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A test for measuring the effects of enzyme inactivation

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Abstract

In the single-enzyme, single-substrate reaction with non-mechanism-based enzyme inactivation, the formation of the product and inactivation of the enzyme occur independently. For this reaction, we show that the steady-state hypothesis is applicable even when degradation of the enzyme occurs. An equation for the rate of product formation has been derived and it shows Michaelis–Menten kinetics with an apparent Michaelis–Menten constant $K_M^{app} = K_M + K_\delta$ where K_δ is the enzyme inactivation constant. Use of a Lineweaver–Burk plot yields values for K_M^{app}, which can be used to estimate K_δ and, consequently, the degree of enzyme inactivation in a particular experiment. We employ this methodology to estimate the inactivation constant for the arsenate reductase catalyzed production of arsenite with appreciable enzyme inactivation.

Keywords: Michaelis–Menten reaction; Enzyme inactivation; Steady-state kinetics; Non-mechanism-based inactivation; Determining kinetics parameters; Enzyme specificity

1. Introduction

Most of the current mechanisms of enzyme action in chemical kinetics assume that the enzyme is stable and maintains its activity during the time course of the reaction [1]. However, it is not uncommon for an enzyme to rapidly lose its activity *in vitro* conditions [2]. Under temperature changes [3], diluted conditions, or changes in the reaction medium (pH or buffer) [4,5], enzymes can undergo progressive loss of activity. Because of this, the current mechanisms are often not adequate for predicting chemical kinetics in industrial or *in vitro* conditions.

Neglecting enzyme inactivation can result in errors in both estimating the kinetics parameters and reporting the mechanisms of enzyme action [2,6]. For example, the observed low enzyme activity due to enzyme inactivation can be incorrectly attributed to cooperativity, substrate or product inhibition, presence of a competitive inhibitor, or two enzymes acting on the same or different substrates [6]. Attempting to describe a system using any of the above mechanisms when the mechanism involved is actually enzyme inactivation can result in grievous errors in describing the behavior of the system, such as incorrect estimation of the kinetics parameters. Therefore it is very important to be able to know when enzyme inactivation is affecting a reaction.

The most commonly used test for identifying enzyme inactivation compares progress curves of the product formation at several different initial enzyme concentrations. In the absence of enzyme inactivation, plots of the synthesized product against time multiplied by the initial enzyme concentration should be superimposable. If the progress curves at different initial enzyme concentrations are not superimposable, it implies that the concentration of active enzyme is varying and that product formation rates are dependent upon the change of enzyme concentration through time. This test was developed by Michaelis and Davidsohn [7], and later systematically adapted by Selwyn [6].

Understanding the effects of enzyme inactivation is important for applications such as predicting the behavior of chemical reactions in the food, chemical, and pharmaceutical industries [8,9]. Since the development processes that involve these essential chemical reactions can be elaborate and expensive, early and accurate predictions of enzyme inactivation have become increasingly important. These predictions are often the result of a variety of *in vitro* assays, in which, as previously mentioned, enzyme inactivation is common. Selwyn's procedure

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is the most commonly used methodology for comparing levels of enzyme stability in different reaction conditions [3,5]. Though it is helpful and can be used to achieve a limited understanding of the effects of enzyme activation, Selwyn's test does not yield the quantitative predictions crucial to predicting the behavior of reactions. While there are numerous models available for mechanism-based inactivation systems or suicide substrates [10,11], there is currently no methodology that yields quantitative predictions for non-mechanism-based enzyme inactivation.

In this paper we derive for the first time an expression for the initial reaction rate of the single-enzyme, single-substrate reaction with non-mechanism-based enzyme inactivation. Unlike in mechanism-based enzyme inactivation, this mechanism considers that the product formation and the inactivation of the enzyme proceed independently. We show that the reaction rate closely follows Michaelis–Menten kinetics (Section 3). We also find that the condition for the validity of steady-state kinetics in an enzyme catalyzed reaction is stronger in the presence of enzyme inactivation (Section 3.1). In Section 4 we use our new methodology to measure an enzyme inactivation parameter, and estimate its value for the *Staphylococcus aureus* arsenate reductase. We conclude our paper with a brief discussion (Section 5).

