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A Century of Enzyme Kinetics: Reliability of the K_M and v_{max} Estimates

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The application of the quasi-steady-state approximation (QSSA) in biochemical kinetics allows the reduction of a complex biochemical system with an initial fast transient into a simpler system. The simplified system yields insights into the behavior of the biochemical reaction, and analytical approximations can be obtained to determine its kinetic parameters. However, this process can lead to inaccuracies due to the inappropriate application of the QSSA. Here we present a number of approximate solutions and determine in which regions of the initial enzyme and substrate concentration parameter space they are valid. In particular, this illustrates that experimentalists must be careful to use the correct approximation appropriate to the initial conditions within the parameter space.

Keywords: enzyme kinetics, Michaelis–Menten, quasi-steady-state approximation, fitting procedures, progress curves

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Many biochemists may find it hard to believe both that there has been an explosion of interest in methods for studying enzyme kinetics and in the importance that biochemical kinetics is acquiring in the postgenomic era, as these have been part of biochemical research for a century.

A consequence of the development of molecular genetics is the great increase in studies of mutant and molecular engineering proteins. At first sight, it does not seem necessary to develop any special methods for studying kinetic behavior in such cases. However, this view has completely changed with the increasing study of mutant variants of proteins and enzymes characteristic of certain diseases or produced by site-directed mutagenesis, because comparison of their kinetic properties with those of their normal analogs can lead to useful conclusions only if performed accurately (Cornish-Bowden 2001).

Although comparisons between the kinetics properties of wild-type and mutant enzyme samples have long been made in the past, it was difficult to reach a biological conclusion since the prevailing methodologies for determining kinetics parameters were not very accurate.

In the nineteenth century, the first scientists studying enzyme kinetics of the single enzyme-substrate reaction experienced a number of difficulties. The experimental practice was to follow the reaction over an extended period of time and to explain observations in terms of the solutions of second-order rate equations used in chemical kinetics. But then, Brown (1892, 1902) and a number of other workers found that the rate of enzyme-catalysed reactions deviated from second-order kinetics. In 1901, Henri (1901a, 1901b, 1902) proposed the following reversible reaction scheme between an enzyme E and a substrate S, giving the enzyme-substrate complex C, which irreversibly yields product P:

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

and developed mathematically the model of enzyme action. Here k_1, k_{-1}, and k_2 are rate constants. Figure 1 illustrates typical time behavior of $[\mathrm{S}](t)$ and $[\mathrm{C}](t)$ in the reaction (1). The difficulty of following the behavior of the enzymatic reaction was largely resolved when Michaelis and Menten (1913) showed that enzymes can be studied by measuring initial rate of product formation under certain conditions. Since then, biochemists usually determine the enzyme kinetics parameters using an expression for the velocity of product formation known as the Michaelis–Menten (MM) equation (Boyde 1980)

$$v_0 = \frac{v_{\max}[\mathrm{S}_0]}{\mathrm{K}_M + [\mathrm{S}_0]} \tag{2}$$

where $v_{\max}$ is the maximum velocity and $K_\mathrm{M} = (k_{-1} + k_2)/k_1$ is the MM constant. For this reason, today the reaction mechanism (1) is conventionally attributed to Michaelis and Menten (1913) although these authors clearly recognized Henri as the originator.

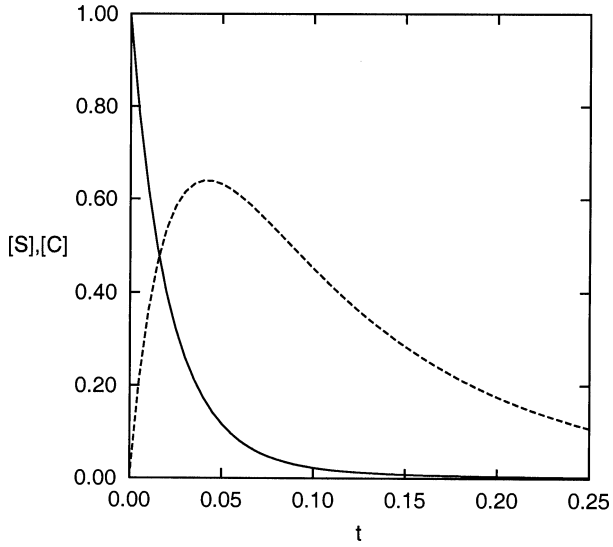

FIGURE 1 Numerical solution of [S](t) (solid curve) and [C](t) (dashed curve) of the reaction (1) for $k_1 = 10,\ k_{-1} = 1,\ k_2 = 10,\ [S_0] = 1,\ [E_0] = 5$.

