Heineken, Tsuchiya and Aris on the Mathematical Status of the Pseudo-steady State Hypothesis: a Classic from Volume 1 of *Mathematical Biosciences*

Marc R. Roussel

Alberta RNA Research and Training Institute, Department of Chemistry and Biochemistry, University of Lethbridge, Lethbridge, Alberta, T1K 3M4, Canada

Abstract

Volume 1, Issue 1 of *Mathematical Biosciences* was the venue for a now-classic paper on the application of singular perturbation theory in enzyme kinetics, "On the mathematical status of the pseudo-steady state hypothesis of biochemical kinetics" by F. G. Heineken, H. M. Tsuchiya and R. Aris. More than 50 years have passed, and yet this paper continues to be studied and mined for insights. This perspective discusses both the strengths and weaknesses of the work presented in this paper. For many, the justification of the pseudo-steady-state approximation using singular perturbation theory is the main achievement of this paper. However, there is so much more material here, which laid the foundation for a great deal of research in mathematical biochemistry in the intervening decades. The parameterization of the equations, construction of the first-order uniform singular-perturbation solution, and an attempt to apply similar principles to the pseudo-equilibrium approximation are discussed in particular detail.

Keywords: Singular perturbation theory, Michaelis-Menten mechanism, pseudo-steady-state approximation, pseudo-equilibrium approximation

1. Introduction

In the very first issue of *Mathematical Biosciences* (*MB*) appeared a paper that has become a classic in the literature on singular perturbation theory in biochemical kinetics: "On the Mathematical Status of the Pseudo-steady State Hypothesis of Biochemical Kinetics", by F. G. Heineken, H. M. Tsuchiya, and R. Aris [1]. While the paper's title promised a study of the mathematical basis of the pseudo-steady-state (PSS) hypothesis, which it did indeed provide, this paper went beyond this immediate objective to develop the singular perturbation solution of the Michaelis-Menten rate equations to first order in the small parameter. The authors also considered a singular-perturbation approach to

---

understanding the Henri-Michaelis-Menten pseudo-equilibrium approximation (PEA) [2, 3], as well as a two-substrate enzyme-catalyzed reaction. As we will see below, this paper has become a cornerstone of the literature in mathematical biochemistry.

The PSS approximation (PSSA, also known as the quasi-steady-state approximation or, more simply, the steady-state approximation, SSA), developed independently by Bodenstein [4] and by Chapman and Underhill [5], requires the identification, usually based on chemical intuition, of "fast" (i.e. highly reactive) chemical species. The idea is that these fast species will accumulate during the initial stages of the reaction, but because of their high reactivity, they will soon reach a state in which they are consumed as fast as they can be made. Thus, for much of the evolution, their rates of change will be "small". The quotation marks here emphasize the *ad hoc* nature of the reasoning used. Formally, starting with an initial-value problem (IVP)

$$\frac{ds}{dt} = p(s, c), \tag{1a}$$

$$\frac{dc}{dt} = q(s, c), \tag{1b}$$

$$(s(0), c(0)) = (s_0, c_0), \tag{1c}$$

where s is a vector of slow variables and c is a vector of fast variables, with constant initial data (s_0, c_0), we set the rates of change of the fast variables equal to zero, replacing the original set of ordinary differential equations (ODEs) by a set of differential-algebraic equations (DAEs):

$$\frac{ds}{dt} = p(s, c), \tag{2a}$$

$$q(s, c) = 0. \tag{2b}$$

In a much-quoted passage, Heineken, Tsuchiya and Aris (henceforth, HTA) wrote of this procedure "To the mathematician this hypothesis, known as the pseudo-steady-state hypothesis (pssh), is somewhat scandalous. For clearly $dc/dt = 0$ in the strict sense at only one instant" [1, p. 97]. Mathematicians are not alone in their discomfort: many generations of chemists have also been bothered by this procedure. For example, Fraser would later write "these approximations are puzzling. [...] SSA implies a constant (time-independent) concentration to obtain its dependence on other changing concentrations, so denying constancy" [6, p. 4732]. Despite the potentially "scandalous" and "puzzling" nature of the PSSA, this approximation is used in one form or another to study a wide variety of problems (e.g. [7–20]). Understanding how, why and when this approximation should be effective is therefore an important problem in applied mathematics.

HTA's rehabilitation of the "scandalous" PSSA involved the then-emerging

theory of singularly perturbed ODEs [21–28], specifically IVPs of the form

$$\frac{dy}{dt} = f(y, z), \tag{3a}$$

$$\mu\frac{dz}{dt} = g(y, z), \tag{3b}$$

$$(y(0), z(0)) = (y_0, z_0), \tag{3c}$$

where μ is a small parameter. A (now) well-known theorem of Tikhonov [21, 23, 28, 29] tells us that, for vanishingly small μ, the solutions of (3) approach those of the differential-algebraic system

$$\frac{dy}{dt} = f(y, z), \tag{4a}$$

$$g(y, z) = 0 \tag{4b}$$

provided the root z^* of equation (4b) is a stable equilibrium point of the adjoined system
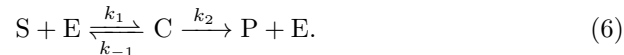
$$\frac{dz}{dt} = g(y, z) \tag{5}$$

with y fixed. A later theorem of Fenichel guarantees that the invariant manifold arising as the solution of Eq. (4b) in the limit $\mu \to 0$ persists for small values of μ [30]. The PSSA looks a lot like an application of Tikhonov's theorem, provided we can write the system (1) in the form (3) with a small parameter μ. There is also the issue of the initial conditions for the system (4) [or (2)], which cannot be the same as the initial conditions of the original IVP, on which more later.

In this perspective, the Heineken, Tsuchiya and Aris (1967) paper is first placed in historical context. A discussion follows of the main points of the paper, to wit the scaling of the equations (Section 3), the construction of the singular perturbation solution (Section 4), the pseudo-equilibrium approximation (Section 5), multi-substrate and multi-enzyme reactions (Section 6), and finally, the determination of kinetic constants from experimental data (Section 7). In a final section, the impact of HTA (1967) through the years is considered.

2. Historical context

When treating enzyme kinetic data, one is soon confronted with the fact that the order of reaction is different in different ranges of substrate concentration: the reaction is of the first order with respect to substrate at low concentrations, but of the zeroth order at high concentrations. The first mathematical resolution of this problem was due to Henri, with the help of Bodenstein [2]. Henri used a pseudo-equilibrium approximation applied to the *reversible* Michaelis-Menten mechanism to obtain a hyperbolic rate law. Michaelis and Menten later simplified Henri's treatment to the irreversible case, designing initial-rate experiments to align their experimental work with their theory, thus avoiding complications due to accumulation of the product [3].

