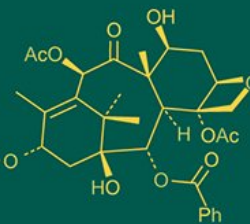
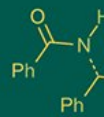


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Influence of enzyme concentration on the michaelis-menten constant: A new hypothesis for isolated cases

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Abstract

The classical Michaelis-Menten equation assumes that the Michaelis constant (K_m) is independent of enzyme concentration. However, this study presents a novel Hypothesis suggesting that K_m increases with enzyme concentration due to electrostatic and steric effects influencing substrate binding. By analyzing kinetic data and mathematical modeling, we demonstrate that traditional approximations such as the Quasi-Steady-State Assumption (QSSA) and Reactant Stationary Approximation (RSA) may not fully describe enzymatic behavior under varying enzyme concentrations. Our findings suggest a re-evaluation in enzyme kinetics, with potential applications in metabolic regulation and drug development. The implications extend to the design of enzyme inhibitors, particularly in therapeutic strategies targeting metabolic pathways in diseases such as cancer and autoimmune disorders. This new perspective challenges certain conventional assumptions in enzymology and provides new perspectives on enzyme-substrate interactions.

Keywords: Michaelis-Menten constant (K_m), enzyme concentration, allosteric enzymes

Introduction

In enzyme kinetics, it has long been accepted that the Michaelis-Menten constant (K_m) is independent of enzyme concentration. This assumption stems from the idea that under standard conditions, K_m reflects the enzyme's affinity for a substrate and is considered a fixed parameter for a given enzyme-substrate interaction. As a result, it has been widely believed that K_m remains constant, irrespective of changes in enzyme concentration, representing an intrinsic property of the enzyme-substrate relationship ^[1]. In 1934, Lineweaver and Burk developed a graphical method for determining K_m and V_{max} , providing researchers with a valuable tool to analyze kinetic data. However, this method has faced criticism, one of the main reasons being that it often shows K_m increasing with enzyme concentration challenging the assumption that K_m is a constant ^[2]. Briggs and Haldane introduced the Quasi-Steady State Approximation (QSSA), which assumes the concentration of the enzyme-substrate complex remains relatively stable during the reaction. This approximation provided the foundation for deriving the Michaelis-Menten equation, which has become a cornerstone of enzymatic kinetics. ^[3]

According to the allosteric hypothesis ^[4], the modulation of enzyme affinity would require a sufficient number of allosteric modulators. If these modulators are insufficient, it would necessitate that only a portion of the enzymes are active while others are inactive, which does not align with observed reality. The proposed theory offers a more plausible explanation, as the decrease in affinity for the modulator is linked to an increase in enzyme concentration.

The mechanism of enzymatic aggregation ^[5], although explainable in a few cases based on electrostatic interactions, is in most cases hindered by these interactions and cannot serve as an explanation for the majority of situations in which it has been proposed as a rationale.

Enzymatic crowding ^[6] may explain variations in K_m only at extremely high enzyme concentrations. However, it does not account for the constant increase in K_m , as observed in Figure 1 of this article. Therefore, this mechanism cannot be utilized to explain the experimental results. The same explanation applies to the diffusion model. Although the model of micro environmental change with enzyme concentration certainly influences the value of K_m if we compare the changes in K_m obtained through enzyme concentration with the effects obtained by other methods, it becomes clear that the. Micro environmental change

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with enzyme concentration model cannot numerically justify such large variations. This paper introduces a novel hypothesis that redefines the traditional understanding of enzyme-substrate interactions by focusing on the role of enzyme concentration in determining affinity. Unlike the classical Michaelis-Menten model, which considers enzyme concentration as irrelevant to affinity, this hypothesis posits that enzyme concentration modulates the attraction between the enzyme and its substrate. This new perspective challenges the long-held assumption that affinity is constant under varying conditions and opens up new possibilities for studying enzyme kinetics, with important implications for biochemical research and therapeutic development.

The relevance of the proposed Hypothesis extends beyond merely reconsidering a fundamental principle in enzyme kinetics. This Hypothesis could have profound implications in various fields of biochemistry and pharmacology, particularly in the context of drug design and understanding mechanisms of drug resistance. Modifying the Michaelis-Menten constant (K_m) based on enzyme concentration not only challenges the current dogma but also provides a new perspective on how enzymatic reactions can be manipulated to achieve desired therapeutic outcomes.