The MM equation has proved to be a simple approach to describe enzyme processes. Its power resides in the time-independent hyperbolic relation of v_0 with $[S_0]$ from which the reaction parameters, K_M and v_{max}, can be determined. v_0 generally follows saturation kinetics with respect to the concentration of substrate, $[S_0]$, as shown in Figure 2. At sufficiently low $[S_0]$, v_0 increases linearly with $[S_0]$ following a relation of the form $v_0 = (v_{max}/K_M)[S_0]$. But as $[S_0]$ is incremented v_0 increases less rapidly than $[S_0]$, until at sufficiently high or saturating $[S_0]$, v_0 reaches a limiting value v_{max}.

During the last 70 years, fitting procedures for determining v_{max} and K_M have been mainly concerned with the development of graphical methods to estimate reaction constants, such as the double reciprocal linear plot (Haldane and Stern 1932; Lineweaver and Burk 1934)

$$\frac{1}{v_0} = \frac{1}{v_{max}}\left(1 + \frac{K_M}{[S_0]}\right) \tag{3}$$

Numerous enzyme kinetics books are dedicated to the important subject of estimating the kinetics parameters and distinguishing between reaction mechanisms with the aid of graphical methods (Schulz 1994; Segel 1975; Dixon and Webb 1979; Cornish-Bowden 1995a; 1995b).

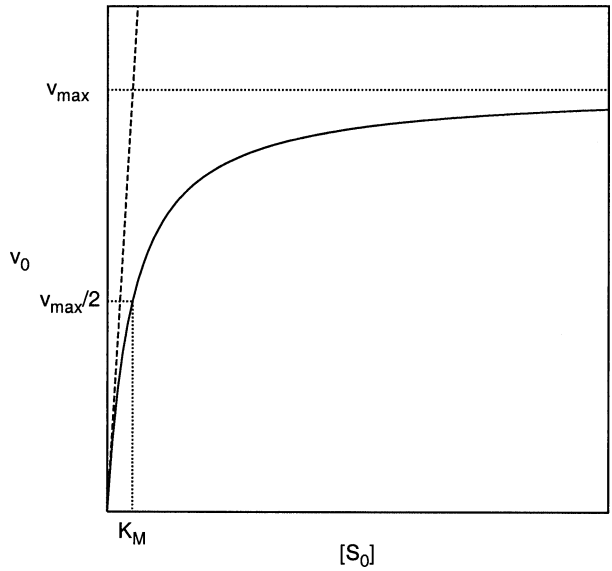

FIGURE 2 Initial velocity plotted against initial substrate concentration for the reaction (1). The dashed curve is $v_0 = (v_{\max}/K_{\mathrm{M}})[\mathrm{S}_0]$.

The application of the MM equation and its linear transformations to estimate kinetic constants does present several problems (Darvey et al. 1975; Schnell and Mendoza 2000a). Despite their virtues in data visualization, diagnostics, and education, such graphical methods of analysis can be tedious, of relatively poor accuracy, and imply acquired expertise (Chan 1995; Dowd and Riggs 1965; Ritchie and Prvan 1996). From the experimental point of view, for instance, it is difficult to measure the reaction velocity, and the information contained in the time evolution is wasted when only the initial velocities are determined. In addition, the complications of the approach are unavoidable: every point for fitting the MM equation requires the timing of a progress curve to estimate the initial velocity (Schnell and Mendoza 1997, 2001). Recently, the accuracy of the determination of kinetic parameters, the MM constant, K_{M}, and the maximum velocity, $v_{\max}$, using the MM equation has been assessed by Monte Carlo modeling (Ritchie and Prvan 1996). It has been concluded that the MM equation can lead to unsatisfactory results. K_{M} estimates made using double-reciprocal plots and the MM equation were consistently inferior to estimates made with nonlinear least-square fitting methods.

Concerns have been also raised regarding the validity of the kinetics parameters obtained using the MM equation and their graphical transformations,

as it seems that these could have been made in numerous experiments under conditions in which the MM equation is not valid (Segel and Slemrod 1989; Schnell and Mendoza 1997; Schnell and Maini 2000). Every biochemistry and enzyme kinetics textbook shows how to derive the MM equation (2) with the aid of the standard quasi-steady-state approximation (sQSSA) and therewith the basis methodology for fitting enzyme kinetics data. However, these texts do not present a clear criterion for the validity of the MM equation and the sQSSA. This is not surprising, because the majority of the research literature for the MM reaction (1) concerning integrated equations or analytic solutions derived from the sQSSA does not contain an analysis of the validity of the assumption and the solutions (Segel and Slemrod 1989). As a consequence, the velocity equations of the catalytic reaction have been employed on a number of occasions outside of the conditions for which they are valid (Schnell and Maini 2000).