3

In 1925, Briggs and Haldane applied the pseudo-steady-state approximation to the Michaelis-Menten mechanism [31]. Their argument anticipates later scaling arguments used in the singular perturbation treatment, so it is worth reviewing briefly. Let us first establish HTA's notation, into which we will translate the Briggs-Haldane argument. The Michaelis-Menten mechanism is

$$S + E \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} C \xrightarrow{k_2} P + E. \tag{6}$$

Following HTA, lower-case letters will denote the concentrations of the corresponding chemical species, and e_0 will denote the total (initial) concentration of enzyme, with s_0 the initial concentration of substrate. The mass-action rate equations governing this mechanism are

$$\frac{ds}{dt} = -k_1 es + k_{-1}c, \tag{7a}$$

$$-\frac{de}{dt} = \frac{dc}{dt} = k_1 es - (k_{-1} + k_2)c, \tag{7b}$$

$$\frac{dp}{dt} = k_2 c. \tag{7c}$$

Two conservation relations allow us to eliminate two of these equations. Since $de/dt + dc/dt = 0$,

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

is a constant. Similarly, since $ds/dt + dc/dt + dp/dt = 0$,

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

is a constant. The latter need not be used explicitly since the irreversibility of the mechanism means that p does not appear in the rate equations (7). Using (8), we obtain the planar dynamical system

$$\frac{ds}{dt} = -k_1 s(e_0 - c) + k_{-1}c, \tag{10a}$$

$$\frac{dc}{dt} = k_1 s(e_0 - c) - (k_{-1} + k_2)c. \tag{10b}$$

Briggs and Haldane start by assuming that e_0 and c are very small compared to s_0 and s. They then point out that the maximum possible value of c is e_0, and that c consequently decreases from a maximum value less than e_0 to zero during the reaction. (They implicitly assume that there is a rapid rise in c to this maximum value, while explicitly recognizing that during this transient period, the PSSA would not apply. The rapid rise of c is a key condition for the validity of the PSSA [32, 33].) Similarly, p increases from zero to s_0 during the reaction. Thus, *on average*,

$$\left\langle \frac{-dc/dt}{dp/dt} \right\rangle < \frac{e_0}{s_0}, \tag{11}$$

4

i.e. dc/dt is relatively small following the establishment of the PSS. One interesting aspect of the Briggs-Haldane argument, incidentally, is that it implicitly shifts the discussion of the PSSA to phase space, since

$$\frac{dc}{dp} = \frac{dc/dt}{dp/dt}. \tag{12}$$

Phase space, which for isothermal kinetics is the space of independent concentrations, is the setting for geometric singular perturbation theory [34] and for a number of approaches to understanding the PSSA [6, 35–39] and improving on it [6, 37, 40, 41]. The Briggs-Haldane argument can therefore be reinterpreted as an argument relating to the slope of the solution in phase space.

As will shortly be seen, scaling is central to HTA's argument [1] and to much of the subsequent literature in singular perturbation theory [42–44]. A scaling argument for the validity of the PSSA appeared in a paper by Swoboda published in 1957 [45]. This paper is, unfortunately, not well known today. In it, Swoboda treated both the usual $e_0 \ll s_0$ case and the more challenging $e_0 \sim s_0$ case, which is often the more relevant regime *in vivo* [46–48]. In a followup paper, he developed equations for the time evolution both in the initial and terminal phases of the reaction [49]. Although HTA appear not to have been aware of this work, it is clear that Swoboda anticipated some aspects of HTA (1967).

Another noteworthy paper in the general spirit of singular perturbation methods is Wong's "unified solution" which, in essence, matches the lowest order solutions for the transient and PSS solutions [50], corresponding respectively to the inner and outer solutions of the singular perturbation treatment.

The first explicit application of singular perturbation theory in chemical kinetics in the Western literature is, to my knowledge, a 1963 paper by Bowen, Acrivos and Oppenheim [51]. These authors tackled a number of two-step mechanisms, including the classic mechanism of the thermal decomposition of ozone. This paper, rich with technical detail, was no doubt an important stepping-stone to the HTA paper, sharing similar aims and carrying out calculations to a similar depth, albeit addressed to different audiences.

Meanwhile, the formal theory of singularly perturbed ordinary differential equations was being developed by Tikhonov, Vasil'eva and others [22, 25, 27].

3. The HTA scaling

The first step is to write the Michaelis-Menten equations in the form (3). HTA achieved this by scaling the variables as follows:

$$y = s/s_0, \qquad\qquad z = c/e_0, \tag{13a}$$
$$\tau = k_1 e_0 t, \tag{13b}$$

as well as defining the dimensionless parameters

$$\mu = e_0/s_0, \tag{14a}$$
$$\kappa = K_M/s_0, \qquad\qquad \lambda = k_2/k_1 s_0, \tag{14b}$$

where

$$K_M = (k_{-1} + k_2)/k_1 \tag{14c}$$

is the well-known Michaelis constant. With these definitions, the mass-action rate equations for the Michaelis-Menten mechanism, taking into account conservation of enzyme, are

$$\frac{dy}{d\tau} = -y + z(y + \kappa - \lambda), \tag{15a}$$

$$\mu\frac{dz}{d\tau} = y - z(y + \kappa), \tag{15b}$$

which is clearly in the form (3) if μ is a small parameter. An application of Tikhonov's theorem to this system immediately justifies the use of the PSSA when $e_0 \ll s_0$, in accordance with Briggs and Haldane's more informal argument [31]. However, the whole argument rests on HTA's scaling, which they offered without justification.

The scalings (13a) are perhaps the most obvious ones available, and were the basis of Briggs and Haldane's reasoning as well: the maximum possible values of s and c are, respectively, s_0 and e_0, so y will certainly be an $O(1)$ variable until the late stage of the evolution, while z will be $O(1)$ through most of the evolution unless $k_{-1} + k_2 \gg k_1 s_0$. (In this context, $O(1)$ indicates a quantity of unit order of magnitude.) In fact, scaling to obtain variables of unit magnitude has become a standard technique in singular perturbation analysis [43], so HTA's scaling of the concentrations is a sensible choice. Interestingly however, textbook accounts of the steady-state approximation often suggest that the approximation ought to be valid precisely when the complex reacts quickly (e.g. [52, p. 363], [53, pp. 275–276], [54, p. 473], [55, p. 6-1]), such that $c \ll e_0$ unless $s \gg K_M$, when HTA's scaling would be expected to break down. This regime is covered by a more refined scaling due to Segel and Slemrod [43].