In particular, applying this theory in the development of enzyme inhibitors could lead to new strategies for blocking critical metabolic pathways in cancer cells or autoimmune diseases by manipulating enzyme concentrations to reduce the enzyme's affinity for its natural substrate. This aspect opens new avenues for further research and provides a solid foundation for the development of innovative therapeutic agents, thus underscoring the practical importance of this Hypothesis in the life sciences.

Our model is based on a physico-chemical mechanism that describes the electrostatic interactions between enzyme and substrate molecules. This mechanistic approach assumes that, as enzyme concentration increases, the forces opposing the substrate's trajectory also increase, leading to a decrease in enzyme affinity for the substrate and, consequently, an increase in K_m . In this paper, we explain this phenomenon by modeling molecular interactions using a geometric simplification, grounding the proposed theory in principles of molecular attraction and repulsion.

Semi-empirical approach

to test and calibrate this Hypothetical model, we used experimental data from the literature. The mechanistic model was adjusted based on experimental results that show variations in K_m as a function of enzyme concentration, offering a semi-empirical method for validating and refining the Hypothesis.

Empirical approach

the experimental data referenced in this study are derived from existing literature on cellulose hydrolysis. These findings have been generalized to apply to all Michaelis-Menten enzymes, clearly illustrating that K_m increases as enzyme concentration rises. This evidence supports the hypothesis that K_m is not a constant parameter, as traditionally accepted in the classical Michaelis-Menten model, but is instead influenced by experimental conditions, particularly enzyme concentration.

Philosophy

The philosophy of science guiding this work is based on the principle that mathematics dictates the relationships in physics, chemistry, and biology. In particular, considering

allosteric enzymes, it can be logically deduced, according to algebraic principles, that $k_{0.5}$ the substrate concentration at which enzymes become activated should increase with the total enzyme concentration. This observation, often intuitively recognized by students, is frequently contradicted in the specialized literature; however, our hypothesis is that this apparent contradiction arises not from the invalidity of the algebraic principle, but from excessive simplifications used in classical models (QSSA, RSA), which generate errors.

To explore this hypothesis, we developed a spherical model that redefines the concept of enzyme affinity for its substrate. Rather than being considered a simple enzyme-substrate interaction, affinity is described as the result of multiple interactions between all enzymes and all substrates at the nanoscale. This approach preserves the classical fundamental relationship between K_m and enzyme affinity, while providing a more realistic mechanism through which affinity manifests collectively and explaining deviations observed in traditional experiments.

Thus, by combining algebraic reasoning with theoretical and experimental validation, our model provides a bridge between the mathematical philosophy of enzyme relationships and experimental observations, demonstrating that the modulation of $k_{0.5}$ with enzyme concentration is a natural consequence of systemic interactions at the molecular level.

Methodology

This study is based on a contradiction between two fundamental principles in biochemistry

1. When enzyme concentration increases, the fractional change in reaction velocity (v) is smaller than the fractional change in V_{max} .^[7]
2. K_m is constant regardless of enzyme concentration.

We propose that the second principle is incorrect. This experimental design tested this hypothesis by using two enzymes for cellulose hydrolysis, and by analyzing how K_m varies with enzyme concentration.

The experimental design described in this study is based on established protocols from the literature, rather than being performed by the authors themselves. The enzymes used for cellulose hydrolysis, including Celluclast CCN and Novozym TN 188 (both provided as gifts by Novo Industries), have their activities and properties documented in previous research. Celluclast CCN is a brown liquid with a density of 1.1989 g/mL and a protein content of 128.6 mg/g, while Novozym TN 188 (β -glucosidase) has a density of 1.1884 g/mL with an activity of 520 units of β -glucosidase per gram of liquid.

The cellulases used in this study were prepared according to methods found in the literature. Specifically, the cellulase enzymes were derived from *Trichoderma reesei* MCG-77 strains via fermentation on lactose (LCC enzyme) and spruce sulfite pulp (D-25 enzyme). The procedures for the preparation and extraction of these enzymes have been previously documented. LCC enzyme exhibited an activity of 0.53 FPU and 0.43 units of β -glucosidase per milligram of protein, while D-25 enzyme showed 0.59 FPU and 0.30 units of β -glucosidase per milligram of protein, consistent with literature values.

