ($v_0$) is measured by monitoring the accumulation of the product for a short period after the reaction has reached the quasi-steady-state. For further details and new insights into modern experimental enzyme kinetics, readers should consult ref 3, Chapters 2 and 3. In the MM mechanism (1), $v_0$ is governed by the MM equation:

$$v_0 = \frac{v_{\max} s_0}{K_\mathrm{M} + s_0} \tag{2}$$

where $v_{\max}$ is the maximum velocity and $K_\mathrm{M} = (k_{-1} + k_2)/k_1$ is the MM constant. During the initial fast transient, the measurements are carried out for a very short period and there is a large excess of substrate, so the substrate concentration ($s$) is considered constant and approximately equal to the initial substrate concentration ($s_0$). Taking $s \approx s_0$ leads to the presence of $s_0$ in MM eq 2. This second assumption, that $s$ remains constant during the fast initial transient, is generally considered as a part of the QSSA. Here, however, we give it a separate name: the reactant stationary approximation (RSA). Côme[2] was the first to define this approximation computationally, though he used a slightly different terminology, as his "S" stands for "stationarity". We chose to avoid this term due to its definition in the field of statistics.

The actual validity of the QSSA was first discussed by Laidler[8] whose theoretical analysis suggested excess substrate concentration to be the main prerequisite for the validity of the QSSA. Heineken et al.[7] then mathematically formalized the application of the QSSA to MM reaction 1 with the aid of singular perturbation theory. This theory relies on the selection of dimensionless variables and parameters to investigate the dynamic behavior of the reaction in two timescales: the fast initial transient and the quasi-steady-state period. A number of authors have proposed different scalings for MM reaction 1. In the majority of cases, these scales have been introduced without motivation. Palsson[12] was one of the first scientists to propose a systematic approach for the derivation of scales to simplify the complex differential equations that describe enzyme cata-

* Corresponding author. Mailing address: Santiago Schnell, Center for Computational Medicine and Biology, University of Michigan Medical School, 2017 Palmer Commons, 100 Washtenaw Avenue, Ann Arbor, MI 48109-2218. E-mail: schnells@umich.edu. Telephone: +1-734-615-8733. Fax: +1-734-615-6553.

† University of Southern California, Los Angeles.

‡ Indiana University School of Informatics and Biocomplexity Institute.

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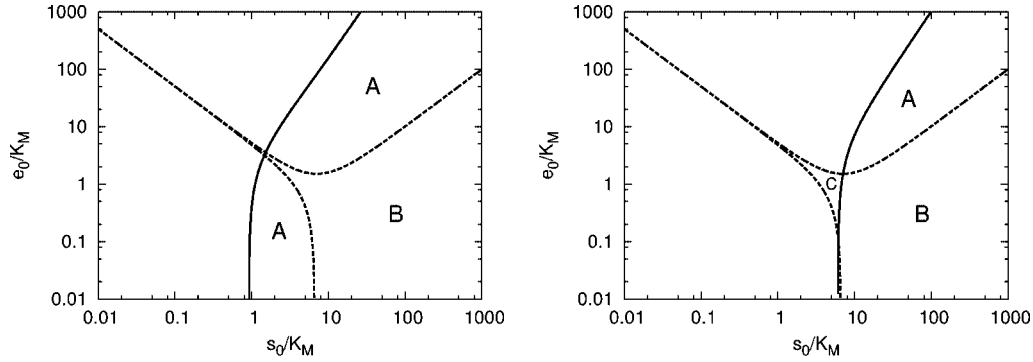


(a) $\delta = 13.5$ (b) $\delta = 1$

**Figure 1.** Conditions of validity of the QSSA and the RSA for the MM reaction mechanism with endogenous substrate ($c_0/K_m = 5$) in the $e_0/K_M - s_0/K_M$ plane for different values of $\delta = (k_{-1} + k_2)/k_2$. The area labeled by the letter B between the two dotted curves is the region of validity of the RSA, while the areas marked by the letter A are the regions in which the QSSA is valid without the RSA being valid. The QSSA is valid in both A and B. In part b, the area labeled by the letter C identifies the region in which the RSA is valid with the QSSA being invalid; in other words, it is the region in which the RSA does not imply the QSSA.

lyzed reactions. A survey of the conditions for the validity of the QSSA can be found in the works of Turányi et al.[18] and Segel and Slemrod.[22]

Irrespective of the scales employed to simplify the reaction dynamics, the derivation of MM equation 2 with the aid of the QSSA generally involves two different assumptions:[10,15,21]