Requiring a small value of μ raises some interesting issues. If we try to make μ small by increasing s_0, then κ and λ also become small parameters [Eqs. (14b)]. This makes the solution of Eqs. (15) a double perturbation problem [56], and one with a nasty singular limit at that. Thus, the only limit that makes sense in this scaling is to let e_0 go to zero. Experimentally, these two limits have correspondingly different effects, illustrated in Fig. 1. Suppose that we start out in a region of experimental design space in which the PSSA does not hold. Because of the irreversibility of product formation in the model (6), the vector field defined by Eqs. (10) is independent of s_0. Thus, increasing s_0 corresponds to moving out along the s axis *within* a stoichiometric compatibility class [57, Section 4.2] (grey surfaces in Fig. 1), which eventually puts the system in a region where the slow manifold, the trajectory approximated by the PSSA [6], has a small slope, and thus the PSSA holds trivially. In a progress curve experiment started from such initial conditions, the time evolution will eventually return the system along the slow manifold to a region where the PSSA does not hold. In this case, we would have to make sure that we don't let the experiment
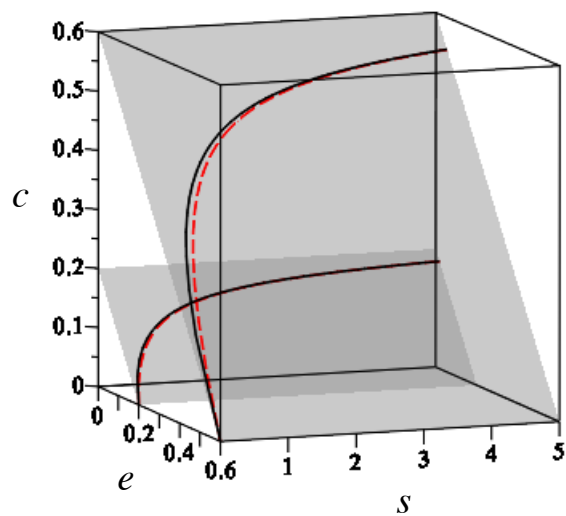
Figure 1: Effects of changing s_0 or e_0 in experimental studies of Michaelian enzymes. The grey surfaces are stoichiometric compatibility classes defined by $e + c = e_0$, for two different values of e_0, namely $e_0 = 0.6$ (in arbitrary units, front) and 0.2 (back). Because the vector field does not depend on s_0 [Eqs. (10)], increasing s_0 corresponds to moving out along the s axis *within* a stoichiometric subspace. On the other hand, choosing a different value of e_0 corresponds to moving to a different stoichiometric subspace. The solid black curves are slow manifolds approximated as the third iterate of Fraser's method [6, 37] (indistinguishable from the exact slow manifold on the scale of this figure). The red dashed curves show the PSSA. Parameters: $K_M = 0.5$, $K_E = k_{-1}/k_1 = 0.05$ (arbitrary units).

run "too long". Needless to say, it would be difficult to define operationally what is "too long". (Note that we are neglecting reversibility, which will also eventually make the Michaelis-Menten equation invalid.) In the more common initial-velocity experiments, we would have the related problem of choosing s_0 values that are "not too small". On the other hand, decreasing e_0 corresponds to moving to a different stoichiometric compatibility class because of the conservation of enzyme. If μ is made sufficiently small by decreasing e_0, the PSSA will hug the slow manifold along its entire length (neglecting reversibility), as illustrated by the smaller-e_0 stoichiometric compatibility class in the figure. This is related to the improved scaling of Segel and Slemrod [43], who discovered a small parameter

$$\epsilon = e_0/(s_0 + K_M). \tag{16}$$

We will have $\epsilon \ll 1$ for any s_0 if $e_0 \ll K_M$. Thus, at sufficiently small e_0, we could follow a trajectory in a progress-curve experiment all the way to equilibrium, or carry out initial-rate experiments over a wide range of s_0 values, all the while remaining within the domain of validity of the PSSA. It is therefore preferable to make μ (or ϵ) small by using small enzyme concentrations than by using large concentrations of substrate. Consequently, it may be particularly difficult to find feasible experimental conditions under which the PSSA is valid for enzymes with tight-binding substrates (i.e. enzyme-substrate systems with small values of K_M). Note that the idea that letting dimensionless groups approach zero can have very different effects depending on which physical quantity is taken to the limit was recently discussed by Goeke and coworkers [58].

The slow time scale implied by Eq. (13b), $t_s = (k_1 e_0)^{-1}$, turns out to be problematic once we have admitted the scalings (13a). As noted above, we require $k_{-1} + k_2 \ggg k_1 s_0$ or, equivalently, $K_M \ggg s_0$, in order for the scaling of c to be valid. A careful estimate of t_s gives [32]

$$t_s = (K_M + s_0)/k_2 e_0. \tag{17}$$

We only recover the HTA time scale if $K_M \gg s_0$ and $k_{-1} \ll k_2$. The latter may or may not be true for any given enzyme, but the former is in clear contradiction to the scaling of c. There is therefore a contradiction built into the HTA scaling. Rather than considering this an error in estimating the slow time scale, it is perhaps best to think of the HTA scaling as not being sufficiently careful in its scaling of c. From the point of view of justifying the PSSA, this incorrect scaling led to limitations in the domain of applicability of the treatment which would only be corrected two decades later by Segel [32], with further refinements by Segel and Slemrod [43]. Note also that the HTA time scale can be obtained consistently from a balancing argument [59, Chapter 7], where the emphasis is on explicitly bringing out the approximate balance that results in the validity of the PSS condition [60]. Both the latter treatment and the Segel-Slemrod work have in common a more careful scaling of c (in fact, the same scaling in both cases). To be fair, the principles of scaling were not stated explicitly, to my knowledge, until Segel's landmark paper [61], which appeared approximately five years after the HTA paper.

Despite the inconsistent scaling, the authors obtained a set of equations in the singular perturbation form. The inconsistent scaling of t affects both equations the same way since the ODEs under study are autonomous. Thus, from the perspective of obtaining a consistent singular perturbation problem valid under the conditions of the concentration scalings (13a), this error is harmless. The key step that yields a small parameter is the scaling of the concentrations, and here it is best to think of the HTA scaling as constraining the validity of the analysis to a particular regime, fortunately the one in which *in vitro* enzyme kinetics typically operates [62, 63].

4. The singular perturbation solution

After transforming the equations, HTA set about constructing the solution using the techniques of singular perturbation theory. The key idea is that equations of the form (3) represent systems with two time scales, a fast time scale during which z tends to the steady state of the adjoined system, and a slow time scale during which the evolution is well approximated by the differential-algebraic system (4). In the jargon of singular perturbation theory, which was borrowed from fluid mechanics [51], the former time scale is associated with an "inner" solution, while the latter corresponds to an "outer" solution.

One of the key ideas in singular perturbation theory is that studying the inner solution requires a rescaling of Eqs. (3). Following HTA, if we define a rescaled time

$$\sigma = t/\mu, \tag{18}$$

then Eqs. (3) become

$$\frac{dy}{d\sigma} = \mu f(y, z), \tag{19a}$$

$$\frac{dz}{d\sigma} = g(y, z). \tag{19b}$$

If μ is small, then $dy/d\sigma \approx 0$, i.e. $y \approx y_0$, while z evolves towards the quasi-stationary point at which $g(y_0, z) = 0$ at large values of σ. As $g(y_0, z) \to 0$, the scaling of Eqs. (19) becomes inappropriate, and we need to switch to Eqs. (3). This will require that the two solutions be matched, i.e. that we ensure a smooth transition from the inner to the outer solution, which resolves the issue of which initial conditions to use in the reduced equations (4).