The hydrolysis procedure followed well-established protocols. The enzyme-buffer mixture, prepared in 0.05 M

citrate buffer (pH 4.8) supplemented with 0.01% Thimerosal as a biocide, was heated to 50 °C in a 300 mL Erlenmeyer flask. Two grams of Sigmacell 50 or pretreated poplar wood were added to the enzyme solution, and the flasks were sealed with aluminum foil and incubated in a rotary shaker at 50 °C for up to 94 hours. At predetermined time intervals, samples were withdrawn and treated according to procedures previously described in the literature. The glucose and cellobiose concentrations in the supernatant were quantitatively analyzed by HPLC using an HPX 87 P column. The hydrolysis yield was calculated based on the concentration of the substrate initially added. The methods applied in this study adhere strictly to previously established and validated experimental procedures from existing literature, ensuring consistency with recognized techniques in the field of enzymatic hydrolysis of cellulose [8].

Theory: To mathematically demonstrate that K_m increases with enzyme concentration, we will express the initial velocity v as a fraction of V_{max} , such that $v = V_{max}/a$ as shown in the equation. 1

$$\text{Equation 1 } \frac{V_{MAX}}{a} = \frac{V_{MAX'}}{a} = a$$

If we substitute in equation 1 $\frac{V_{max}}{a}$ with $\frac{V_{MAX} \times S}{K_m + S}$, we obtain

$$\text{Equation 2 } \frac{K_{m1} + [S]}{[S]} = \frac{K_{m2} + [S]'}{[S]'} = a \Rightarrow \text{Equation 3 } K_{m1} = (a - 1)[S] \text{ and Equation 4 } K_{m2} = (a - 1)[S]'$$

Since when enzyme concentration increase the fractional change in v is less than the fractional change in V_{max} [7] and v varies hyperbolically with the substrate

V_{MAX} represents the maximum velocity before the enzyme concentration increase.

V_{MAX}' represents the maximum velocity after the enzyme concentration increase.

$[S]$ Represents the substrate concentration at V_{MAX}/a ,

$[S]'$ represents the substrate concentration at V_{MAX}'/a ,

a is a number greater than 1, belonging to the set of real numbers ($\mathbb{R}$)

Concentration, it follows that $[s] < [s]'$ As long as the enzymes follow the kinetics in Figure

1. $\Rightarrow K_m$ increase with enzyme concentration. (Equation 5 $K_{m2}/K_{m1} = S'/S$) To obtain the mathematical relationship between K_m and E , we will perform the following mathematical modeling:

$$\text{Equation 6 } \frac{dS}{dt} = -\frac{V_{max} \times S}{K_m + S} \Rightarrow \text{Equation 7 } \frac{K_m + S}{S} dS = -V_{max} \times dt \Rightarrow \text{Equation 8 } \left(1 + \frac{K_m}{S}\right) dS = -V_{max} dt$$

If we integrate on both sides of the equation, we obtain

$$\text{Equation 9 } \int_{S_0}^S \left(1 + \frac{K_m}{S}\right) dS = - \int_{t_0}^t V_{max} dt \Rightarrow$$

$$\text{Equation 10 } S - S_0 + K_m (\ln S - \ln S_0) = -V_{max} (t - t_0) \Rightarrow \text{If we replace } V_{max} \text{ with } K_{cat} \times$$

$$\text{Equation 11 } \frac{S_0 - S + K_m \times \ln \frac{S_0}{S}}{K_{cat}} = E(t - t_0)$$

K_m is treated as a constant because, during integration, the variation of substrate over time is taken into account while considering the enzyme concentration to be constant. Thus, equation 11 reflects the relationship between K_m and enzyme concentration.

I have verified Equation 11 of this article using various enzymatic incubation data from the literature, and for the same enzyme concentration, K_m remains constant, while it increases with higher enzyme concentrations. Therefore, I invite the reader to search for articles and test Equation 11 with any data they choose. Any dataset that does not yield results in accordance with this Hypothesis would invalidate the Hypothesis.

Thus, having this equation, which yields results very close to the Lineweaver-Burk linearization, provides the

possibility to measure K_m without the need to satisfy the QSSA because S is obtained from $S_0 - P$.

For this mathematical model, I would like to propose this Hypothesis

Let's consider, for simplicity, an ideal case: a circle containing a solution of enzyme and substrate, simplifying from a sphere to a circle. On each substrate, a force acts that is equal to the sum of the attraction forces between each enzyme and its respective substrate, plus the sum of the repulsion forces between each substrate and its respective substrate. Since these are evenly distributed on the surface of a circle, any deviation from the center of the circle in the substrate's position will result in an increase in the repulsion and attraction forces acting on the substrate from the opposite side of the deviation and a decrease in the forces from the same side as the deviation. Since the substrate concentration is much higher than the enzyme concentration, it means that the repulsion force dominates. Therefore, as the substrate deviates more from the center of the circle, the electrostatic force that drives it toward the periphery of the circle increases, causing the substrate to collide with an enzyme located closer to the periphery than the substrate. From this, we can conclude that with an increase in enzyme concentration, the forces opposing the substrate's trajectory increase, thus decreasing the enzyme's affinity for the substrate.

