Influence of enzyme concentration on the Michaelis-Menten constant: A new theory for izolated cases

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ABSTRACT:

This study proposes a novel theory that challenges the traditional understanding of the Michaelis-Menten constant (Km) as a fixed value for a given enzyme-substrate pair. We explore the possibility that Km can be modulated by enzyme concentration. By analyzing the relationship between initial reaction velocity and substrate concentration at different enzyme concentrations, we observed a change in the apparent affinity constant (designated as Km1 and Km2). This observation contradicts established theory, leading us to propose a new model to explain this variation.

INTRODUCTION:

In enzyme kinetics, it has long been accepted that the Michaelis-Menten constant (Km) is independent of enzyme concentration. This assumption stems from the idea that under standard conditions, Km 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 Km remains constant, irrespective of changes in enzyme

22 concentration, representing an intrinsic property of the enzyme-substrate relationship.

23 (1)

24 In 1934, Lineweaver and Burk developed a graphical method for determining Km and

25 Vmax, providing researchers with a valuable tool to analyze kinetic data. However, this

26 method has faced criticism, one of the main reasons being that it often shows Km

27 increasing with enzyme concentration—challenging the assumption that Km is a constant.

28 (2)

29 Briggs and Haldane introduced the quasi-steady state approximation (QSSA), which

30 assumes the concentration of the enzyme-substrate complex remains relatively stable

31 during the reaction. This approximation provided the foundation for deriving the

32 Michaelis-Menten equation, which has become a cornerstone of enzymatic kinetics. (3)

33 According to the allosteric hypothesis (4), the modulation of enzyme affinity would

34 require a sufficient number of allosteric modulators. If these modulators are insufficient,

35 it would necessitate that only a portion of the enzymes are active while others are

36 inactive, which does not align with observed reality. The proposed theory offers a more

37 plausible explanation, as the decrease in affinity for the modulator is linked to an increase

38 in enzyme concentration.

39 situations as well.

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

44 Enzymatic crowding (6) may explain variations in Km only at extremely high enzyme
45 concentrations. However, it does not account for the constant increase in Km, as observed

46 in Figure 1 of this article. Therefore, this mechanism cannot be utilized to explain the
47 experimental results. The same explanation applies to the diffusion model.

48 Although the model of microenvironmental change with enzyme concentration certainly
49 influences the value of Km, if we compare the changes in Km obtained through enzyme
50 concentration with the effects obtained by other methods, it becomes clear that the
51 microenvironmental change with enzyme concentration model cannot numerically justify
52 such large variations.

53

54

55 This paper introduces a novel theory that redefines the traditional understanding
56 of enzyme-substrate interactions by focusing on the role of enzyme concentration in
57 determining affinity. Unlike the classical Michaelis-Menten model, which considers
58 enzyme concentration as irrelevant to affinity, this theory posits that enzyme
59 concentration modulates the attraction between the enzyme and its substrate. This new
60 perspective challenges the long-held assumption that affinity is constant under varying
61 conditions and opens up new possibilities for studying enzyme kinetics, with important
62 implications for biochemical research and therapeutic development.

63 The relevance of the proposed theory extends beyond merely reconsidering a
64 fundamental principle in enzyme kinetics. This theory could have profound implications in
65 various fields of biochemistry and pharmacology, particularly in the context of drug design
66 and understanding mechanisms of drug resistance. Modifying the Michaelis-Menten
67 constant (Km) based on enzyme concentration not only challenges the current dogma but
68 also provides a new perspective on how enzymatic reactions can be manipulated to achieve
69 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 theory 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 Km. 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 theoretical model, we used experimental data from the literature . The mechanistic model was adjusted based on experimental results that show variations in Km as a function of enzyme concentration, offering a semi-empirical method for validating and refining the theory.

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 Km increases as enzyme concentration rises. This

evidence supports the hypothesis that Km is not a constant parameter, as traditionally accepted in the classical Michaelis-Menten model, but is instead influenced by experimental conditions, particularly enzyme concentration.

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 Vmax. (7)
2. Km 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 Km 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.