In this article, we begin by briefly reviewing how kinetics parameters are calculated using progress curves and present a new solution recently derived by one of us (Schnell and Mendoza 1997, 2001). We then carefully consider the parameter space in which the approximation underlying the derivation of these progress curves is valid. We show that approximations can be found that are valid in other areas of parameter space. In particular, we discuss the importance of using the correct approximation depending on the initial conditions for fitting the kinetic parameters. We conclude that some of the errors that have arisen in calculating kinetic parameters may be due to inappropriate use of these approximations.

ANALYSIS OF PROGRESS CURVES

In the introduction we mentioned that nowadays biochemists characterize enzymatic reactions by measuring the rate or velocity of the catalytic reaction. The rate equations are derived by applying the sQSSA to the system of nonlinear ordinary differential equations (ODEs) set up using the law of mass action on the reaction (1) (Schnell and Mendoza 1997). This simplification and its consequences are generally studied in elementary enzyme kinetics courses, but in the next section we discuss them further.

Biochemists usually perform experimental measurements of the rate of enzyme-substrate reactions after a relatively short and fast initial transient but before the substrate concentration decays appreciably. However, experimental measurements rarely determine rates directly. Rather, substrate or product concentrations are determined at various times, and rates are calculated from the change in concentration with time. This process of differentiating the data is necessarily inexact (Schnell and Mendoza 1997, 2000b). Furthermore, if the assay method is discontinuous and the change in concentration is not linear with time, the rate determined may be unreliable

(Duggleby 2001). This limits the quantitative information that can be deduced from an enzymatic reaction.

There is an alternative to using the velocity equations to determine the kinetics parameters and that is to solve the differential equations governing the biochemical reaction. This involves creating a mathematical description that is formulated in the same terms as the experimental measurements, that is, concentrations are written as functions of time (Schnell and Mendoza 1997).

Apart from resolving the basic problem of the inaccuracy of the velocity determination, measurements of time courses, or progress curves as they are often known, have other advantages: (1) A single reaction experimental assay can yield multiple experimental points, allowing more data to be collected. In contrast, when velocities are measured it is usual to determine one velocity from each experimental assay. Using progress curves usually decreases the number of experimental assays by at least a factor of five (Schnell and Mendoza 2002). (2) The kinetic data collected from a single experiment are obtained at exactly the same concentration of all the elements present in the reaction assay. (3) Low-affinity enzymes are easily studied because it is possible to measure the reaction over a period during which a substantial fraction of substrate is used. In contrast, velocity measurements must be restricted to a timescale where substrate depletion is negligible so that its concentration is nearly constant and the sQSSA is valid (Duggleby 2001).

Given these advantages, why do the vast majority of enzyme studies continue to focus on velocities rather than the progress curves? Presently, there are three main ways to fit progress curves. First, the MM equation (2) is integrated and then rearranged as a linear plot to determine the kinetic parameters. This analysis ignores the fact that the variable associated with the experimental error is a concentration and not the velocity; thus, the linear transformation distorts the experimental error leading to biased parameter estimates (Schnell and Mendoza 2001). The second approach is the numerical integration of the differential equations describing the enzyme reactions, but in general this is computationally intensive and can result in misleading conclusions due to the use of root-finding methods during the integration and a nonlinear least-squares fitting procedure (Duggleby 1995; Schnell and Mendoza 2000a; Zimmerle and Frieden 1989).

The third approach consists of deriving analytical approximations. This is the most effective method and analytic progress curves have so far been derived for various reactions (Duggleby 1995 and references therein). Schønheyder (1952) and Duggleby and Morrison (1977) derived analytic progress curves for the single MM reaction with and without product inhibition. Alberty and Koerber (1957) and Walter (1963) obtained progress curves for the reversible MM reaction. Darvey and Williams (1964), Boeker (1984), and Duggleby and Wood (1989) have derived progress curves for enzyme reactions with alternative substrates. Duggleby (1986) derived analytic progress curves for enzyme inactivation. Orsi and Tipton (1979) and Szedlacsek

and collaborators (1990) obtained progress curves for an enzyme reaction in which a linear inhibitor is present. Tsou (1988), Topham (1990), and Wang and Zhao (1997) have derived progress curves for enzymatic reactions affected by modifiers, and Di Cera and collaborators (1996) obtained progress curves for allosteric reactions. Most of these progress curves have in common the property that they are implicit algebraic approximations based on the sQSSA. To obtain the best-fit values for K_M and v_{max}, numerical methods for root-finding need to be applied (Duggleby 2001).