Thermal agitation does not negate this mechanism because the electrostatic forces involved in this phenomenon will orient the enzyme's face with active sites toward the center of the circle due to the electrostatic attraction forces acting

on them from the opposite side of the deviation. Thus, the collisions between substrates and the enzymes positioned toward the center of the circle will not lead to the formation of ES complexes.

Semi-empirical and empirical validation

- When enzyme concentration increases, the fractional change in v is less than the fractional change in $V_{\max}$ [7] as we can see in figure 1! This statement is consistent with the Hypothesis as it confirms that the activity of each enzyme decreases with an increase in enzyme concentration for the same substrate concentration.
- The disproportion between the fractional change in v and the fractional change in $V_{\max}$ decreases with an

increase in substrate concentration [7] as we can see in figure 1! This is another statement consistent with the theory as it confirms the decreasing relevance of the attraction forces between each enzyme and substrate, compared to the repulsion forces between each substrate and its reference substrate with substrate concentration.

- These two experimental pieces of evidence demonstrate the Hypothesis through inductive and abductive reasoning: the first proves that the phenomenon actually occurs, and the second that the explanation is the proposed mechanism.

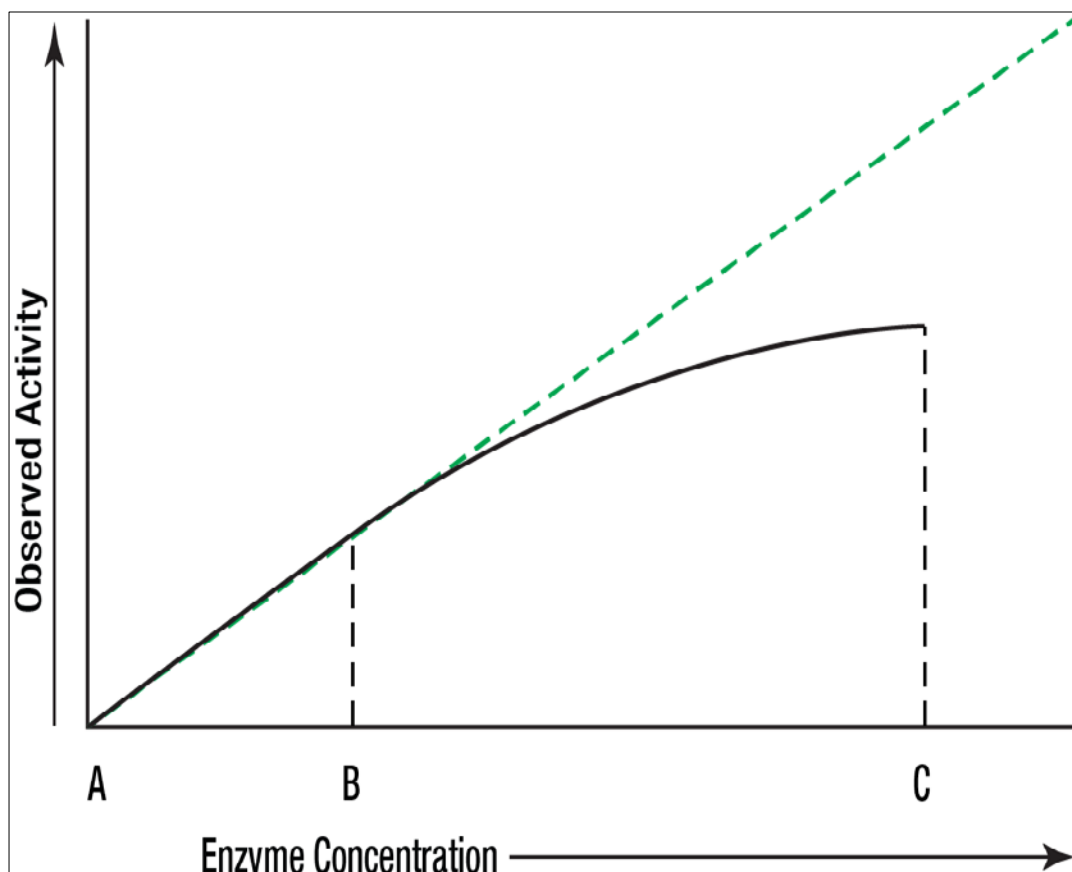


Fig 1: The variation of the reaction rate with enzyme concentration valid for all enzymes with Michaelis-Menten kinetics [7]

This graph is semi-empirical, obtained by generalizing the results of studies on the variation of velocity with enzyme concentration at a constant substrate value.