If we have a small parameter, then we can build a perturbation series for the solution. In accordance with the foregoing statements, we in fact build two perturbation series in μ, one for the inner solution, and one for the outer. The details of these calculations will not be reproduced here, and readers are referred to the original text [1] or to Lin and Segel's exposition [64, Chapter 10] for details. In a nutshell, the two solutions were developed, and the long-time limit of the inner solution was used to generate an initial condition for the outer solution. A uniform approximation was obtained by, as Lin and Segel would

9

later explain, "add[ing] the inner and outer approximations and subtract[ing] their common part" [64, p. 310].

HTA carried out this process to first order in μ, and then showed some numerical solutions of the full system along with the zero-order and first-order uniform approximations using parameters estimated for the hydrolysis of benzoyl-L-arginine ethyl ester catalyzed by trypsin. Even for $\mu = 1$, which is of course not a small value, the first-order solution is reasonably—or perhaps unreasonably?—accurate.

Coming up with a "good" scaling, and the related problem of estimating the time scales operating in a kinetic system [44, 65], continues to be an active research topic. (Note however that Goeke and coworkers have challenged the idea that scaling *per se* is necessary beyond the identification of a small parameter [58].) Recent work has focused on the tQSSA [66, 67], in which the slow variable is $s + c$ rather than s [68–70].

5. The pseudo-equilibrium approximation

While the work of Bowen, Acrivos and Oppenheim showing how singular perturbation theory could be used to justify the PSSA [51] predated HTA's paper, to my knowledge no one had yet tried to apply similar reasoning to rigorously justify the pseudo-equilibrium approximation which formed the basis of Henri's [2] and Michaelis and Menten's work [3]. Despite the inconsistencies noted above in their PSS scaling, it is clear that HTA understood the importance of scaling as they proposed a different scaling to study the PEA. Their reasoning, only partly explained in their paper, ran as follows: "the reversible reaction forming the complex C [is] very fast compared to its decomposition" [1, p. 108], therefore k_2^{-1} is a slow time scale. Following this line of reasoning, they retain the concentration scalings (13a), to which they add the slow time scaling

$$\tau = k_2 t, \tag{20}$$

and the new parameter

$$\nu = k_{-1}/k_1 s_0. \tag{21}$$

The definitions of λ and μ are unchanged. Now however the authors' assumption that the rate of decomposition of the complex is relatively small implies that λ is the small parameter. The rate equations transform to

$$\lambda \frac{dy}{d\tau} = \mu \left[-y + z(y + \nu) \right], \tag{22a}$$

$$\lambda \frac{dz}{d\tau} = y - z(y + \nu) - \lambda z. \tag{22b}$$

Clearly, this form leads us into a mathematical difficulty since the small parameter multiplies both derivatives. In the limit $\lambda \to 0$, both equations degenerate to

$$z = \frac{y}{y + \nu}, \tag{23}$$

10

which is the PEA. However, as HTA point out, we don't have a differential equation left into which to substitute this approximation. HTA go on to explain that the standard approach consists of substituting this expression into the rate of product formation, and then pretending that it is still reasonable to write

$$v = \frac{dp}{dt} = -\frac{ds}{dt} \tag{24}$$

even though the PEA makes ds/dt formally equal to zero. HTA indicated that they find this procedure unsatisfactory given the lack of rigorous mathematical justification.

There is a way out of this mathematical difficulty based on geometric singular perturbation theory, which would only be developed a decade later [34]. The PSSA and PEA both seek to discover an algebraic relationship such as Eq. (4b), which defines, at least implicitly, a function $h(y)$ such that $z \approx h(y)$ after decay of transients. The dynamics is then constrained to the curve in phase space defined by $z = h(y)$ [6]. If we go back to the original IVP (3), we can now ask whether such a relationship exists between y and z in general. The answer is, trivially, yes: along every trajectory, there is some functional relationship between y and z. However in model reduction approaches related to the PSSA, we are not looking for a general solution, but for one that represents the slow time evolution. Since the slow time evolution is just motion along a special trajectory, namely the trajectory reached from arbitrary initial conditions after the decay of transients, then the slow time evolution must also be characterized by a functional relationship $z = h(y)$. Differentiating this relation with respect to time, we get

$$\frac{dz}{dt} = \frac{dh}{dy}\frac{dy}{dt}. \tag{25}$$

This equation is known as the invariance equation. All trajectories of Eqs. (3) are solutions of this equation. The special trajectory representing the global slow time evolution is known as the slow invariant manifold [6, 71–73]. Substituting the rate equations (22) into the general invariance equation, we get

$$y - (y + \nu)h(y) - \lambda h(y) = \mu \frac{dh}{dy}\left[-y + (y + \nu)h(y)\right]. \tag{26}$$

Note that the factors of λ multiplying the derivatives have cancelled out. The non-standard singular problem posed by the time evolution equations (22) has therefore become a *non-singular* perturbation problem in λ for the phase-space geometry of the slow manifold [56, 74]. Specifically, write

$$h(y) = \sum_{i=0}^{\infty} \lambda^i \phi_i(y), \tag{27}$$

where the $\phi_i(y)$ are unknown functions to be determined. Substituting this

series into the invariance equation (26), we get, after a bit of work,

$$\phi_0(y) = \frac{y}{y + \nu}, \tag{28a}$$

$$\phi_1(y) = \frac{-y}{(y + \nu)^2 + \mu\nu}, \tag{28b}$$

$$i > 1 : \phi_i(y) = \frac{-(y + \nu)\left\{\phi_{i-1} + \mu(y + \nu)\sum_{j=1}^{i-1}\phi_j'\phi_{i-j}\right\}}{(y + \nu)^2 + \mu\nu}, \tag{28c}$$

where the function arguments on the right-hand side have been dropped for clarity, and ϕ_j' denotes $d\phi_j/dy$. Note that the $i = 0$ term is the PEA. Thus we see that the PEA emerges as the zero-order approximation of a regular perturbation problem in λ and *not*, as HTA believed, as the leading term in a singular perturbation problem. In fact, the appearance of λ as a multiplicative factor in front of both derivatives in Eqs. (22) implies that both y and z are governed by a single time scale in this parameterization.