Recently we have derived a refined version of the MM equation for the time evolution of the substrate concentration that allows the determination of the kinetics constants through progress curves (Schnell and Mendoza 1997). This formalism has been extended to deal with other classes of irreversible MM type reactions (Schnell and Mendoza 2001) by solving the kinetics equations with the aid of the sQSSA. The time evolution of the apparent reduced substrate concentration $[S''] \equiv [S]/\widetilde{K}_M$,

$$[S''](t) = W\{[S''_0]\ \exp([S''_0] - \widetilde{\kappa}t)\} \tag{4}$$

is regulated by the apparent first-order rate constant $\widetilde{\kappa} \equiv \widetilde{v}_{max} / \widetilde{K}_M$. In this equation $\widetilde{K}_M$ and $\widetilde{v}_{max}$ are now referred to as the ''apparent'' MM constant and maximum velocity, respectively. They are defined as functions of the activator, inhibitor, or competitor initial concentrations, depending on the reaction type (Schnell and Mendoza 2001). The W-function is defined as a solution of the equation

$$W(x)\ \exp[W(x)] = x \tag{5}$$

As recently discussed by Segel and Slemrod (1989), the sQSSA solutions can be regarded as the term of lowest order in the asymptotic expansion of the solution of the MM reaction. In this respect, the closed-form solution (4) for the substrate derived by Schnell and Mendoza (2001) corresponds to the lowest order term in the outer solution obtained with a singular perturbation technique. The first-order correction term can also be determined in terms of this closed-form solution and thus in terms of the W-function. Although the closed form solutions for the basic enzyme reaction (1) given by Schnell and Mendoza (1997) are not exact, they have nonetheless been shown to fit experimental behavior accurately by Goudar et al. (1999) in experiments with the enzyme pyruvate kinase and prephenate dehydratase. The solution in terms of the W-function is highly accurate in describing the substrate depletion and product formation with an accuracy of the order of 10^{-16} when double-precision arithmetic is used to fit the experimental data (Goudar et al. 1999). In addition, the range of validity of these solutions has been studied. It has been found that the solution (4) and those derived before with the aid of the sQSSA are valid subject to a condition that depends on the enzymatic reaction (Schnell and Mendoza 1997, 2000a, 2000b). For

the case of the MM reaction (1), the closed-form solution is accurate for $[E_0]/(K_M + [S_0]) \ll 1$, where $[E_0]$ and $[S_0]$ are, respectively, the initial enzyme and substrate concentrations.

Therefore, one of the key problems associated with the MM reaction is deriving analytical approximations with the aid of the sQSSA and identifying parameter regimes in which various analytical approximations hold, as these approximations are then used to calculate kinetic parameters from experimental data.

VALIDITY OF THE QUASI-STEADY-STATE APPROXIMATION

The actual validity of the sQSSA was first discussed by Laidler (1955), who suggested through a theoretical analysis an excess substrate concentration to be a main prerequisite for the validity of the sQSSA. Laidler also made an early attempt to determine an approximate solution for the pre-steady-state or initial transient of the reaction. In particular, Laidler found that the initial substrate concentration has to greatly exceed that of the enzyme

$$\frac{[E_0]}{[S_0]} \ll 1 \tag{6}$$

Bowen and et al. (1963) were the first to examine the sQSSA with the aid of singular perturbation theory. After reviewing previous efforts to estimate the error in the sQSSA, they analyzed a number of biochemical reactions, not including the MM reaction (1). In the analysis of Bowen and coworkers the timescales and dimensionless variables applied to the sQSSA were introduced without motivation and do not provide a general condition for the application of the sQSSA.

Using the early analog computers, Hommes (1962), Walter and Morales (1964), and Walter (1966) mapped the range of validity of the sQSSA for both the irreversible and reversible MM reactions, showing notable shortcomings for cases with large reverse bimolecular constants (k_{-1}).

Wong (1965) made an attempt to develop a continuous description of the initial transient and the steady-state phases of the reaction and concluded that the initial transient must be brief for the sQSSA to be applicable. This can be obtained by increasing the $[S]/[E]$ ratio. Stayton and Fromm (1979) found the sQSSA to generally hold for

$$\frac{[S_0]}{[E_0]} > 100 \tag{7}$$

by means of simulation modeling on a digital computer.