- This figure illustrates how the reaction velocity (v) changes as enzyme concentration increases, at a constant substrate concentration.
- The graph shows that the fractional increase in reaction velocity (v) is smaller than the fractional increase in $V_{\max}$, indicating that as enzyme concentration rises, the enzyme's activity becomes less efficient relative to $V_{\max}$.

- The disproportion between these fractions decreases as substrate concentration increases

Any dataset from the literature that does not yield results consistent with Figure 1 also negates the hypothesis, this being the second method of falsification. I do not use new experiments for falsification, because the two existing experiments are the most robust ways to validate such a hypothesis; in fact, this is precisely how experiments validating such a hypothesis. Ought to appear

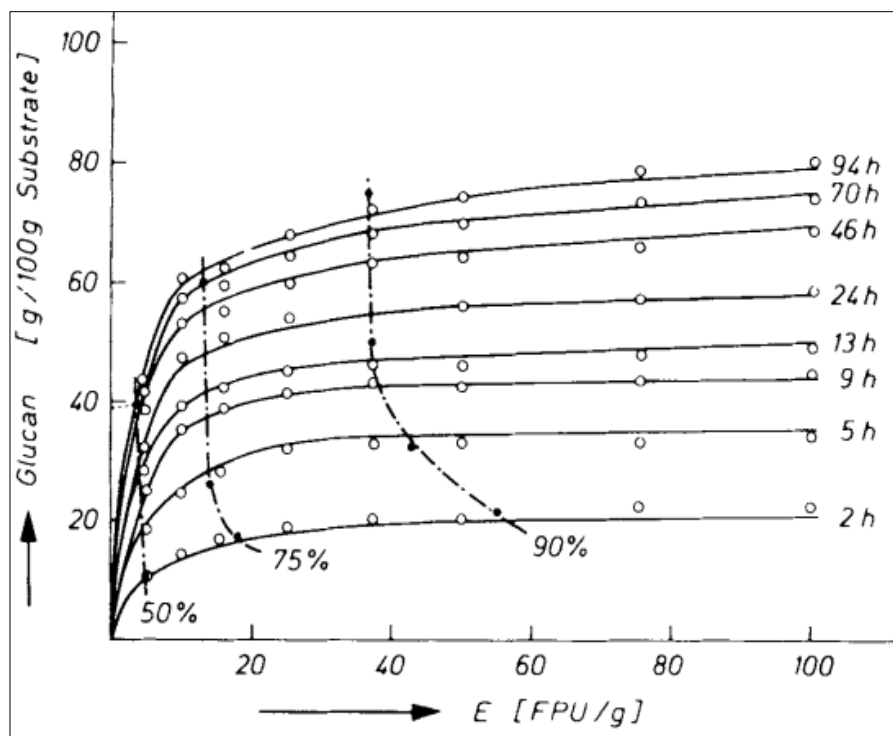


Fig 2: the effect of enzyme concentration on the Michaelis-Menten constant obtained from the experiment mentioned in methodology ^[8]

This figure demonstrates how K_m varies with enzyme concentration based on experimental data. As enzyme concentration increases, K_m also increases, signifying a reduction in enzyme affinity for the substrate. The graph provides evidence for the Hypothesis that K_m is not constant, but instead is influenced by enzyme concentration, contradicting the classical Michaelis-Menten model.

If we try to take into account the conditions under which the experiments were conducted that resulted in the graph in Figure 1 and 2, and calculate the velocity when the enzyme concentration tends to infinity with the substrate concentration constant, considering two cases for verification: the first where K_m remains constant regardless of the enzyme concentration, and the second where K_m also tends to infinity with the enzyme concentration.

$$1) \frac{v_{max}[s]}{K_m + [s]} = \infty \quad 2) \frac{v_{max}[s]}{K_m + [s]} = L'Hopital \rightarrow [s]$$

As can be seen, only when $K_m \rightarrow \infty$ is the result consistent with reality, with all the substrate reacting instantly. Currently, the accepted hypothesis for this graph is that enzymes become unsaturated due to a decrease in substrate competition for the enzyme, but this cannot be true for the following reasons

1. The linear section of the graph in Figure 1 is a linearization, but it still has a slight curvature.
2. The linearization of segment AB in Figure 1 is equivalent to the linearization of the plateau phase, meaning that any point in this section belongs to the plateau phase of the Michaelis-Menten graph. Therefore, we cannot speak of a low ratio between substrate concentration and enzyme concentration, as the points at the beginning of section BC are in the immediate vicinity of the plateau phase.