The time evolution implied by Eqs. (22a), (27) and (28) also highlights a central difficulty in the PEA problem, namely that the lowest-order approximation to the graph of the slow manifold is given by $z \approx \phi_0(y)$, but this is not sufficient to define the time evolution. If we substitute this lowest-order approximation into Eq. (22a), $dy/d\tau$ vanishes. This is not surprising since the zero-order term (28a) is just the PEA, in which y is assumed to be in pseudo-equilibrium and thus static at the level of this approximation. Thus, we must include the $O(\lambda)$ term of the perturbation series to obtain the time evolution of y on the slow manifold to lowest order:

$$\begin{aligned}
\frac{dy}{d\tau} &\approx \frac{\mu}{\lambda}\left[-y + (\phi_0 + \lambda\phi_1)(y + \nu)\right] \\
&= \mu\phi_1(y + \nu) \\
&= \frac{-\mu y(y + \nu)}{(y + \nu)^2 + \mu\nu}.
\end{aligned} \tag{29}$$

This observation would apply to any method in which the PEA appears as a leading-order approximation, and resolves the conundrum raised by HTA regarding the meaning to be attached to ds/dt when the PEA formally makes this quantity zero.

Having hit a roadblock in their first attempt, HTA decided to change variables. Instead of scaled versions of s and c, they considered scaled versions of e and p, viz.

$$w = e/e_0, \qquad\qquad x = p/s_0. \tag{30}$$

Using the mass conservation relation for the substrate, $s_0 = s + c + p$, the scaled

12

equations become

$$\frac{dx}{d\tau} = \mu(1 - w), \tag{31a}$$

$$\lambda\frac{dw}{d\tau} = \kappa - w(1 + \kappa - \mu - x) - \mu w^2. \tag{31b}$$

Now the small parameter λ multiplies just one of the derivatives, and Tikhonov's theorem applies. The catch is that applying Tikhonov's theorem to these equations leads to a quadratic in w, so the equation obtained is more complex than the traditional PEA. Note that quadratic expressions for the rate of catalysis of a single-substrate Michaelian enzyme have appeared in the literature from time to time [68, 75–77].

Suppose that we do go ahead and apply Tikhonov's theorem to Eqs. (31), i.e. we set the right-hand side of Eq. (31b) equal to zero. We can transform the resulting equation back to the usual (y, z) variables using the mass conservation relations, which in HTA's parameterization read

$$w + z = 1, \tag{32a}$$

$$x + y + \mu z = 1. \tag{32b}$$

Using these equation to eliminate w and x in favour of y and z, Eq. (31b) reduces to

$$z = \frac{y}{y + \kappa}. \tag{33}$$

In attempting to derive the PEA rigorously, HTA in fact derived an equation equivalent to the PSSA.

As can be seen from HTA's struggles, understanding the PEA in the Michaelis-Menten mechanism is actually more difficult than understanding the PSSA. Various approaches would later be used to tackle this problem. Schauer and Heinrich developed a method in which reaction time scales near a steady state were used to identify sets of fast and slow reactions [78], setting up a PEA calculation. Segel and Slemrod would tackle the PEA briefly in their landmark paper [43], with a thorough treatment coming only in 2000 from Schnell and Maini [79], as well as interesting recent contributions to this problem by Prescott and Papachristodoulou [80] and by Walcher and coworkers [58, 81, 82].

6. Multi-substrate and multi-enzyme reactions

HTA briefly tackled multi-substrate and multi-enzyme reactions as well. The work they presented on this problem was a sketch, but they did correctly point out that the PSSA can be justified using Tikhonov's theorem in more complicated cases. It would be left to others to work out the details [78, 83].

7. The determination of kinetic constants

Because of the two time scales inherent in singularly perturbed systems, measurements on one time scale do not give the experimentalist access to all of the rate constants. Thus, the traditional steady-state enzyme kinetics experiment provides $v_{\max} = k_2 e_0$ and K_M [Eq. (14c)], from which we cannot calculate any of the rate constants without additional information. Knowing the enzyme concentration would at least give us k_2, but then we are stuck. The final section of HTA's paper discusses this issue, pointing out however that if we measure the kinetics on both the slow and fast time scales, we then have enough information to extract all of the parameters of the Michaelis-Menten mechanism (rate constants and enzyme concentration). The experiment on the fast time scale implied by their discussion, measuring $c(t)$ during the pre-steady-state period, is difficult because the enzyme-substrate complex does not usually have a convenient spectroscopic feature that is clearly distinguished from the spectroscopic features of the enzyme and substrate, with some exceptions [84–86]. Having said that, with the tremendous advances in rapid kinetics methods and in spectroscopic instrumentation in the last few decades, it may be interesting to take another look at this idea. Note also that an analogous procedure is entirely feasible with cell growth data [87], which often follow similar (Monod) kinetics [88], but with much longer time scales.

Incidentally, another approach to extracting a full set of rate constants emerges from HTA's work: the outer solution (slow invariant manifold), expanded to first order in μ, may provide enough information to recover all of the parameters [89].

8. Impact of HTA (1967) and concluding statements

It seems inevitable these days to carry out a bibliometric analysis as one way to understand the impact of a paper. HTA (1967) has so far accumulated 178 citations (Web of Knowledge search, October 23, 2019). For comparison, I carried out a Web of Knowledge search of all papers published in *Mathematical Biosciences* in the period 1967 to 1970 inclusive. This provides a comparison group of 199 contemporary papers. The average *MB* paper from this period has collected just over 21 citations from publication to the present day. HTA (1967), a technical paper addressed to an audience of specialists in theoretical biochemistry, has therefore performed far above the norm. It is in fact the 5th most cited *MB* paper from this period. Interestingly, H. M. Tsuchiya is also a coauthor of the 4th most cited *MB* paper in this set [90].

When looking at the HTA (1967) citations in detail, it becomes clear that the paper is undergoing a renaissance (Fig. 2). From 1970 to 1994, HTA (1967) was cited at a fairly constant rate of about 3.7 citations per year. Then from 1995 to 2009, there was a clear decline in citation rate, to an average of just over 2 citations per year. In the last decade however, citations have returned to their original rate, and in fact the paper was cited more often in the last five years than in any previous five year period; and note that 2019 is not yet done
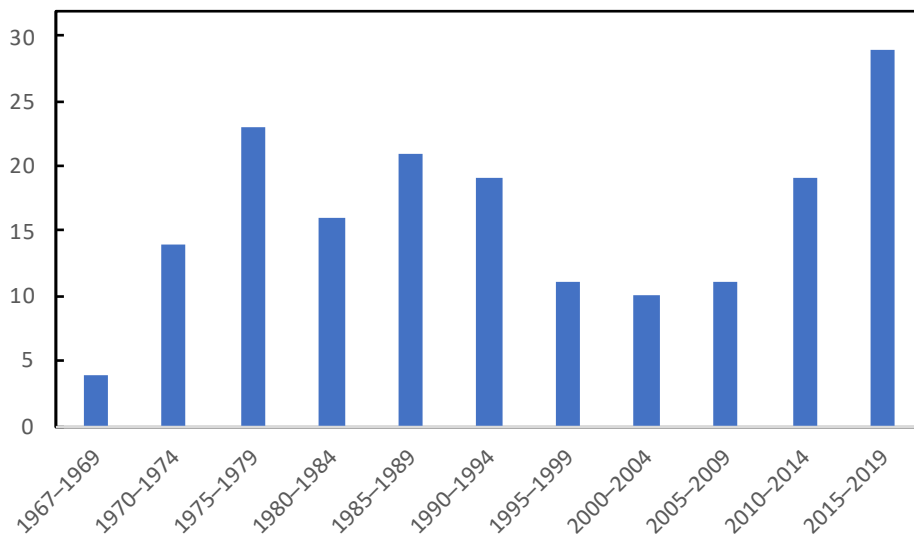
Figure 2: Histogram of citations to HTA (1967) [1] by five-year period. Note the shortened period represented by the leftmost bar. Web of Knowledge search, October 23, 2019.

as I write these words. Clearly, a new generation is discovering and appreciating HTA (1967).