Heineken and coworkers (1967) provided a careful discussion of the singular perturbation treatment of the MM reaction (1), giving references to early efforts in this direction. Seshadri and Fritzsch (1980, 1981), possibly unaware of the results of Heineken et al. (1967), studied the more general situation wherein the reversible nature of the formation of the product was taken into account. They employed dimensionless variables that differ from the standard set suggested by Heineken et al. (1967) in that K_M is taken as the scale for the enzyme concentration. This yielded

$$\frac{[E_0]}{K_M} \ll 1 \tag{8}$$

as the natural small parameter. As the source of their choice of dimensionless variables, Seshadri and Fritzsch (1980) cited Reich and Sel'kov (1976), but the latter authors provided no motivation for their choice. Palsson (1987; Palsson and Lightfoot 1984) derived $[E_0]/K_M$ as the small parameter from a linear and modal analysis of the MM reaction. de la Selva et al. (1996) obtained the same small parameter by studying the asymptotic slope at equilibrium of the rate of product formation versus substrate depletion.

Schauer and Heinrich (1979) gave a detailed analysis of the errors resulting from the sQSSA by considering the time-dependent change in the substrate and complex concentration. They proposed three criteria: the smallness of the relative relaxation deficit, of the relative relaxation time, and of the relative relaxation error. In Klonowski (1983) there is a general discussion of timescales and the Russian literature concerning approximations for chemical kinetics describing the Tihonov theorem (1952). However, the time scales selected to study the sQSSA are introduced without motivation.

The importance of scaling in the numerical simulation of chemical reactions was stressed by Dahlquist et al. (Dahlquist 1985; Dahlquist et al. 1982). In particular, they pointed out that scaling gives appropriate weights in the norm for measuring the local error and makes it possible to write the systems of equations describing the MM reaction in a partitioned form, where [S] and [C] should be interpreted as vectors. In this context, the scaling of a biochemical system should be carried out in terms of quantities that are inherent to the process, which usually requires prior knowledge of the solutions by experimentation, physical intuition, or numerical computation. The book by Lin and Segel (1988) and the review by Segel (1972) provides the essential points that are necessary for scaling.

More recently, Segel (1988) and Segel and Slemrod (1989) showed that a more general condition for the sQSSA to be valid is

$$\frac{[E_0]}{K_M + [S_0]} \ll 1 \tag{9}$$

Note that conditions (6) and (8) are special cases of (9).

In the case of most in vitro assays, condition (9) is satisfied easily for the basic enzyme reaction (1). It is normally assumed that the formation of the enzyme-substrate complex C does not diminish significantly the concentration of the substrate S. Thus, the purpose of assumption (9) is to guarantee that there is not a significant fraction of the substrate bound to the enzyme during the assay (Reiner 1969; Schulz 1994; Segel 1975, 1988). According to Schulz (1994), the presumption is not that the enzyme must be saturated with substrate; this is a misinterpretation of the assumption, although this condition does imply that the concentration of the intermediate complex is in a quasi-steady state (QSS) with regard to the substrate and the product, due to enzyme saturation (Schnell and Mendoza 1997; Segel 1988; Segel and Slemrod 1989).

However, the sQSSA condition breaks down in some in vivo conditions (Albe et al. 1990; Sols and Marco 1970). Intracellular concentrations of enzyme are usually higher or at least of the same magnitude as their substrates and, consequently, a significant fraction of S can be bound as C complexes. Substrate concentration within cells are in the neighborhood of their K_M values (these values range from about 10^{-6} to 10^{-2} M); otherwise the full potential of the enzyme would not be realized (Cha 1970; Goldstein 1944; Segel 1975; Srere 1967). Furthermore, it is recognized that high affinity of an enzyme for a substrate may lead to binding of a significant proportion of substrate to the enzyme. Under these conditions, the MM equation (2), its double-reciprocal plot (3), and equation (4) become increasingly invalid (Cha 1970; Schnell and Mendoza 1997; Segel 1988; Straus and Goldstein 1943). Some expressions have been developed that allow the determination of the kinetic parameters for high enzyme concentration or high affinity of an enzyme for a substrate (Dixon 1972; Goldstein 1944; Henderson 1973). The equation most widely used is the generalized rate equation for the formation of product derived by Goldstein (1944), Cha and Cha (1965), and Reiner (1969),

$$v = \frac{k_2}{2}\left\{(K_M + [E_0] + [\bar{S}]) - \sqrt{(K_M + [E_0] + [\bar{S}])^2 - 4[\bar{S}][E_0]}\right\} \qquad (10)$$