3. The decrease in substrate competitiveness for the enzyme begins at the start of the graph in Figure 1 if segment AB is not linearized, which means that the Decrease in competitiveness occurs due to a decrease in affinity.
4. The proposed hypothesis is more plausible

The formulation of the error that led the empirical data to indicate That K_m is constant regardless of enzyme concentration.

According to this mechanism, the variation of K_m with enzyme concentration is challenging to identify when the $[S] \gg [E]$ condition holds. This is because the factors influencing K_m become less apparent at high substrate concentrations. The small increase in reaction rate in the plateau phase, often ignored when determining V_{max} and K_m , reflects these factors. Linearizing the plateau phase from a high substrate concentration (as required by QSSA) mask the variation of K_m . While this approach simplifies data analysis, it neglects the fact that the slope of the line is steeper at higher enzyme concentrations. This is because the fractional change in v is less than the fractional change in V_{max} , and the disproportion between them decrease with substrate concentration. If the substrate concentration is too high, the variation of K_m with enzyme concentration may be underestimated. This is because any small increase in v will lead to a more significant decrease in K_m at high substrate concentrations. By addressing this error in data interpretation, it becomes possible to observe an increase in K_m with enzyme concentration. Figure3 Thus, all values indicating that K_m is constant regardless of enzyme concentration, when using this simplification, are actually evidence that K_m increases with enzyme concentration this being the only method by which K_m remains constant regardless of enzyme concentration.

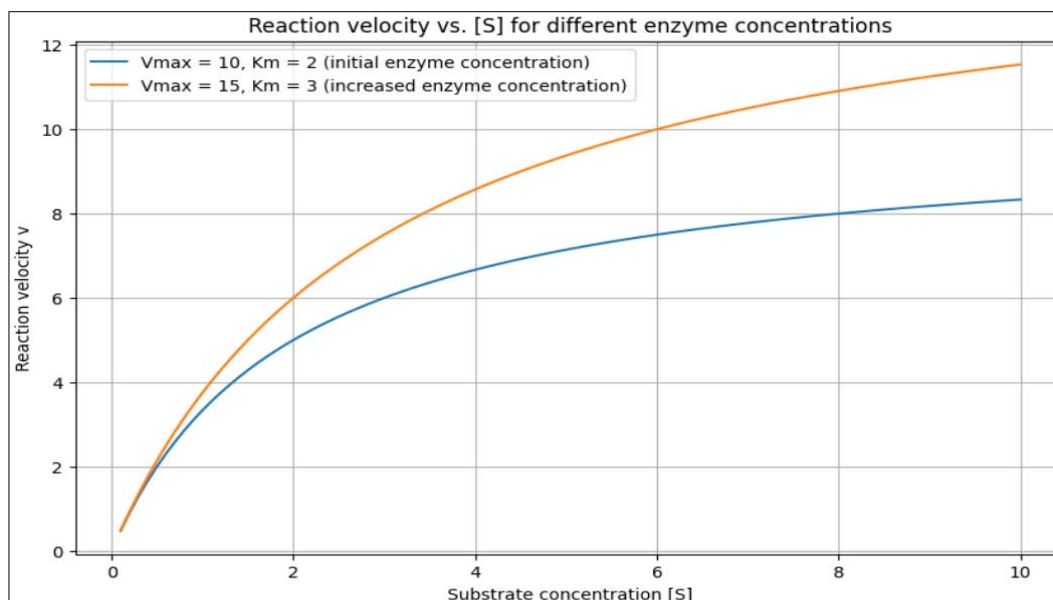


Fig 3: The variation of K_m with enzyme concentration

This image illustrates two hypothetical graphs for different enzyme concentrations so that the results are consistent with the graph in Figure 1

a) It is observed that for the graphs to be consistent with the graph in Figure 1, it is necessary for K_m to increase with enzyme concentration since the slope of the hyperbola is given by K_m .

b) Linearizing the plateau phase leads to ignoring the difference between the fraction of increase in v and the fraction of increase in V_{max} with enzyme concentration.

The mechanism described in this Hypothesis contradicts the premises of the demonstration for the old theory, showing that k_1 , k_2 , and k_3 also vary with enzyme concentration.