A good paper answers a question. A really great paper answers a question and opens up many other questions. HTA (1967) is a really great paper, which explains its enduring appeal. It likely introduced many theoretical biochemists to some of the ideas of singular perturbation theory, which were not well known in the West at the time. It thereby answered the question implied by its title, i.e. it explained how the PSSA could be derived rigorously, and explained how the conditions for its validity could be explored in a systematic manner. It also left many open problems for its readers: How should the parameterization of the equations be chosen? Is there an analogous treatment that would give the PEA but wouldn't lead to the mathematical problems encountered by HTA? And how do we generalize these ideas to more complex reactions and networks of reactions? The references on these problems cited in this perspective give a bit of an idea, incomplete though it may be, of the literature stimulated by HTA's paper.

Heineken, Tsuchiya and Aris left us an ambitious paper that challenges its readers to think about the relationships between the structure of a set of equations, the values of the parameters appearing in these equations, and the consequent geometry of the solution. We know a great deal more about these relationships now than we did at the time they wrote their paper, but if we do, it is precisely because their paper pushed us to study these questions intensely. And yet, echoing a similar comment by Borghans, De Boer and Segel [68, p. 59],

15

there is still much to learn, as evidenced by the ongoing interest in HTA's classic paper.

Acknowledgements

References

[1] F. G. Heineken, H. M. Tsuchiya, R. Aris, On the mathematical status of the pseudo-steady state hypothesis of biochemical kinetics, Math. Biosci. 1 (1967) 95–113. doi:10.1016/0025-5564(67)90029-6.

[2] V. Henri, Théorie générale de l'action de quelques diastases, C. R. Acad. Sci. 135 (1902) 916–919.

[3] L. Michaelis, M. L. Menten, Die Kinetik der Invertinwirkung, Biochem. Z. 49 (1913) 333–369.

[4] M. Bodenstein, Eine Theorie der photochemischen Reaktionsgeschwindigkeiten, Z. Phys. Chem. 85 (1913) 329–397. doi:10.1515/zpch-1913-8512.

[5] D. L. Chapman, L. K. Underhill, LV.—The interaction of chlorine and hydrogen. The influence of mass, J. Chem. Soc., Trans. 103 (1913) 496–508. doi:10.1039/CT9130300496.

[6] S. J. Fraser, The steady state and equilibrium approximations: A geometrical picture, J. Chem. Phys. 88 (1988) 4732–4738. doi:10.1063/1.454686.

[7] N. G. van Kampen, Elimination of fast variables, Phys. Rep. 124 (1985) 69–160. doi:10.1016/0370-1573(85)90002-X.

[8] M. Frankowicz, M. Moreau, P. P. Szczęsny, J. Tóth, L. Vicente, Fast variables elimination in stochastic kinetics, J. Phys. Chem. 97 (1993) 1891–1895. doi:10.1021/j100111a029.

[9] A. Kumar, P. Daoutidis, Control of Nonlinear Differential Algebraic Equation Systems with Applications to Chemical Processes, Chapman & Hall/CRC, Boca Raton, 1999.

[10] J. C. Schaff, B. M. Slepchenko, Y.-S. Choi, J. Wagner, D. Resasco, L. M. Loew, Analysis of nonlinear dynamics on arbitrary geometries with the Virtual Cell, Chaos 11 (2001) 115–131. doi:10.1063/1.1350404.

[11] J. D. Murray, Mathematical Biology I: An Introduction, 3rd Edition, Springer, New York, 2002.

[12] A. Korobeinikov, E. Shchepakina, V. Sobolev, Paradox of enrichment and system order reduction: Bacteriophages dynamics as case study, Math. Med. Biol. 33 (2016) 359–369. doi:10.1093/imammb/dqv025.

[13] N. N. Novikova, V. A. Yakovlev, I. V. Kucherenko, V. S. Vinogradov, Y. A. Aleschenko, A. V. Muratov, G. Karczewski, S. Chusnutdinow, Infrared Reflection Spectra of Pb1-x Sn (x) Se (x=0.2, 0.34) Topological Insulator Films on a ZnTe/GaAs Substrate and the Vibrational Modes of Multilayer Structures, Semiconductors 52 (2018) 34–40. doi:10.1134/S1063782618010177.

[14] K. Chayambuka, G. Mulder, D. L. Danilov, P. H. L. Notten, A modified pseudo-steady-state analytical expression for battery modeling, Solid State Commun. 296 (2019) 49–53. doi:10.1016/j.ssc.2019.04.011.

[15] C.-Y. Chang, Simulation of dynamic mold compression and resin flow for force-controlled compression resin transfer molding, J. Polym. Eng. 39 (2019) 844–851. doi:10.1515/polyeng-2019-0180.

[16] M. Gortych, Z. Lipnicki, B. Weigand, An experimental and theoretical study of the solidification process of phase change materials in a horizontal annular enclosure, Appl. Therm. Eng. 161 (2019) 114140. doi:10.1016/j.applthermaleng.2019.114140.

[17] E. P. Ivanova, X-ray lasers in cluster flows and in nanostructured targets, Opt. Spectrosc. 127 (2019) 69–76. doi:10.1134/S0030400X19070117.

[18] S. M. Jo, O. J. Kwon, J. G. Kim, Electronic-state-resolved analysis of high-enthalpy air plasma flows, Phys. Rev. E 100 (2019) 033203. doi:10.1103/PhysRevE.100.033203.

[19] H. Khan, M. A. Bazaz, S. A. Nahvi, Singular perturbation-based model reduction of power electronic circuits, IET Circ. Devices Syst. 13 (2019) 471–478. doi:10.1049/iet-cds.2018.5234.

[20] T. Zhang, Z. Zhang, C. Li, H. Xia, H. Liu, A new method to determine reasonable well pattern density in low-permeability gas reservoirs, J. Pet. Explor. Prod. Technol. 9 (2019) 2165–2174. doi:10.1007/s13202-019-0620-2.

[21] A. N. Tikhonov, Systems of differential equations containing small parameters in the derivatives, Mat. Sb. (N. S.) 31 (1952) 575–586.

[22] A. B. Vasil'eva, Asymptotic behaviour of solutions to certain problems involving non-linear differential equations containing a small parameter multiplying the highest derivatives, Russ. Math. Surv. 18 (3) (1963) 13–84. doi:10.1070/RM1963v018n03ABEH001137.