where $[\bar{S}]$ is a new variable, the total substrate concentration, given by what is called the substrate mass balance (Reiner 1969; Schulz 1994; Segel 1975),

$$[\bar{S}] = [S] + [C] \qquad (11)$$

In spite of these attempts to study enzyme kinetics at high enzyme concentrations, the latter rate equation (10) has been developed in accordance with the sQSSA for the complex C without examining if the sQSSA holds for this case. Lim (1973) showed that expression (11) is not the substrate mass

balance, it is only a definition for the sum of C and S. The correct conservation law is

$$[\bar{S}] = [S] + [C] = [S_0] - [P] \tag{12}$$

Substituting (12) into (10) leads to (2) (see Lim 1973, p. 660, for details). Therefore, the general rate equation is equivalent to the MM equation. In addition, Lim (1973) analyzed the discrepancies between the numerical solution for [S] and the sQSSA solution. The agreement between the sQSSA solution and the numerical solution is quite good when $[E_0] \leq 0.01[S_0]$. However, when $[E_0]/[S_0]$ becomes large the error of the sQSSA solution becomes intolerably high. Furthermore, the author illustrates that the error involved is particularly high during the initial stages of the reaction. These results suggest that the assumption $d[C]/dt \approx 0$ of the sQSSA could be inappropriate at high enzyme concentration. This clearly illustrates the importance of studying the constraint for the kinetics equations presently employed in the literature. It also shows that errors in calculations of kinetics parameters may arise by employing expression (10) in experiments in which the initial enzyme concentration is higher than the substrate concentration.

In a previous paper (Schnell and Maini 2000), we challenged the basic assumption $d[C]/dt \approx 0$ of the sQSSA with the aid of the reverse QSSA (rQSSA) when the enzyme reaction (1) occurs under the following condition:

$$\frac{[E_0]}{[S_0]} \gg 1 \tag{13}$$

The rQSSA considers the substrate S in a QSS with respect to the enzyme-substrate complex C by assuming $d[S]/dt \approx 0$. From a biophysical point of view, it seems reasonable to state that the enzyme-substrate complex C is in a QSS when the concentration of the substrate S is high enough, because the free enzyme E will immediately combine with another molecule of S. However, when there is an excess of enzyme E, this condition does not hold (Borghans et al. 1996; Segel and Slemrod 1989). In the latter case, all the molecules of substrate S will immediately combine with the molecules of E. This implies that the substrate will be depleted, and the approximation $d[S]/dt \approx 0$ can be valid for a considerable period of time. Therefore, instead of C being in a QSS with respect to S, at high enzyme concentration it seems to be more reasonable to propose that S is in QSS with respect to C.

We have also shown that the velocity expression

$$v = \frac{d[P]}{dt} = \frac{v_{max}[S]}{K_S + [S]} \tag{14}$$

where $K_S = k_{-1}/k_1$ is the equilibrium dissociation constant of the substrate from the complex is the appropriate kinetics for studying the enzyme reaction (1) at high enzyme concentration. This comes as a surprise, because the

velocity equation (14), derived by a number of enzyme kinetics textbooks (Cornish-Bowden 1995b; Schulz 1994; Segel 1975), is well known but was considered invalid for high enzyme concentrations. This result has three important consequences:

1. Enzyme reactions at high enzyme concentration can operate at thermodynamic equilibrium. Indeed, conditions can be derived to ensure thermodynamic equilibrium.
2. The commonly stated condition in the literature (Briggs and Haldana 1925; Cornish-Bowden 1995b; Michaelis and Menten 1913; Schulz 1994; Segel 1975) that there is a thermodynamic equilibrium only if $k_2 \ll k_{-1}$ is a necessary but not sufficient condition to ensure thermodynamic equilibrium.
3. For high enzyme concentrations a very simple fitting procedure can now be used to determine the kinetic parameters K_S and v_{max} without involving the more complicated methods presently being employed. The latter have been used with (10) to determine K_M and hence information on the reaction constants k_1, k_{-1}, and k_2. However, the appropriate equation is (14), so this fitting procedure can only be used to determine information on the two rate constants k_1 and k_{-1}.

In addition, we have obtained a uniformly valid approximation for the total time evolution ($0 < t < \infty$) of the reactant concentration that can be employed in progress curve analysis. The time evolution of the substrate depletion may be approximated by

$$[S](t) \approx [S_0] \ \exp(-k_1[E_0]t) \tag{15}$$

The velocity equation (14) can also be integrated to give $[P](t) = [S_0](1 - \exp[-k_2 t])$, and again we can use this for fitting the concentration of product after the initial transient.