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

120 The hydrolysis procedure followed well-established protocols. The enzyme-buffer mixture,
121 prepared in 0.05 M citrate buffer (pH 4.8) supplemented with 0.01% Thimerosal as a
122 biocide, was heated to 50 °C in a 300 mL Erlenmeyer flask. Two grams of Sigmacell 50 or
123 pretreated poplar wood were added to the enzyme solution, and the flasks were sealed with
124 aluminum foil and incubated in a rotary shaker at 50 °C for up to 94 hours. At
125 predetermined time intervals, samples were withdrawn and treated according to procedures
126 previously described in the literature. The glucose and cellobiose concentrations in the
127 supernatant were quantitatively analyzed by HPLC using an HPX 87 P column. The
128 hydrolysis yield was calculated based on the concentration of the substrate initially added.

129 The methods applied in this study adhere strictly to previously established and validated
130 experimental procedures from existing literature, ensuring consistency with recognized
131 techniques in the field of enzymatic hydrolysis of cellulose.

132 (8)

133

134 ## THEORY:

135

136 $$\frac{VMAX}{\frac{VMAX}{a}} = \frac{VMAX'}{\frac{VMAX'}{a}} = a \Rightarrow \frac{Km + [S]}{[S]} = \frac{KM + [S]'}{[S]'} = a \Rightarrow Km1 = (a-1)[S] \text{ and } Km2 = (a-1)[S]'$$

> VMAX represents the maximum velocity before the enzyme concentration increase.
>
> VMAX’ represents the maximum velocity after the enzyme concentration increase.
>
> [S] represents the substrate concentration at VMAX/a,
>
> ’[S]’ represents the substrate concentration at VMAX’/a,
>
> a is a number greater than 1, belonging to the set of real numbers (R)

137 Since when enzyme concentration increse the fractional change in v is less than the
138 fractional change in vmax[7] , and v varies hyperbolically with the substrate
139 concentration, it follows that [s]<[s]'. ⇨Km increase with enzyme
140 concentration.(km2/km1=S'/S)
141 For this mathematical model, I would like to propose this theory.

142

143 Let's consider, for simplicity, an ideal case: a circle containing a solution of
144 enzyme and substrate, simplifying from a sphere to a circle. On each substrate, a force
145 acts that is equal to the sum of the attraction forces between each enzyme and its
146 respective substrate, plus the sum of the repulsion forces between each substrate and its
147 respective substrate. Since these are evenly distributed on the surface of a circle, any
148 deviation from the center of the circle in the substrate's position will result in an increase
149 in the repulsion and attraction forces acting on the substrate from the opposite side of
150 the deviation and a decrease in the forces from the same side as the deviation. Since the

151 substrate concentration is much higher than the enzyme concentration, it means that the
152 repulsion force dominates. Therefore, as the substrate deviates more from the center of
153 the circle, the electrostatic force that drives it toward the periphery of the circle
154 increases, causing the substrate to collide with an enzyme located closer to the periphery
155 than the substrate. From this, we can conclude that with an increase in enzyme
156 concentration, the forces opposing the substrate's trajectory increase, thus decreasing
157 the enzyme's affinity for the substrate.
158 Thermal agitation does not negate this mechanism because the electrostatic forces
159 involved in this phenomenon will orient the enzyme's face with active sites toward the
160 center of the circle due to the electrostatic attraction forces acting on them from the
161 opposite side of the deviation. Thus, the collisions between substrates and the enzymes
162 positioned toward the center of the circle will not lead to the formation of ES complexes.

163 Experimental biochemistry data supporting the theory and analysis of existing literature

164 1. When enzyme concentration increases, the fractional change in v is less than the
165 fractional change in Vmax [7] as we can see in figure 1! This statement is consistent with
166 the theory as it confirms that the activity of each enzyme decreases with an increase in
167 enzyme concentration for the same substrate concentration.
168 2. The disproportion between the fractional change in v and the fractional change
169 in Vmax decreases with an increase in substrate concentration[7] as we can see in figure
170 1! This is another statement consistent with the theory as it confirms the decreasing
171 relevance of the attraction forces between each enzyme and substrate, compared to the

172 repulsion forces between each substrate and its reference substrate with substrate

173 concentration.

174 These two experimental pieces of evidence demonstrate the theory through inductive

175 reasoning: the first proves that the phenomenon actually occurs, and the second that the

176 explanation is the proposed theory.