Experimental confirmation based on pH

After reviewing the data on the pH at which K_m has minimum values, I was able to observe that for enzymes with substrates that have ionizable functional groups, the pH favors the ionization of the substrate rather than the ionization of the enzyme. When the pH favors the ionization of a substrate with functional groups that have charges opposite to those of the enzyme, this enhances substrate ionization at the expense of enzyme ionization. According to this Hypothesis, the optimal pH for an enzyme-substrate interaction is one where the substrate becomes ionized more readily than the enzyme. This contrasts with classical models, which suggest that maximum affinity is achieved when the enzyme is fully ionized. In my Hypothesis, maximum affinity is reached when the substrate is ionized, thereby optimizing the attraction between enzyme and substrate through favorable electrostatic interactions. The first two references in the supplementary file confirm this phenomenon, and additional data can be found in the literature addressing this topic.⁴⁰

QSSA and RSA

In enzyme kinetics, two fundamental approximations are often used: the quasi-steady state approximation (QSSA) and the reactant stationary approximation (RSA). Although both approximations are widely employed, it is important to understand the distinction between them, as their applicability and assumptions differ.

In a study by Hanson and Schnell (2008)^[9], and later by Schnell (2013)^[10], it was demonstrated that QSSA does not necessarily imply RSA, and that they are distinct approximations. This difference is crucial for the interpretation of kinetic data and is consistent with the theory proposed in this paper.

- QSSA assumes that the concentration of the enzyme-substrate complex remains constant over time after a brief initial period. This assumption allows simplification of the kinetic equations and is commonly used to derive the Michaelis-Menten equation. The approximation is valid when the substrate concentration $[S]$ is much larger than the enzyme concentration $[E]$, ensuring that $[ES]$ reaches a steady state quickly and remains nearly constant during the reaction.

Reactant Stationary Approximation (RSA)

RSA, on the other hand, is applied under different conditions, where the concentrations of both the substrate and the enzyme are considered to be nearly constant throughout the reaction. RSA is valid when the substrate concentration is not only large but also changes very slowly over time, ensuring that the concentrations of the reactants do not vary significantly during the reaction course.

QSSA vs RSA

The key difference between these two approximations lies in the assumption about the reactant concentrations. While QSSA focuses on the stability of the enzyme-substrate complex, RSA assumes that both the substrate and enzyme concentrations remain nearly unchanged. As a result, RSA is typically applied in cases where the substrate concentration is even higher than what is required for QSSA.

In the context of this hypothesis, we can conclude that:

- When $k_1[E][S] \approx k_2[ES]$ RSA is valid and because $[ES]$ is constant, as assumed by QSSA.
- However, when only QSSA is applied, we assume $k_1[E][S] > k_2[ES]$, meaning that the concentration of $[ES]$ is in steady state.

For RSA to hold, the substrate concentration must increase beyond what is required for QSSA, implying that variations

in the rate constants k_1 and K_2 may occur. This is consistent with the proposed Hypothesis, which posits that substrate concentration affect K_1 and K_2 .

Biotechnology

In a study examining the overexpression of PPAR α in tumor cells, it was observed that this led to a paradoxical inhibition of cell proliferation.^[11] Peroxisome proliferator-activated receptor alpha (PPAR α) is a nuclear receptor that regulates the expression of genes involved in lipid metabolism. When activated by ligands, such as fatty acids, PPAR α binds to PPAR response elements (PPREs) in the DNA and promotes the transcription of genes responsible for fatty acid oxidation and other metabolic processes.

In the context of the proposed hypothesis, the overexpression of enzymes induced by PPAR α would lead to an increase in enzyme concentration. According to our findings, this would cause an increase in the Michaelis-Menten constant (K_m) for allosteric enzymes. As K_m increases, a much higher substrate concentration is required for the enzyme to maintain its catalytic efficiency. This suggests that metabolic activation would only occur at very high substrate concentrations, as the enzyme's affinity for its substrate decreases with increasing K_m .

More examples of strong agonists that have led to apoptosis, which can be explained by the increase in K_m for allosteric enzymes with enzyme concentration, can be found in the supplementary file.

I have not found in the literature, after a careful search, any strong agonists that lead to proliferation; proliferation occurs with weak or moderate agonization.