[23] A. N. Tikhonov, A. B. Vasil'eva, A. G. Sveshnikov, Differential Equations, Springer, Berlin, 1985.

[24] J. D. Cole, The development of perturbation theory at GALCIT, SIAM Rev. 36 (1994) 425–430. doi:10.1137/1036098.

[25] A. B. Vasilieva, On the development of singular perturbation theory at Moscow State University and elsewhere, SIAM Rev. 36 (1994) 440–452. doi:10.1137/1036100.

[26] R. S. Johnson, Singular Perturbation Theory, Springer, Boston, 2005.

[27] R. E. O'Malley, Historical Developments in Singular Perturbations, Springer, Cham, 2014. doi:10.1007/978-3-319-11924-3.

[28] E. Shchepakina, V. Sobolev, M. P. Mortell, Singular Perturbations: Introduction to System Order Reduction Methods with Applications, Vol. 2114 of Lecture Notes in Mathematics, Springer, Cham, 2014. doi:10.1007/978-3-319-09570-7.

[29] W. Klonowski, Simplifying principles for chemical and enzyme reaction kinetics, Biophys. Chem. 18 (1983) 73–87. doi:10.1016/0301-4622(83)85001-7.

[30] N. Fenichel, Persistence and smoothness of invariant manifolds for flows, Indiana U. Math. J. 21 (1971) 193–226.

[31] G. E. Briggs, J. B. S. Haldane, A note on the kinetics of enzyme action, Biochem. J. 19 (1925) 338–339. doi:10.1042/bj0190338.

[32] L. A. Segel, On the validity of the steady state assumption of enzyme kinetics, Bull. Math. Biol. 50 (1988) 579–593. doi:10.1007/BF02460092.

[33] S. M. Hanson, S. Schnell, Reactant stationary approximation in enzyme kinetics, J. Phys. Chem. A 112 (2008) 8654–8658. doi:10.1021/jp8026226.

[34] N. Fenichel, Geometric singular perturbation theory for ordinary differential equations, J. Differential Equations 31 (1979) 53–98. doi:10.1016/0022-0396(79)90152-9.

[35] I. G. Darvey, R. F. Matlak, An investigation of a basic assumption in enzyme kinetics using results of the geometric theory of differential equations, Bull. Math. Biophys. 29 (1967) 335–341. doi:10.1007/BF02476904.

[36] M. Schauer, R. Heinrich, Analysis of the quasi-steady-state approximation for an enzymatic one-substrate reaction, J. Theor. Biol. 79 (1979) 425–442. doi:10.1016/0022-5193(79)90235-2.

[37] A. H. Nguyen, S. J. Fraser, Geometrical picture of reaction in enzyme kinetics, J. Chem. Phys. 91 (1989) 186–193. doi:10.1063/1.457504.

[38] S. J. Fraser, M. R. Roussel, Phase-plane geometries in enzyme kinetics, Can. J. Chem. 72 (1994) 800–812. doi:10.1139/v94-107.

[39] M. S. Calder, D. Siegel, Properties of the Michaelis-Menten mechanism in phase space, J. Math. Anal. Appl. 339 (2008) 1044–1064. doi:10.1016/j.jmaa.2007.06.078.

[40] M. Okuda, Inflector analysis of the second stage of the transient phase for an enzymatic one-substrate reaction, Prog. Theor. Phys. 68 (1982) 1827–1840. doi:10.1143/PTP.68.1827.

[41] U. Maas, S. B. Pope, Simplifying chemical kinetics: Intrinsic low-dimensional manifolds in composition space, Combust. Flame 88 (1992) 239–264. doi:10.1016/0010-2180(92)90034-M.

[42] P. S. Crooke, R. D. Tanner, R. Aris, The role of dimensionless parameters in the Briggs-Haldane and Michaelis-Menten approximations, Chem. Eng. Sci. 34 (1979) 1354–1357. doi:10.1016/0009-2509(79)80031-7.

[43] L. A. Segel, M. Slemrod, The quasi-steady-state assumption: A case study in perturbation, SIAM Rev. 31 (1989) 446–477. doi:10.1137/1031091.

[44] S. Schnell, Validity of the Michaelis-Menten equation — steady-state or reactant stationary assumption: That is the question, FEBS J. 281 (2014) 464–472. doi:10.1111/febs.12564.

[45] P. A. T. Swoboda, The kinetics of enzyme action, Biochim. Biophys. Acta 23 (1957) 70–80. doi:10.1016/0006-3002(57)90286-X.

[46] P. A. Srere, Enzyme concentrations in tissues, Science 158 (1967) 936–937. doi:10.1126/science.158.3803.936.

[47] A. Sols, R. Marco, Concentrations of metabolites and binding sites. Implications in metabolic regulation, Curr. Topics Cell. Reg. 2 (1970) 227–273.

[48] K. R. Albe, M. H. Butler, B. E. Wright, Cellular concentrations of enzymes and their substrates, J. Theor. Biol. 143 (1990) 163–195. doi:10.1016/S0022-5193(05)80266-8.

[49] P. A. T. Swoboda, The kinetics of enzyme action. II. The terminal phase of the reaction, Biochim. Biophys. Acta 25 (1957) 132–135. doi:10.1016/0006-3002(57)90428-6.

[50] J. T.-F. Wong, On the steady-state method of enzyme kinetics, J. Am. Chem. Soc. 87 (1965) 1788–1793. doi:10.1021/ja01086a032.

[51] J. R. Bowen, A. Acrivos, A. K. Oppenheim, Singular perturbation refinement to quasi-steady state approximation in chemical kinetics, Chem. Eng. Sci. 18 (1963) 177–187. doi:10.1016/0009-2509(63)85003-4.

[52] I. Tinoco, Jr., K. Sauer, J. C. Wang, Physical Chemistry: Principles and Applications in Biological Sciences, 3rd Edition, Prentice Hall, Englewood Cliffs, NJ, 1995.

[53] M. R. Roussel, A Life Scientist's Guide to Physical Chemistry, Cambridge University Press, Cambridge, 2012.

[54] T. Engel, P. Reid, Thermodynamics, Statistical Thermodynamics, & Kinetics, 3rd Edition, Pearson, Boston, 2013.

[55] C. Vallance, An Introduction to Chemical Kinetics, IOP Concise Physics, Morgan & Claypool, San Rafael, CA, 2017. doi:10.1088/978-1-6817-4664-7.

[56] S. J. Fraser, Double perturbation series in the differential equations of enzyme kinetics, J. Chem. Phys. 109 (1998) 411–423. doi:10.1063/1.476578.

[57] J. Tóth, A. L. Nagy, D. Papp, Reaction Kinetics: Exercises, Programs and Theorems, Springer, New York, 2018.