I. After an initial transient, $c$ remains approximately constant. That is, in the steady-state regime, it can be taken that $dc/dt \approx 0$. From the biophysical point of view, this approximation can be made when the time ($t_s$) taken for a significant change in $s$ is much bigger than the time ($t_c$) taken for a significant buildup of $c$ during the initial fast transient. In this case, $t_s$ characterizes the QSS period, while $t_c$ is the timescale of the initial fast transient. By estimating the relevant timescales for MM mechanism 1, Segel[21] found that

$$\frac{t_c}{t_s} \ll 1, \quad \text{is equivalent to} \tag{3}$$

$$\frac{e_0}{K_M + s_0} \ll \left(1 + \frac{K_S}{K}\right)\left(1 + \frac{s_0}{K_M}\right) \tag{4}$$

In this expression $e_0$ is the initial enzyme concentration, $K_S = k_{-1}/k_1$ is the dissociation constant of S from C, and $K = k_2/k_1$ is the Van Slyke−Cullen constant.[14]

II. During the initial transient, that is for $t < t_c$, the substrate concentration $s$ is approximately constant. Therefore, there must be only a negligible depletion of the substrate concentration during the initial fast transient. Segel[21] proposed this decrease ($\Delta s$) must be less than the product of the timescale $t_c$ and the initial maximal rate of substrate depletion. This yields

$$\left|\frac{\Delta s}{s_0}\right| = \frac{t_c}{s_0}\left|\frac{ds}{dt}\right|_{max} \ll 1 \tag{5}$$

$$= \frac{e_0}{K_M + s_0} \ll 1 \tag{6}$$

These two inequalities, which follow naturally from the two assumptions, are generally considered the conditions for validity of the QSSA. It is easy to see that when eq 6 is valid then eq 4 must be valid. This relationship led Segel[21] to propose that the second condition regarding the initial transient, which we call here the RSA, is the single criterion for the validity of the QSSA. The fact that the RSA implies the QSSA has been confirmed for more complex enzyme catalyzed reactions.[16,17]

Segel[21] was not the first to propose the separation of the QSSA into these two different assumptions (eqs 3 and 5). Earlier, Schauer and Heinrich[13] had proposed that the QSSA could only be applied if the error caused by the first (eq 3) or second assumption (eq 5) did not exceed a tolerated value. Segel[21] then used scaling arguments to estimate the measurements proposed by Schauer and Heinrich.[13]

Since it is assumed that reactant concentrations are constant during the initial transient period if the QSSA is applied, the RSA is not usually considered to be independent of the QSSA. However, whether the RSA should be considered independent of the QSSA has never been directly addressed. The difference between the two approximations is worth investigating particularly if we approach them in more realistic reaction scenarios. For example, the substrate depletion is affected by the reactant propagation in long chain pathways.[2] Also, a crude cell-free extract of an enzyme may contain endogenous substrate in the form of enzyme−substrate complex.[19] Endogenous substrate is also present in reactions occurring in vivo conditions. This extra substrate can affect the reaction dynamics and the application of the QSSA and RSA. As has been discussed before,[15] the correct application of approximations in enzyme kinetics is of great importance for the determination of reaction parameters.

In this paper, we investigate the conditions for the application of the QSSA and RSA to reaction 1 with endogenous substrate in the form of enzyme−substrate complex. In section 2, we derive the system of ODEs governing the reaction and determine the characteristic timescales of the reacting species. By applying the scaling arguments proposed by Segel[21] to determine the conditions for validity of the QSSA and RSA, we find that the RSA and QSSA are two different assumptions and that one can be valid without the other and vice versa (section 3). However, we have also found that it is rare to find the RSA valid without the QSSA being valid, which means that the assumption that the RSA implies the QSSA can be made mostly without concern in the MM reaction with endogenous substrate. In the light of these results, we establish the conditions under which one should carefully consider the relationship between the RSA and QSSA and the implications that this has on the use of MM equation 2.

## 2. Single-Enzyme, Single-Substrate Reaction in the Presence of Endogenous Substrate

Let us consider MM reaction mechanism 1 with endogenous substrate. In an experimental assay, the endogenous substrate

can exist in two forms: free substrate S and enzyme−substrate complex C. Endogenous substrate could be present if the enzyme solution was not purified. It is possible to find endogenous substrate in the enzyme extract if the reaction requires additional substrates or cofactors that are not present in the enzyme extract.[19]

When the law of mass action is applied to mechanism 1, we obtain a well-known set of coupled nonlinear ODEs. In the presence of endogenous substrate, the initial condition for this system is the following:

$$(s, e, c, p)(t = 0) = (s_0, e_0, c_0, 0) \tag{7}$$

Here, we denote the concentrations with lower case. Assuming that the reaction is a closed and isolated thermodynamic system, it obeys the enzyme conservation law

$$e + c = e_0 + c_0 \tag{8}$$

and the substrate conservation law

$$s + c + p = s_0 + c_0 \tag{9}$$

These conservation laws along with the following ODEs describe the time course of the reaction mechanism (1):

$$\frac{ds}{dt} = k_1(-(e_0 + c_0 - c)s + K_S c) \tag{10}$$

$$\frac{dc}{dt} = k_1((e_0 + c_0 - c)s - K_M c) \tag{11}$$

with initial conditions $(s, c)(t = 0) = (s_0, c_0)$.