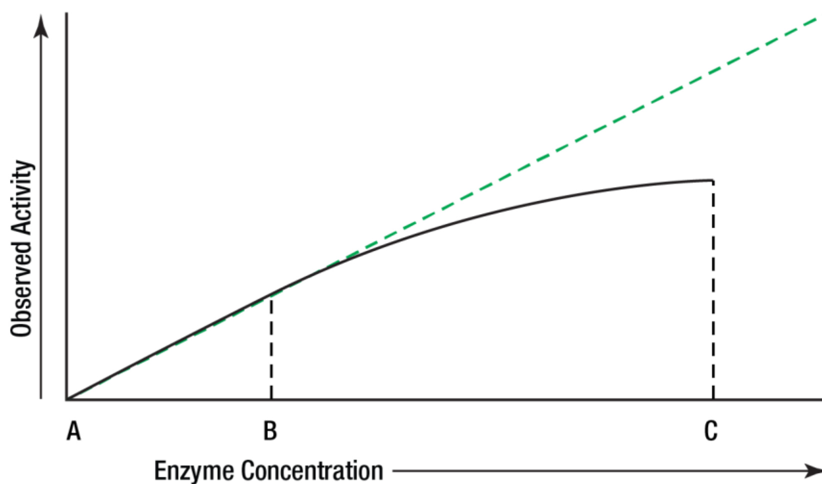

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Figure1 The variation of the reaction rate with enzyme concentration valid for all enzymes with michaelis menten kinetics[7]

a)This figure illustrates how the reaction velocity (v) changes as enzyme concentration increases, at a constant substrate concentration.

b)The graph shows that the fractional increase in reaction velocity (v) is smaller than the fractional increase in Vmax, indicating that as enzyme concentration rises, the enzyme's activity becomes less efficient relative to Vmax.

c)The disproportion between these fractions decreases as substrate concentration increases.

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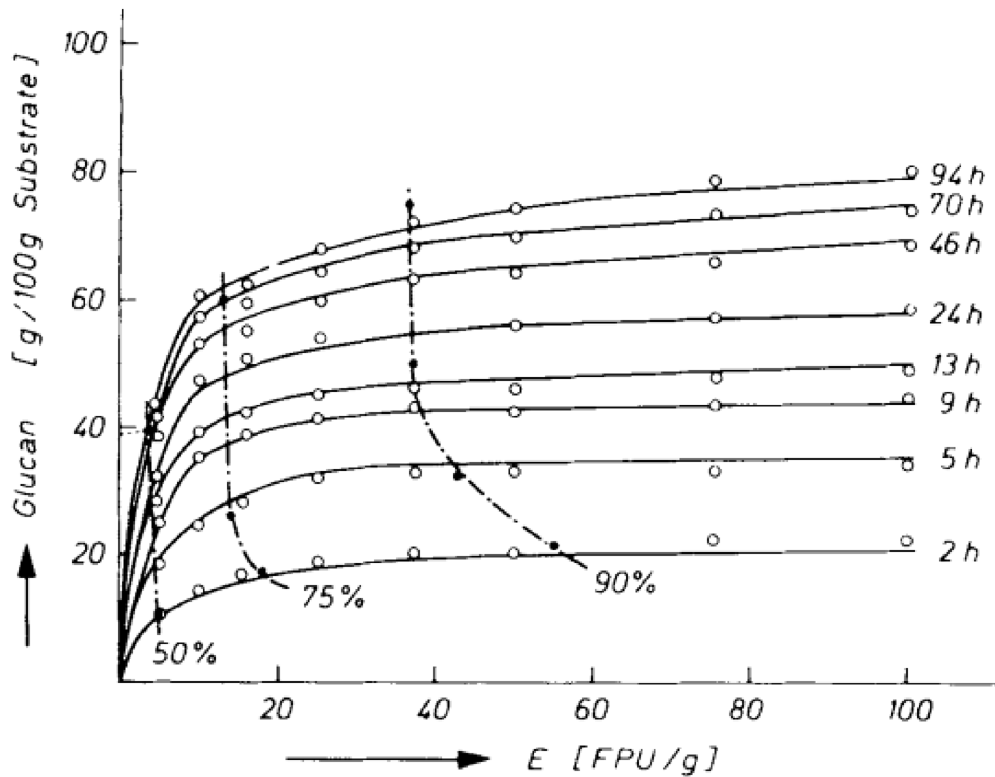

Figure 3. Effect of enzyme dosage on the extent of hydrolysis at different reaction times. The dashed lines give the enzyme dosage leading to 50, 75, and 90% maximum hydrolysis which could theoretically be reached at the indicated times. The solid lines are those calculated with the individual constants given in Table III using eq. (6). The ordinate is $[100(Y/C_0)]$.