[58] A. Goeke, C. Schilli, S. Walcher, E. Zerz, Computing quasi-steady state reductions, J. Math. Chem. 50 (2012) 1495–1513. doi:10.1007/s10910-012-9985-x.

[59] M. R. Roussel, Nonlinear Dynamics: A Hands-On Introductory Survey, IOP Concise Physics, Morgan & Claypool, San Rafael, CA, 2019. doi:10.1088/2053-2571/ab0281.

[60] A. C. Fowler, Mathematical Models in the Applied Sciences, Cambridge University Press, Cambridge, 1997.

[61] L. A. Segel, Simplification and scaling, SIAM Rev. 14 (1972) 547–571. doi:10.1137/1014099.

[62] R. D. Allison, D. L. Purich, Practical considerations in the design of initial velocity enzyme rate assays, Meth. Enzymol. 63 (1979) 3–22. doi:10.1016/0076-6879(79)63003-3.

[63] H. Bisswanger, Practical Enzymology, Wiley-Blackwell, Weinheim, 2011.

[64] C. C. Lin, L. A. Segel, Mathematics Applied to Deterministic Problems in the Natural Sciences, Vol. 1 of Classics in Applied Mathematics, SIAM, Philadelphia, 1988.

[65] J. Eilertsen, W. Stroberg, S. Schnell, Characteristic, completion or matching timescales? An analysis of temporary boundaries in enzyme kinetics, J. Theor. Biol. 481 (2019) 28–43. doi:10.1016/j.jtbi.2019.01.005.

[66] G. Dell'Acqua, A. M. Bersani, A perturbation solution of Michaelis-Menten kinetics in a "total" framework, J. Math. Chem. 50 (2012) 1136–1148. doi:10.1007/s10910-011-9957-6.

[67] A. M. Bersani, E. Bersani, G. Dell'Acqua, M. G. Pedersen, New trends and perspectives in nonlinear intracellular dynamics: One century from Michaelis-Menten paper, Continuum Mech. Thermodyn. 27 (2015) 659–684. doi:10.1007/s00161-014-0367-4.

[68] J. A. M. Borghans, R. J. De Boer, L. A. Segel, Extending the quasi-steady state approximation by changing variables, Bull. Math. Biol. 58 (1996) 43–63. doi:10.1007/BF02458281.

[69] A. R. Tzafriri, Michaelis-Menten kinetics at high enzyme concentrations, Bull. Math. Biol. 65 (2003) 1111–1129. doi:10.1016/S0092-8240(03)00059-4.

[70] A. R. Tzafriri, E. R. Edelman, The total quasi-steady-state approximation is valid for reversible enzyme kinetics, J. Theor. Biol. 226 (2004) 303–313. doi:10.1016/j.jtbi.2003.09.006.

[71] C. E. Leith, Nonlinear normal mode initialization and quasi-geostrophic theory, J. Atmos. Sci. 37 (1980) 958–968.
URL http://doi.org/fcwfs4

[72] E. N. Lorenz, On the existence of a slow manifold, J. Atmos. Sci. 43 (1986) 1547–1557.
URL http://doi.org/bsbnnt

[73] A. N. Gorban, I. V. Karlin, Method of invariant manifold for chemical kinetics, Chem. Eng. Sci. 58 (2003) 4751–4768. doi:10.1016/j.ces.2002.12.001.

[74] M. R. Roussel, S. J. Fraser, Geometry of the steady-state approximation: Perturbation and accelerated convergence methods, J. Chem. Phys. 93 (1990) 1072–1081. doi:10.1063/1.459171.

[75] K. J. Laidler, Theory of the transient phase in kinetics, with special reference to enzyme systems, Can. J. Chem. 33 (1955) 1614–1624. doi:10.1139/v55-195.

[76] R. B. Fisher, Significance of the Michaelis constant, Nature 197 (1963) 799. doi:10.1038/197799a0.

[77] S. Cha, Kinetic behavior at high enzyme concentrations, J. Biol. Chem. 245 (1970) 4814–4818.
URL http://www.jbc.org/content/245/18/4814

[78] M. Schauer, R. Heinrich, Quasi-steady-state approximation in the mathematical modeling of biochemical reaction networks, Math. Biosci. 65 (1983) 155–170. doi:10.1016/0025-5564(83)90058-5.

[79] S. Schnell, P. K. Maini, Enzyme kinetics at high enzyme concentration, Bull. Math. Biol. 62 (2000) 483–499. doi:10.1006/bulm.1999.0163.

[80] T. P. Prescott, A. Papachristodoulou, Layered decomposition for the model order reduction of timescale separated biochemical reaction networks, J. Theor. Biol. 356 (2014) 113–122. doi:10.1016/j.jtbi.2014.04.007.

[81] L. Noethen, S. Walcher, Tikhonov's theorem and quasi-steady state, Discrete Contin. Dyn. Syst. B 16 (2011) 945–961. doi:10.3934/dcdsb.2011.16.945.

[82] A. Goeke, S. Walcher, E. Zerz, Classical quasi-steady state reduction—a mathematical characterization, Physica D 345 (2017) 11–26. doi:10.1016/j.physd.2016.12.002.

[83] F. Battelli, C. Lazzari, On the pseudo-steady-state approximation and Tikhonov theorem for general enzyme systems, Math. Biosci. 75 (1985) 229–246. doi:10.1016/0025-5564(85)90039-2.

[84] K. G. Stern, Spectroscopy of an enzyme reaction, Nature 136 (1935) 335. doi:10.1038/136335a0.

[85] K. Hayashi, T. Imoto, M. Funatsu, The enzyme-substrate complex in a muramidase catalyzed reaction. I. Difference spectrum of complex, J. Biochem. 54 (1963) 381–387. doi:10.1093/oxfordjournals.jbchem.a127803.

[86] R. M. Paton, E. T. Kaiser, Detection of a "Michaelis" complex by spin labeling in a model enzyme system, J. Am. Chem. Soc. 92 (1970) 4723–4725. doi:10.1021/ja00718a040.

[87] R. D. Tanner, A. C. Loo, J. L. Shisler, M. W. Reed, R. D. Rowlett, J. W. Morris, G. W. Schlossnagle, J. R. Overley, Mapping the lag phase and bounding the growth phase in fermentation reactions, AIChE Symp. Ser. 73 (167) (1976) 55–65.

[88] J. Monod, Recherches sur la croissance des cultures bactériennes, Hermann, Paris, 1942.

[89] V. G. Gorskii, S. I. Spivak, T. N. Shvetsova-Shilovskaya, R. Kh. Bakhitova, M. D. Ramazanov, Asymptotic approximation and parameter identifiability for a system of differential equations in chemical kinetics, Dokl. Phys. Chem. 316 (1991) 47–50.

[90] A. G. Fredrickson, D. Ramkrishna, H. M. Tsuchiya, Statistics and dynamics of procaryotic cell populations, Math. Biosci. 1 (1967) 327–374. doi:10.1016/0025-5564(67)90008-9.