**2.1. Determining the Timescales.** Since the timescales are crucial to estimating the validity of the QSSA, it is necessary to estimate the duration of the initial fast transient and the duration of the QSS period. Following Segel,[21] we can use simple scaling arguments to estimate the values of these timescales.

To estimate the initial fast transient timescale, $t_c$, we solve eq 11 by setting $s \approx s_0$. We obtain

$$c(t) = \bar{c}[1 - \exp(-kt)] + c_0[\exp(-kt)] \tag{12}$$

where

$$\bar{c} = \frac{(e_0 + c_0)s_0}{K_M + s_0} \tag{13}$$

$$k = k_1(K_M + s_0) \tag{14}$$

For first-order reactions, which are described by linear differential equations, the timescale is defined as the absolute value of the reciprocal of the eigenvalue for the chemical species in consideration.[23] As a consequence, we can estimate $t_c$ from eq 14:

$$t_c = k^{-1} = \frac{1}{k_1(K_M + s_0)} \tag{15}$$

Note that this timescale is identical to that of the MM reaction in the absence of endogenous substrate (see eq 22 of ref 21).

The estimation of $t_s$ requires employing the characterization (see p 56 of ref 20)

$$t_s = \frac{s_0}{|ds/dt|_{max}} \tag{16}$$

We obtain the value of $|ds/dt|_{max}$ from eq 10 with $s = s_0$ and $c = (e_0 + c_0)s/(K_M + s)$. This value of $c$ is obtained by assuming $dc/dt \approx 0$ in eq 11. Substituting these values into eq 16 gives

$$t_s = \frac{K_M + s_0}{k_2(e_0 + c_0)} \tag{17}$$

Unlike $t_c$, this timescale differs from that reported for the reaction without endogenous substrate (see eq 24 of ref 21). The expression now reflects the enzyme concentration in its two forms: free ($e_0$) and occupied ($c_0$) enzyme. In the next section, we show that this difference is crucial to a further analysis of the QSSA and RSA.

## 3. Conditions for Validity of the QSSA and the RSA

We are now ready to find the criteria for the validity of the QSSA and RSA in the MM mechanism with endogenous substrate. In the introduction (section 1), we established that the condition for the validity of the QSSA is that the initial fast transient must be much shorter than the QSS period, eq 3. Substituting eqs 15 and 17 into eq 3 gives

$$\frac{e_0 + c_0}{K_M + s_0} \ll 1 + \frac{K_S + s_0}{K} \tag{18}$$

The criterion for the validity of the RSA was also presented in the introduction (section 1) as eq 5. We estimate $|ds/dt|_{max}$ as the absolute value of eq 10 at $c = c_0$ and $s = s_0$. After substituting this and $t_c$ into eq 5, the condition for validity of the RSA becomes

$$\left|\frac{-e_0 + K_S\frac{c_0}{s_0}}{K_M + s_0}\right| \ll 1 \tag{19}$$

Traditionally, when $c_0$ is zero (in the absence of endogenous substrate), the absolute value is easily eliminated, but in our case, it remains as a peculiar feature. Notice also that with a nonzero $c_0$ it is no longer obvious which condition (eq 18 or eq 19) is stronger. While mathematically it is not evident that the RSA implies the QSSA, plotting the conditions for validity shows that the RSA mostly implies the QSSA (see Figure 1a). The regions denoted in the figures correspond to the following:

$$\frac{e_0 + c_0}{K_M + s_0} - \frac{K_S + s_0}{K} \leq 0.1 \tag{20}$$

$$\left|\frac{-e_0 + K_S\frac{c_0}{s_0}}{K_M + s_0}\right| \leq 0.1 \tag{21}$$

There is a small region, however, in which the RSA is valid without implying the QSSA. This region is generally negligible except at certain ratios of the rate constants (see Figure 1b). While these graphs are both given at a constant value of endogenous substrate $c_0$, the behavior of this system remains largely the same as $c_0$ increases. However, as $c_0$ decreases, the assumption that the RSA implies the QSSA becomes overwhelmingly valid, which is consistent with the previous treatment of the QSSA (see Figure 2).

## 4. Discussion

In enzyme kinetics, the application of the QSSA generally assumes that there is an initial transient during which the substrate concentration remains approximately constant while the complex concentration builds up. This assumption is known as the RSA, and it has been considered part of the QSSA. Segel[21] demonstrated that the RSA is a sufficient condition for the application of the QSSA to MM mechanism 1.