Figure 2 The effect of enzyme concentration on the Michaelis-Menten constant obtained from the experiment mentioned in methodology[8]."

This figure demonstrates how Km varies with enzyme concentration based on experimental data. As enzyme concentration increases, Km also increases, signifying a reduction in enzyme affinity for the substrate. The graph provides evidence for the theory that Km 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,

189 considering two cases for verification: the first where Km remains constant regardless of

190 the enzyme concentration, and the second where Km also tends to infinity with the

191 enzyme concentration.

192 1)$\lim_{vmax\to\infty}\frac{vmax\,[s]}{km+[s]}$=∞ 2) $\lim_{\substack{vmax\to\infty\\ km\to\infty}}\frac{vmax\,[s]}{km+[s]}\xrightarrow{L'Hopital}[s]$

193 As can be seen, only when Km→ ∞is the result consistent with reality, with all

194 the substrate reacting instantly.

195 3. According to this theory, the variation of Km with enzyme concentration is

196 challenging to identify when the [S]>>[E] condition holds. This is because the factors

197 influencing Km become less apparent at high substrate concentrations. The small increase

198 in reaction rate in the plateau phase, often ignored when determining Vmax and Km,

199 reflects these factors. Linearizing the plateau phase from a high substrate concentration

200 mask the variation of Km. While this approach simplifies data analysis, it neglects the fact

201 that the slope of the line is steeper at higher enzyme concentrations. This is because the

202 fractional change in v is less than the fractional change in Vmax, and the disproportion

203 between them decrease with substrate concentration . If the substrate concentration is too

204 high, the variation of Km with enzyme concentration may be underestimated. This is

205 because any small increase in v will lead to a more significant decrease in Km at high

206 substrate concentrations. By addressing this error in data interpretation, it becomes

207 possible to observe an increase in Km with enzyme concentration. figure3

208

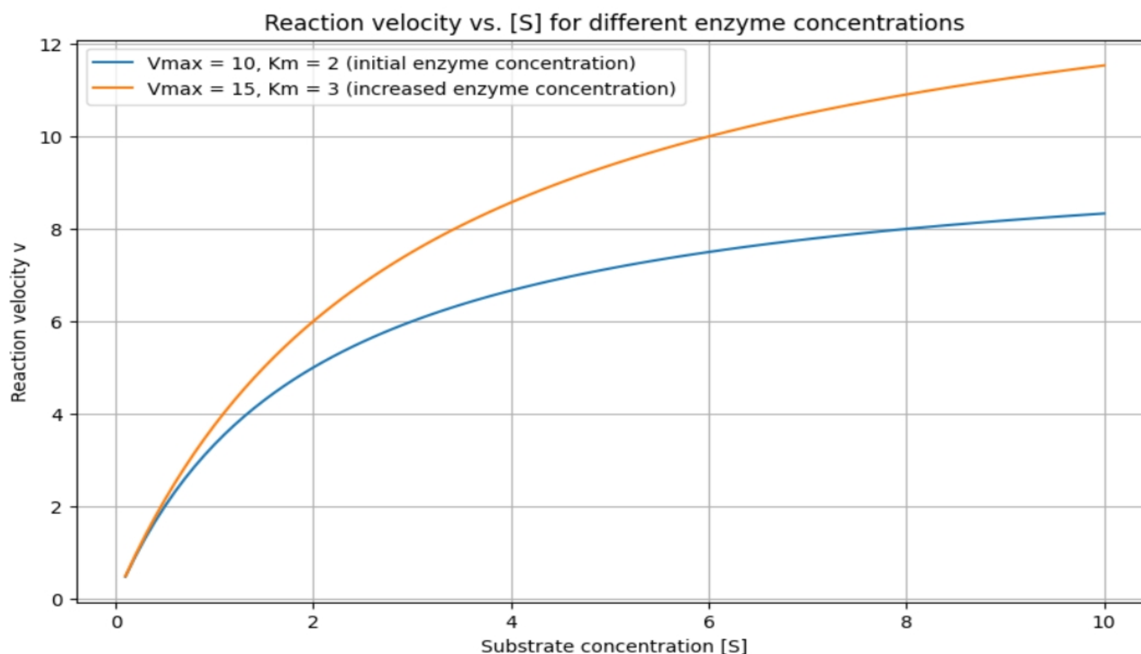

Figure 3 The variation of Km with enzyme concentration:

209 .This image illustrates two hypothetical graphs for different enzyme concentrations so that the results are consistent with

210 the graph in Figure 1.