2. The single-enzyme, single-substrate reaction with non-mechanism-based enzyme inactivation

To set the stage, consider the reaction mechanism, originally proposed by Henri [12], in which an enzyme E and substrate S combine to form, as an intermediate, the enzyme-substrate complex C, which then yields the product P and again the enzyme E. This reaction is known as the Michaelis–Menten mechanism of enzyme action [13]. The enzyme is normally considered more stable while incorporated in C than when in free E form. From this, we can add to the Michaelis–Menten mechanism that free E decays into its inactive form E_i. This reaction scheme is represented

$$S + E \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} C \overset{k_{\text{cat}}}{\rightarrow} P + E, \tag{1}$$

$$E \overset{k_3}{\rightarrow} E_i. \tag{2}$$

Note that the enzyme inactivation is irreversible in this mechanism. In the reversible case, known as slowly reverting enzyme inactivation mechanism [14], the enzyme undergoes a reversible conformation change to an inactive form. These enzymes are called "hysteretic" [15], and are common among regulatory enzymes [16].

The time evolution, and governing equations, of the Reaction (1)–(2) are obtained by applying the law of mass action to yield the set of coupled nonlinear differential equations

$$\frac{ds}{dt} = k_1(-es + K_S c), \tag{3}$$

$$\frac{de}{dt} = k_1(-es + K_M c - K_\delta e), \tag{4}$$

$$\frac{dc}{dt} = k_1(es - K_M c), \tag{5}$$

$$\frac{dp}{dt} = k_{\text{cat}} c, \tag{6}$$

$$\frac{de_i}{dt} = k_1 K_\delta e, \tag{7}$$

with initial conditions $(s,e,c,p,e_i)=(s_0,e_0,0,0,0)$ at time $t=0$, where lower-case letters represent concentrations and the subscript 0 denotes initial concentration. In this system, $K_S=k_{-1}/k_1$ is the equilibrium dissociation constant for the enzyme–substrate complex, $K_M=(k_{-1}+k_{\text{cat}})/k_1$ is the Michaelis–Menten constant (see Schnell and Maini [1] for a review), and $K_\delta=k_3/k_1$ is a constant we have defined for the inactivation of the enzyme.

This reaction mechanism obeys two conservation laws: the enzyme conservation law obtained by adding Eqs. (4), (5) and (7)

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

and the substrate conservation law obtained by adding Eqs. (3), (5) and (6):

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

With the help of these conservation laws we can fully describe the reaction mechanism using only three differential equations

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

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

$$\frac{de_i}{dt} = k_1 K_\delta(e_0 - c - e_i), \tag{12}$$

with initial conditions $(s,c,e_i)=(s_0,0,0)$ at time $t=0$. In the next section, we will simplify the system further by exploiting steady-state kinetics and obtain an expression for estimating the kinetics parameters.

3. Steady-state kinetics of the reaction

The most common type of assay in enzyme kinetics studies is the initial rate experiments [2,17]. When an enzyme is mixed with a large excess of substrate, the enzyme–substrate intermediate builds up in a fast initial transient. Because of the large excess of substrate and the short period over which the rate is measured, the approximation $s \approx s_0$ can be made. At the end of this fast transient, the reaction achieves steady-state kinetics and c remains approximately constant over time as the reaction rate changes relatively slowly. This rate is typically measured by monitoring the accumulation of product with time for a short period after the reaction attains steady-state kinetics. Experiments following this format are common because they are simple to perform and analyze, being relatively free of

complications such as back-reaction (the formation of substrate from product).

Following the methodology originally proposed by Briggs and Haldane [18], we can now reduce the complexity of the governing equations by assuming the steady-state kinetics described above for the enzyme inactivation mechanism (Reaction (1)–(2)). There are two main characteristics of a reaction for which steady-state kinetics are assumed [19]:

I. After the fast initial transient ($t>t_c$), we assume that c remains approximately constant. That is, in the steady-state regime it can be taken that

$$\frac{dc}{dt} \approx 0 \quad for \quad t>t_c. \tag{13}$$

Applying this condition to Eq. (11), we can solve for c in terms of s and e_i

$$c = \frac{(e_0-e_i)s}{K_M+s} \quad for \quad t>t_c. \tag{14}$$

Substituting Eq. (14) into Eq. (10) leads to

$$\frac{ds}{dt} = -\frac{k_{cat}(e_0-e_i)s}{K_M+s}. \tag{15}$$

Note that the rate of product formation in steady-state regime is

$$\frac{dp}{dt} = -\frac{ds}{dt}. \tag{16}$$

We now need make another assumption of steady-state kinetics to determine e_i at the beginning the steady-state regime.