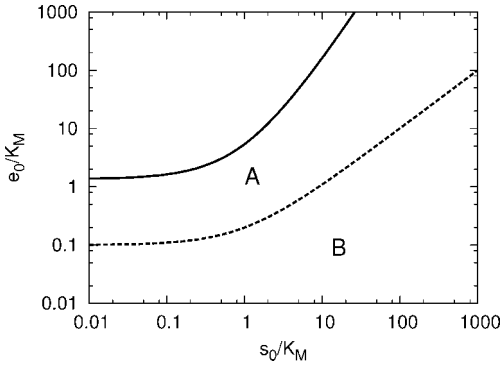


**Figure 2.** Conditions of validity of the QSSA and the RSA for the MM reaction mechanism in the absence of endogenous substrate ($c_0/K_m = 0$) in the $e_0/K_M - s_0/K_M$ plane. The area labeled by the letter B underneath the dotted curve is the region of validity of the RSA, while the area marked by the letter A is the region in which the QSSA is valid without the RSA being valid. The QSSA is valid in both A and B. Parameter: $\delta = (k_{-1} + k_2)/k_2 = 13.5$.

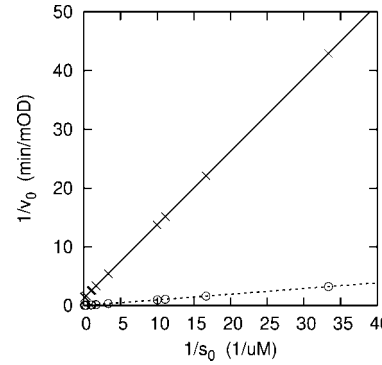


**Figure 3.** Double-reciprocal plots illustrating the poor estimates of the kinetics parameters when the QSSA is valid without the RSA. We numerically simulated the initial velocities for a single enzyme, single substrate reaction with $K_M = 1$ $\mu$M and $v_{max} = 0.8$ mOD/min. The expected MM equation with these values is shown with a solid line. The numerical simulation of the initial velocities leads to an estimate of $K_M = 44$ $\mu$M and a $v_{max} = 455$ mOD/min (dotted line). Parameters for the numerical simulations are the following: $k_1 = 10.8$ $\mu$M/min, $k_{-1} = 10$ 1/min, $k_2 = 0.8$ 1/min, and $v_{max} = 0.8$ mOD/min.

In this work, we have shown that the RSA and QSSA are two separate approximations. We have investigated the relationship between the RSA and QSSA in MM reaction 1 in the presence of endogenous substrate. Our analysis shows two important results: (i) there are instances when the RSA does not imply the QSSA and (ii) the QSSA can be valid without the RSA. As we illustrate in Figure 1b, the first result concerns a small parameter domain area in the $e_0/K_M - s_0/K_M$ plane. Since this situation corresponds to such a small area of the parameter domain, it does not seem to be significant experimentally. In addition there is a general consensus that the enzyme−substrate complex is a short-lived intermediate; therefore, its effects may not be significant in the reaction.

However, the second result of our work has important consequences in the determination of kinetics parameters using MM eq 2 derived with the aid of the Briggs−Haldane approach, King−Altman method,[3] or alternative approaches.[9] For the single enzyme, single substrate reaction 1, the MM equation assumes $s \approx s_0$, which is invalid when the QSSA is valid without the RSA. As a consequence, the application of MM eq 2 can lead to inaccuracies of 10−1000-fold in the estimation of $K_M$ and $v_{max}$ when the QSSA is valid without the RSA. In Figure 3, we illustrate an example of these deviations. In a numerical simulation of the initial velocities for a single enzyme, single substrate reaction with $K_M = 1$ $\mu$M and $v_{max} = 0.8$ mOD/min, we found that the double-reciprocal plot of the MM equation leads to an incorrect estimate of $K_M = 44$ $\mu$M and a $v_{max} = 455$ mOD/min (dotted line). Note the difference between the simulated curve and the expected double-reciprocal plot of MM eq 2 with $K_M = 1$ $\mu$M and $v_{max} = 0.8$ mOD/min (solid line).

The results of this paper illustrate that experimentalists must be careful to use the correct approximation that is appropriate to the initial conditions within the parameter space. It also brings to our attention that MM eq 2, which is generally considered valid with the QSSA, can only be employed accurately to determine the kinetics parameters when the RSA is valid. These potential problems can be avoided by using a radically different treatment of enzyme kinetics: progress curve analysis. With the advent of computers, the estimation of kinetics parameters can be made using sophisticated nonlinear regression algorithms, which numerically integrate the differential equations governing MM reaction 1.[1,24] These new methods do away with the QSSA and RSA approximations.

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