What happens when enzymes operate at around the same concentration as that of their substrates? A number of biochemists suggest that most enzymes work on the edge of a catastrophe or chaos in such conditions and the velocity expression of product formation such as (2) and (14) are no longer valid (Atkinson 1977; Cornish-Bowden 1999). However, the evidence for this is not conclusive.

Segel (1988); Segel and Slemrod (1989) and Frenzen and Maini (1988) have shown that the sQSSA can provide a good approximation even when $[S_0] \approx [E_0]$ as long as $[E_0]$ is small compared to K_M. The positive $[S_0]$–$[E_0]$ plane can be divided into regions in which these approximations hold, but in certain circumstances there remains a region where neither holds.

We illustrate this in Figure 3a, from which we note that in the shaded region, when both the sQSSA and rQSSA are invalid, the initial enzyme and substrate concentrations are comparable.

Borghans et al. (1996) reexamined the problem when there is an excess of enzyme and K_M is small, so that (9) does not hold. In their paper, by changing variables from free substrate S to total substrate concentration $[\bar{S}]$, they extend the parameter domain for which it is permissible to employ the classical assumption $d[C]/dt \approx 0$, with the following condition

$$\frac{K[E_0]}{(K_M + [S_0] + [E_0])^2} \ll 1 \tag{16}$$

where $K = k_2/k_1$ is the Van Slyke–Cullen constant (Schnell and Maini 2000). This new condition is the criterion for the validity of the total quasi-steady-state approximation (tQSSA). By including the tQSSA in the plot (see Figure 3b), the positive $[S_0]$–$[E_0]$ plane is divided into six regions. It can be seen that the region in which none of the assumptions are valid is reduced considerably due to the tQSSA.

Interestingly, the total substrate concentration ($[\bar{S}]$) is an aggregated variable. The tQSSA is an aggregation method based directly on the restatement

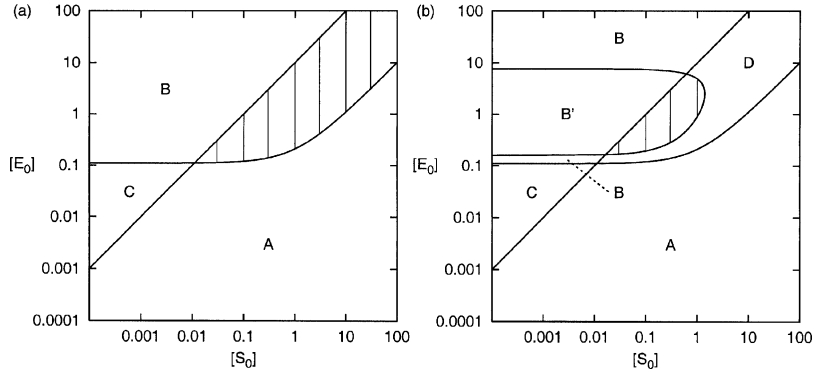

FIGURE 3 (a) Regions of validity of the sQSSA and rQSSA for the enzyme-substrate reaction (1) plotted using the conditions (9) and (13). There are four regions: A where only the sQSSA is valid, B where only the rQSSA is valid, C where both assumptions are valid, and the shaded region where both are invalid. Note that in the latter region the initial enzyme and substrate concentrations are comparable. (b) Regions of validity of the sQSSA, rQSSA and tQSSA plotted using the conditions (9), (13), and (16). The positive $[S_0]$–$[E_0]$ plane is now divided into six regions. The sQSSA, rQSSA, and tQSSA are not valid in the shaded region. In region B′, the sQSSA and tQSSA are both invalid, but the rQSSA is valid. In region D, only the tQSSA is valid. The regions A, B, and C are as in (a), but here the tQSSA is also valid. Parameter values used are $k_1 = 10,\ k_{-1} = 1,\ k_2 = 10$ ($K = 1$, $K_S = 0.1$, $K_M = 1.1$).

of the chemical mechanism. By changing the substrate variable to the total substrate ($\bar{S}$), the mechanism of the reaction is of the form

$$\bar{S} \xrightarrow{k_2[E]} P \tag{17}$$

Aggregation techniques have been used in a number of areas, such as chemistry, control theory, economy, and ecology, to reduce systems in the following way: The differential equations that model the system are transformed to a lower dimension of pseudo-species, in which the new dependent variables are functions of the original variables chosen such that the kinetics equations are easier to solve, and fewer parameters need to be experimentally determined.