II. The concentration of the substrate does not change significantly during the fast initial transient. Therefore, we can assume that the initial condition of the substrate for the steady-state period is

$$s \approx s_0 \quad for \quad t\le t_c. \tag{17}$$

Substituting Eq. (17) into Eqs. (11)–(12), we find a general solution for these differential equations that has the form

$$e_i = e_0 + a\ \exp(\lambda_1 t) - b\ \exp(\lambda_2 t), \tag{18}$$

$$c = ab\frac{(\lambda_1-\lambda_2)}{k_1 K_\delta e_0}(\exp(\lambda_2 t)-\exp(\lambda_1 t)), \tag{19}$$

with

$$a = \frac{e_0(\lambda_2/k_1+K_\delta)}{(\lambda_1-\lambda_2)/k_1}, \tag{20}$$

$$b = \frac{e_0(\lambda_1/k_1+K_\delta)}{(\lambda_1-\lambda_2)/k_1}, \tag{21}$$

and

$$\lambda_1,\lambda_2 = \frac{-k_1(K_M^{app}+s_0)}{2}\left(1\mp\sqrt{1-\frac{4K_\delta K_M}{(K_M^{app}+s_0)^2}}\right). \tag{22}$$

In this expression

$$K_M^{app} = K_M + K_\delta.$$

It is physically realistic to assume (see Appendix)

$$\epsilon = \frac{4K_\delta K_M}{(K_M^{app}+s_0)^2} << 1, \tag{23}$$

which yields

$$\lambda_1 = -\frac{k_1 K_\delta K_M}{K_M^{app}+s_0} + O(\epsilon^2), \tag{24}$$

$$\lambda_2 = -k_1(K_M^{app}+s_0) + O(\epsilon^2). \tag{25}$$

In our case, $t_c=|1/\lambda_2|$,

$$t_c \approx \frac{1}{k_1(K_M^{app}+s_0)}. \tag{26}$$

We can now obtain the approximation for the concentration of e_i at time t_c. Substituting Eqs. (24)–(26) into Eq. (18), we obtain

$$e_i(t_c) = e_0 + a\ \exp\left(\frac{K_M K_\delta}{(K_M^{app}+s_0)^2}\right) + b\ \exp(-1). \tag{27}$$

From Eq. (23) the inequality $(K_M^{app}+s_0)^2 \gg K_M K_\delta$ is strong (see Appendix), and allows the above expression to be simplified to

$$e_i(t_c) = (1-\exp(-1))\frac{e_0 K_\delta}{K_M^{app}+s_0} \approx \frac{e_0 K_\delta}{K_M^{app}+s_0}. \tag{28}$$

We are now in a position to derive an expression for v_0, the initial rate, of the single-enzyme, single-substrate reaction with enzyme inactivation. After the initial fast transient, during which the substrate concentration hardly changes, the initial rate measurements are carried out for a very short period. Therefore, the previous approximation $s \approx s_0$ is still valid. After substituting this approximation into Eq. (15) with Eq. (28), we obtain the following Michaelis–Menten type expression for the initial velocity of the reaction

$$v_0 = \left.\frac{dp}{dt}\right|_{s=s_0} = -\left.\frac{ds}{dt}\right|_{s=s_0} = \frac{v_{max}s_0}{K_M^{app}+s_0}, \tag{29}$$

where $v_{max}=k_{cat}e_0$ is the maximum velocity of the reaction.

The above expression shows that enzyme inactivation increases the apparent Michaelis–Menten constant while leaving the apparent maximum velocity unchanged. In Section 4 we illustrate how the use of a Lineweaver–Burk, or double-reciprocal plot [20], with experimental data supports this theory. We also show how experimentalists can estimate K_δ. However, let us first turn our attention to the conditions for the validity of steady-state kinetics.