Independent validation

The validity of the Hypothesis proposing that the Michaelis-Menten constant (K_m) varies with enzyme concentration has been independently supported by an external experimental study (Pineda, 2024)^[12]. In this work, the authors investigated lactose hydrolysis using lactase across a wide range of enzyme concentrations and explicitly referred to Yassin (2024)^[13], reporting experimental behavior consistent with the Hypothetical model presented in this article. As stated in their analysis:

"The study by Yassin^[56] proposes an alternative theory, demonstrating that K_m can vary with enzyme concentration. The experimental work presented in the article shows that, as $[E]$ increases, the specific activity decreases, meaning that the increase in reaction rate is not proportional to the increase in V_{max} . This implies an apparent decrease in affinity, even when the substrate concentration is constant." This observation provides independent experimental confirmation that K_m does not remain constant when enzyme concentration changes. At high $[E]$, the specific activity decreases and an apparent loss of affinity is observed a kinetic behavior that aligns precisely with the predictions of the present Hypothesis.^[12]

Implications in Autoimmune Diabetes: The Role of Allosteric Enzymes

The hypothesis that the Michaelis-Menten constant (K_m) (or $K_{0.5}$ for allosteric enzymes) increases with enzyme concentration offers a novel and highly plausible explanation for the onset of autoimmune diabetes mellitus (Type 1). Diabetogenic events are often preceded by a

period of sustained hyperglycemia, a key factor in this proposed mechanism.

Our model suggests the following sequence involving an allosteric enzyme that regulates a critical metabolic pathway in the beta cells

- The persistent increase in blood glucose concentration (the primary metabolic substrate) drives the activation or upregulation (increased synthesis) of a key allosteric enzyme responsible for glucose processing in the beta cells.
- According to our hypothesis, this increase in the concentration of the allosteric enzyme ($[E]$) leads directly to an increase in its $K_{0.5}$ (the substrate concentration at which the enzyme is half-activated). The philosophical basis of this work supports the deduction that $K_{0.5}$ should increase with total enzyme concentration.
- This significantly elevated $K_{0.5}$ drastically reduces the enzyme's affinity/activation for its substrate. Consequently, despite the presence of high substrate concentration ($[S]$) (hyperglycemia), the ratio $K_{0.5}/[S]$ becomes unfavorable.
- The outcome is a critical slowdown or effective blockage of the metabolic pathway due to reduced affinity (high $K_{m0.5}$), leading to profound cellular stress.
- This metabolic distress, occurring despite the abundance of substrate, can trigger apoptosis (programmed cell death) of the beta cells. Beta cell apoptosis is a recognized event that can initiate the autoimmune response and the subsequent formation of diabetes-specific autoantibodies.

This perspective highlights the practical importance of the Hypothesis in pharmacology. It aligns with the idea that by increasing the concentration of an allosteric enzyme (e.g., via strong agonism), the physiological substrate concentration may no longer exceed the new, higher K_m or $K_{0.5}$, resulting in metabolic pathway blockage. This mechanism provides a new avenue or research into therapeutic agents targeting cancer and autoimmune diseases.

Results

1. k_m increase with enzyme concentration
2. considering the plateau phase as linear will lead to significant errors in the calculation of K_m
3. as the concentration of the enzyme increases, the force with which the substrate moves towards the enzyme decreases
4. It is experimentally confirmed that the fact that K_m is constant regardless of enzyme concentration is an error of the QSSA approximation.
5. All studies that assume that K_m is constant regardless of enzyme concentration should be reconsidered, as this assumption is likely an artifact of the QSSA approximation

Discussions

This article suggests that literature and data based on the assumption that K_m is constant regardless of enzyme concentration should be re-evaluated and viewed within the context of this mechanism. This hypothesis can be used for the further development of a new class of drugs by

increasing the concentration of the allosteric enzyme (for example, through strong agonization of a receptor) until the concentration of the physiological substrate no longer exceeds K_m , resulting in metabolic pathway blockage. These drugs could be useful in cases of cancer or autoimmune diseases, for example.

Limitations

The limitation of this hypothesis is that it is currently formulated for isolated cases, meaning systems where enzyme-substrate interactions can be approximated by simplified geometric models and where specific electrostatic effects dominate. We recognize that this model does not yet account for the full complexity of *in vivo* systems, such as non-isolated metabolic pathways, crowding, or rapid turnover, where multiple competing factors influence affinity. Further experimental studies are recommended to categorize metabolic pathways based on charge sign changes and applicability of this mechanism in non-isolated, *in vivo* settings.

Conclusion

This study challenges the traditional view of K_m as a fixed constant and demonstrates that it can vary with enzyme concentration. These findings have important implications for how enzyme kinetics is understood and suggest that future studies must account for the influence of enzyme concentration when interpreting kinetic data.

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Author Yassin Fadel is an inventor on a pending patent application (Application No. A/00520) related to the subject matter of this manuscript.

Consent

- **Clinical trial number:** not applicable.
- **Ethics, Consent to Participate, and Consent to Publish declarations:** Not applicable

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