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

212 b) Linearizing the plateau phase leads to ignoring the difference between the fraction of increase in v and the fraction of

213 increase in Vmax with enzyme concentration.

214

215 The mechanism described in this theory contradicts the premises of the

216 demonstration for the old theory, showing that k1, k2, and k3 also vary with enzyme

217 concentration.

218 After reviewing the data on the pH at which Km has minimum values, I was able
219 to observe that for enzymes with substrates that have ionizable functional groups, the pH
220 favors the ionization of the substrate rather than the ionization of the enzyme. This
221 phenomenon is consistent with the proposed theory alll these values being direct

222 experimental validations of the theory and contradiction fir the old theory, according to
223 which the optimal pH should favor the ionization of the enzyme.

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

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

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

238 Reactant Stationary Approximation (RSA):

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

244 QSSA vs RSA:

245 The key difference between these two approximations lies in the assumption about the
246 reactant concentrations. While QSSA focuses on the stability of the enzyme-substrate
247 complex, RSA assumes that both the substrate and enzyme concentrations remain nearly
248 unchanged. As a result, RSA is typically applied in cases where the substrate concentration
249 is even higher than what is required for QSSA.
250 In the context of this theory, we can conclude that:

251 - When $k1[E][S] \approx k2[ES]$, RSA is valid and because [ES] is constant, as assumed by
252 QSSA.
253 - However, when only QSSA is applied, we assume $k1[E][S] > k2[ES]$, meaning that
254 the concentration of [ES] is in steady state.

255 For RSA to hold, the substrate concentration must increase beyond what is required for
256 QSSA, implying that variations in the rate constants k1 and k2 may occur. This is
257 consistent with the proposed theory, which posits that substrate concentration affect K1
258 and K2
259 In a study examining the overexpression of PPARα in tumor cells, it was observed that this
260 led to a paradoxical inhibition of cell proliferation. (11), Peroxisome proliferator-activated
261 receptor alpha (PPARα) is a nuclear receptor that regulates the expression of genes
262 involved in lipid metabolism. When activated by ligands, such as fatty acids, PPARα binds
263 to PPAR response elements (PPREs) in the DNA and promotes the transcription of genes
264 responsible for fatty acid oxidation and other metabolic processes.

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

272

273

274 ## Results:

275 1)km increse with enzyme concentration

276 2) considering the plateau phase as linear will lead to significant errors in the calculation
277 of Km

278 3) as the concentration of the enzyme increases, the force with which the substrate
279 moves towards the enzyme decreases

280

281

282 ## Discussions:

283 This theory can be used for the further development of a new class of drugs by
284 increasing the concentration of the allosteric enzyme (for example, through strong
285 agonization of a receptor) until the concentration of the physiological substrate no longer

286 exceeds Km, resulting in metabolic pathway blockage. These drugs could be useful in
287 cases of cancer or autoimmune diseases, for example.
288

289 The limitations of this theory are that due to the numerous factors to be
290 considered and the fact that the way in which Km varies with the enzyme concentration
291 depends on the specific characteristics of each enzyme-substrate pair, it is unrealistic to
292 believe that we can create a mathematical model for predictions, and it will require
293 numerous practical experiments.

294 Another limitation of this theory is that it is studied for isolated studies, but
295 starting from the principles of this mechanism, non-isolated cases (as they occur in vivo)
296 can also be studied by categorizing metabolic pathways into those where the substrate
297 changes its charge sign and those where this does not happen, It is recommended that these
298 studies be conducted experimentally, given the complexity of the phenomena.

299 CONCLUSION:

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

304

305 Funding and competing interest:
306 This article did not require any financial support and no financial interest so far.

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