3.1. Validity of steady-state kinetics assumption

We have employed steady-state kinetics to simplify the system (10)–(12), however using this simplification has its

consequences: the derived Michaelis–Menten expression (29) is only valid when the conditions for the validity of steady-state kinetics are met [1]. These conditions have been systematically studied [21–23] in recent years. That is, in order to apply steady-state kinetics, we have made two assumptions:

I. From a biophysical point of view, it is reasonable to assume that steady-state kinetics are valid when the time, t_s, taken for a significant change in s is much bigger than the time, t_c, taken for a significant build-up of c during the initial fast transient. Therefore, a necessary condition is $t_c \ll t_s$.

We have estimated t_c with Eq. (26). Now we estimate t_s by dividing the total change in substrate concentration by the maximum rate of substrate change in the steady-state period [19]

$$t_s = \frac{s_0}{|\mathrm{d}s/\mathrm{d}t|_{\max}}. \tag{30}$$

The maximum value of $\mathrm{d}s/\mathrm{d}t$ for the steady-state period at a given initial substrate concentration is v_0 (see Eq. (29)). Hence we have

$$t_s = \frac{K_M^{app} + s_0}{v_{\max}}. \tag{31}$$

After substituting Eqs. (31) and (26) into the condition $t_c \ll t_s$ we obtain the first condition for the validity of steady-state kinetics:

$$\frac{e_0}{K_M^{app} + s_0} << 1 + \frac{k_1(K_S + K_\delta + s_0)}{k_{cat}}. \tag{32}$$

II. We can assume steady-state kinetics to be valid only if the change in substrate concentration is extremely small compared to the initial substrate concentration in the initial transient. This can be quantitatively determined [19] by

$$\frac{\Delta s}{s_0} \approx \frac{t_c}{s_0}\left|\frac{\mathrm{d}s}{\mathrm{d}t}\right|_{\max} << 1. \tag{33}$$

In contrast with the derivation of Eq. (31), we are now referring to the initial transient and determine the maximum velocity of substrate depletion using Eq. (10) with $c = e_i = 0$ and $s = s_0$. Substituting Eq. (26) into Eq. (33), we find our second necessary condition to be

$$\frac{e_0}{K_M^{app} + s_0} << 1. \tag{34}$$

Eq. (34) implies the first condition, Eq. (32), therefore Eq. (34) is the condition for the validity of steady-state kinetics for the single-enzyme, single-substrate reaction with enzyme inactivation. Note that this derived condition is very similar to the derived condition for the Michaelis–Menten reaction mechanism [19,21] and the single-enzyme reaction with alternative substrates [24]. Our derived condition shows that enzyme inactivation increases the apparent Michaelis–Menten constant, making the validity of steady-state kinetics stronger with increased inactivation.

4. Measuring the effects of enzyme inactivation

Now that we have determined the conditions for the validity of our initial rate expression, we can discuss the experimental methodology for estimating the enzyme inactivation constant K_δ.

The effects of enzyme inactivation can be measured using any of the plots biochemists currently employ to determine the enzyme kinetics parameters $v_{\max}$ and K_M. However, the most commonly employed graphical method [25] is the double-reciprocal plot. Performing an enzyme kinetics experiment first requires determining whether the conditions of the assay (pH, temperature, and other conditions) maintain enzyme stability during the period of observation. We have found that this can be observed graphically using the double-reciprocal plot. When an enzyme is maintained in a stable condition, plots of $1/v_0$ against $1/s_0$ will show a line that crosses the $1/v_0$ axis at $1/v_{\max}$ and the $1/s_0$ axis at $-1/K_M$. However if an enzyme is degrading, the double-reciprocal plot will create a line that still crosses the $1/v_0$ axis at $1/v_{\max}$, but crosses the $1/s_0$ axis at $-1/K_M^{app}$ instead of $-1/K_M$. The enzyme inactivation constant is determined as:

$$K_\delta = K_M^{app} - K_M. \tag{35}$$

A recent example in the literature of enzyme inactivation is the kinetics study of the *S. aureus* arsenate reductase [5,26–28]. This enzyme reduces intracellular arsenate to a more toxic component arsenite in *S. aureus*. Arsenate reductase is very labile to oxidative inactivation. Messens et al. [5] studied the effectiveness of oxyanions as stabilizing agents for arsenate reductase. They found that the best stabilizing oxyanions for arsenate reductase are its substrate arsenate and the tetrahedral oxyanions phosphate, sulfate and perchlorate. We extracted Messens and co-workers' kinetics data for the arsenate reductase oxidation of arsenate into arsenite in both stable and non-stable enzyme conditions. As there is no convenient way to monitor the formation of arsenite directly, the reaction is followed indirectly by observing the rate of NADP formation. The initial velocity of NADPH oxidation was measured in 50 mM Tris (pH 8.0), 50 mM K_2SO_4, 0.1 mM EDTA (argon flushed). The substrate arsenate was used as a stabilizing agent in these assays. The initial rate of NADPH oxidation coupled with arsenate reduction shows typical Michaelis–Menten kinetics for the assays with stable and unstable enzyme conditions (see Fig. 1). Both double-reciprocal plots show the same apparent maximum velocities: $v_{\max}^{app} = 62.5$ mOD/min. However, the apparent Michaelis–Menten values varied substantially. At low arsenate concentrations $K_M^{app} = 91$ μM (dotted line), while at high arsenate concentrations $K_M^{app} = 508$ μM (solid line). As there is no difference in the maximum velocities, but significant difference in the Michaelis–Menten constants, we can now measure the effects of enzyme inactivation in this reaction under our new theory. The inactivation constant under unstable assay conditions is equal to the difference between the apparent Michaelis–Menten constants, that is $K_\delta = 417$ μM.

Although we did not find theoretical changes in the values of the apparent maximum velocity for the enzyme inactivation, in several instances unexpected changes have been observed in the maximum velocities. For example, the stabilizing effects of tetrahedral oxyanions in arsenate reductase can result in an apparent k_{cat} value [5,27] that will inevitably change the apparent maximum velocity. The selection of stabilizing factors that

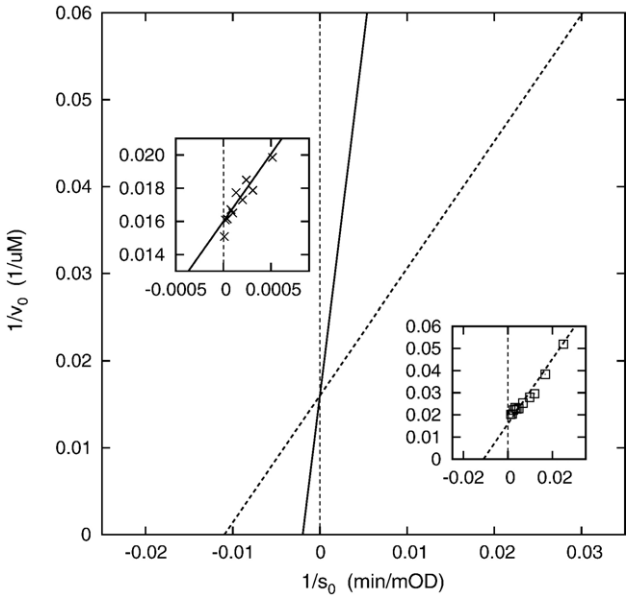

Fig. 1. Double reciprocal plots of the initial rate velocity of NADPH oxidation as a function of arsenate concentration. The substrate arsenate was used as a stabilizing agent. The data of Messens et al. [5] is shown in the insets with the same double reciprocal plots of the figure. The cross symbols (solid line) corresponds to the experiment at high arsenate concentration. The square symbols (dotted line) corresponds to the experiment at low arsenate concentration. The initial velocity of NADPH oxidation was measured in 50 mM Tris (pH 8.0), 50 mM K_2SO_4, 0.1 mM EDTA (argon flushed). The substrate, arsenate, was used as a stabilizing agent to the enzyme arsenate reductase in these assays. Under these experimental conditions, and as predicted by our theory, the enzyme inactivation affects the apparent Michaelis–Menten constant. The enzyme inactivation constant K_δ is determined by the difference between the apparent Michaelis–Menten constants. In this case, K_δ=417 μM.

do not affect k_{cat} values is essential for obtaining reliable quantitative estimates of K_δ.