We have derived (Schnell and Maini 2002) velocity equations for the substrate depletion and product formation with the aid of the tQSSA and an aggregation technique for cases where neither the more normally employed standard nor reverse quasi-steady-state approximations are valid. The velocity of product formation can be written as

$$v \approx \frac{v_{\max}[S]}{K_M + [S]} + \frac{K_M}{k_1} \frac{v_{\max}^2 [S]}{(K_M + [S])^4} \tag{18}$$

Hence, in principle the kinetic parameters can be determined from progress curves by numerically integrating (18). This type of fitting procedure has been carried out previously for the MM reaction (1) (Zimmerle and Frieden 1989). In addition, we have found that if the sQSSA is valid, the tQSSA velocity equation for product formation is almost indistinguishable from the sQSSA, and if the rQSSA is valid, the tQSSA velocity equation for product formation is in very close agreement with the rQSSA velocity equation.

The tQSSA velocity equation (18) allows us to enhance the regions in parameter space for which analytical approximations are valid to fit experimental data. In some cases, the tQSSA provides a good approximation in regions where either one or other of the sQSSA and the rQSSA (or thermodynamic equilibrium approximation) holds. Therefore, the tQSSA velocity equation provides a convenient way of assessing the kinetics behavior of enzymes accurately and is valid in a parameter domain that covers most of the initial enzyme and substrate concentration parameter space.

CONCLUSION

Presently many biochemists characterize enzymatic reactions by measuring the rate or velocity of the catalytic reaction. Experimental measurements are carried out after a relatively short and fast initial transient but before the substrate concentration decays appreciably for the enzymatic reactions. After the initial transient, if the rate of the substrate depletion or product formation

measured is approximately constant, then the enzyme-substrate complex concentration [C] is approximately constant. This implies that $d[C]/dt \approx 0$. With this approximation the rate or velocity of the catalytic reaction is determined.

However, many biochemists have devoted their attention to the art of accurately determining the kinetics parameters by employing the velocity expression rather than studying the conditions under which the velocity expression can be used. As a consequence, the velocity equations of the catalytic reaction have been employed on a number of occasions outside of the conditions for which they are valid.

Furthermore, experimental measurements rarely determine rates directly. As we have seen in the second section, this process is inexact and limits the quantitative information that can be deduced from an enzymatic reaction. There is an alternative to using the velocity equations to determine the kinetics parameters and that is the analysis of progress curves, which allows accurate measurements of the kinetic parameters. A review of the literature shows that previous analyses of progress curves have in common the property that they are implicit algebraic approximations based on the sQSSA. To obtain the best-fit values for K_M and v_{max}, numerical methods for root-finding need to be applied that are computationally intensive and can result in misleading conclusions (Schnell and Mendoza 2000a).

Schnell and Mendoza 1997 have introduced, with the aid of the sQSSA, a closed-form solution to describe the time-dependent evolution of the basic MM reaction. This innovative solution provides a new fitting procedure for calculating kinetic parameters from progress curves without the need for employing root-finding methods. The solution has proven (Goudar et al. 1999) to be highly accurate in describing the substrate depletion and product formation with an accuracy of the order of 10^{-16} when double-precision arithmetic is used to fit the experimental data. This solution has been extended to multiple alternative substrates (Schnell and Mendoza 2000a; 2000b) and enzymatic reactions with modifiers, such as linear inhibitors and activators (Schnell and Mendoza 2001).

Furthermore, Schnell and Maini (2000, 2002) have extended the solutions for the basic MM reaction (1) to fit experimental data in a broader parameter domain in which the sQSSA is not valid. These new solutions challenge the validity of expressions previously developed that allow the determination of the kinetic parameters for high enzyme concentration or high affinity of an enzyme for a substrate. They also raise concerns about the validity of the kinetic parameters determined with these equations.

The conclusions presented in Schnell and Maini (2000, 2002) suggest that investigators have to be cautious in interpreting kinetics parameters determined and the conditions under which these are estimated. This is extremely important for the postgenomic era that is now beginning. The flood of new information is highly relevant to enzyme kinetics methods, as it brings a mass of gene sequences of unknown functions. Understanding genomes as fast as they are being sequenced will thus require major attention to

functional genomics. This involves a revival of attention to methods of characterizing enzymes and other proteins kinetically, because kinetics analysis is an essential step not only in understanding enzyme mechanisms, but also for understanding better how the kinetic properties of individual enzymes or proteins in a network system combine to produce the kinetic properties of the biological system under study (Cornish-Bowden 2001).

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