5. Discussion

The double-reciprocal plot using

$$\frac{1}{v_0} = \frac{1}{v_{max}^{app}} + \frac{K_M^{app}}{v_{max}^{app}} \frac{1}{s_0}, \tag{36}$$

is the most common method for determining the enzyme kinetics parameters. In this expression, v_{max}^{app} and K_M^{app} are the apparent values of the maximum velocity v_{max} and the Michaelis–Menten constant K_M that appear in reaction mechanisms. In this paper, we show for the first time that the apparent values of v_{max} and K_M in a single-enzyme, single-substrate reaction with non-mechanism-based enzyme inactivation are given by

$$v_{max}^{app} = v_{max}, \tag{37}$$

$$K_M^{app} = K_M + K_\delta. \tag{38}$$

Therefore, inactivation decreases the apparent value of the pseudo-first-order constant (or specificity time),

$$\frac{v_{max}^{app}}{K_M^{app}} = \frac{v_{max}/K_M}{1 + \frac{K_\delta}{K_M}}, \tag{39}$$

by the factor $1+K_\delta/K_M$, while leaving the v_{max} unchanged. The pseudo-first-order constant v_{max}/K_M is the fraction of substrate converted into product per unit time [17] if the enzyme reaction is under pseudo-first-order conditions [29]. A similar decrease is observed in the specificity constant (or pseudo-second-order constant), which is defined as k_{cat}/K_M^{app}.

This shows that the primary effect of non-mechanism-based enzyme inactivation is to decrease the specificity constants. It would appear then that the appropriate name for the reaction mechanism described by Eqs. (1)–(2) is specific enzyme inactivation. With this inactivation mechanism, we would expect a diminution of the total synthesized product and remaining substrate in the reaction medium. It can easily be confirmed from the governing equations of the system Eqs. (3)–(6) that the substrate concentration has a non-zero equilibrium value.

In this paper we find that we can use double-reciprocal plots and Eqs. (37)–(38) to determine the inactivation constant (K_δ) and possibly to diagnose enzyme inactivation (see Section 4). Before estimating the inactivation constant in enzyme inactivation experiments, two primary steps must be taken. The first is to detect enzyme inactivation with the aid of the Selwyn plot. Once appreciable enzyme inactivation has been detected during the period of observation of the reaction, the next step is to use the Selwyn plot to find conditions that will stabilize the enzyme. The enzyme inactivation constant can then be determined from the values of the apparent Michaelis–Menten constants of the enzyme catalyzed reaction under stable and unstable enzyme conditions. The enzyme inactivation constant is simply the difference between the apparent Michaelis–Menten constants. To obtain reliable estimates it is essential that the reactions under stable and unstable conditions do not appreciably change the apparent k_{cat} values.

To derive Eqs. (36)–(38) it is assumed that the steady-state kinetics is valid. Interestingly, we find the condition for validity of steady-state kinetics in the enzyme inactivation mechanism, $K_M+K_\delta+s_0 \gg e_0$, is stronger than the condition for validity in the Michaelis–Menten mechanism, $K_M+s_0 \gg e_0$.

Current experimental practice does not consider that the single-enzyme, single-substrate reaction with enzyme inactivation would follow the expected Michaelis–Menten behavior. In fact, it was previously considered that enzyme inactivation leads to deviations from the expected behavior and inaccurate estimates of the kinetics parameters [2,6]. We have shown that even with enzyme inactivation the Michaelis–Menten equation with steady-state kinetics remains useful as a first order approximation in enzyme kinetics and a tool for estimating reaction parameters.

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opinions, findings, and conclusions or recommendations expressed in this paper are those of the authors and do not necessarily reflect the views of their universities, the National Science Foundation or the United States government.

Appendix A

The derivation of a simpler expression for the fast timescale, t_c, relies on assuming

$$\frac{4K_\delta K_M}{(K_M^{app}+s_0)^2} << 1. \quad (40)$$

By scaling Eq. (40) with respect to K_M, we obtained

$$\frac{4\hat{K}_\delta}{(1+\hat{K}_\delta+\hat{s_0})^2} << 1, \quad (41)$$

with $\hat{K_\delta}=K_\delta/K_M$ and $\hat{s_0}=s_0/K_M$. This expression can be written

$$2\sqrt{\hat{K}_\delta} << 1+\hat{K}_\delta+\hat{s_0}. \quad (42)$$

If we re-arrange this inequality, we have

$$\hat{s_0} \gg 2\sqrt{\hat{K}_\delta}-(1+\hat{K}_\delta). \quad (43)$$

Note that the right-hand side of the expression is always less than or equal to zero for any value of K_δ and K_M. Since $\hat{s_0}$ must be greater than zero, it is safe to assume that Eqs. (40) and (43) are generally valid.

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