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Where Initial Rates are Directly Proportional to Substrate Concentrations with Application in Molar-Mass Determination, Zero-Order Specificity Constant is Inappropriate

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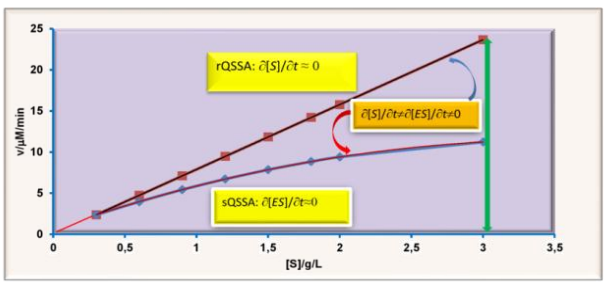
Abstract

"High-ranking scientists" use the initial rate (v_i) equation regardless of the conditions under which it can be applied. This approach incorrectly suggests that v_i is directly proportional to the substrate concentration, $[S_o]$, where the proportionality constant is V_{max} (which is an inappropriate maximum velocity) divided by Michaelis-Menten constant, K_M instead of enzyme-substrate complex dissociation, K_d. The main objectives are threefold: 1) demonstrating that v_i is not equal to $V_{max}\ [S_o]/K_M$, 2) establishing that K_d is directly proportional to the enzyme concentration ($[E_o]$), and 3) illustrating that the standard quasi-steady-state assumptions (sQSSA) and reverse QSSA (rQSSA) have a limited range of validity. The study utilized both experimental and theoretical approaches, following the Bernfeld method for enzyme assay. A K_M-like value was determined to be 2.569 g/L, higher than the putative K_d of 2.482 g/L. Other K_M-like values recorded were 2.396 and 2.407 g/L, corresponding to K_d values of 2.288 and 2.299 g/L, respectively. The molar mass of insoluble potato starch ranged from 62.296 to 65.616 exp. (+6) g/mol. When the $[S_o]$ is much lower than K_M, the derived equations contradict the assumption that v_i equals V_{max}/K_M. Additionally, K_d was found to be directly proportional to $[E_o]$. Furthermore, the molar mass of starch could be determined using the given equations. Graphical and mathematical evidence showed that the sQSSA and rQSSA domains are valid only within certain limits. The equation for determining the second-order rate constant based on rQSSA validity does not apply to sQSSA. The K_M-like value may exceed the putative K_d value.

Keywords: *Aspergillus Oryzae* Alpha-Amylase, Quasi-Steady-State Assumptions, Michaelis-Menten Constant, Enzyme-Substrate Complex Dissociation Constant, Molar Mass, Insoluble Potato Starch

Graphical abstract



The graphical abstract illustrates three zones: the zone in which the sQSSA is valid, the zone in which the rQSSA is valid, and the zone in which neither assumption is exclusively valid. The curved arrow (oxblood) pointing to the red line depicts a tendency towards conditions that validate the rQSSA if the assay is conducted with an appropriate $[S_o]/[E_o]$ ratio (< 1 to ≪1) while the red curved arrow pointing to the blue line depicts a tendency towards conditions that validate the sQSSA if the assay is conducted with an appropriate $[S_o]/[E_o]$ ratio (>1 to ≫1). The enzyme-substrate complex (ES) is in a quasi-steady state with respect to S as depicted by $\partial[ES]/\partial t \approx 0$, the sQSSA case, while in the rQSSA, it is the S that is in a quasi-steady state with respect to ES as depicted by $\partial[S_o]/\partial t \approx 0$.The double-headed arrow merely shows, artistically, the limit of the data points.

1. Introduction

For highly skilled and advanced scholars, three years of residence at a preprint server is sufficient for fault-finding!

The well-known constancy of the rate of product production, such that $d[ES]/dt$ (or $\Delta[ES]/\Delta t$) and $d[S_0]/dt$ (or $\Delta[S_0]/\Delta t$) are both ≈ 0, is one of the outstanding research areas related to the derivation of correct kinetic parameters. In this case, $[S_0]$ is the initial substrate concentration and $[ES]$ is the enzyme-substrate complex concentration. One such question is, "Are there parameter domains in which, instead of the $[ES]$ being in a quasi-steady state with respect to the substrate (S), there is a 'reverse quasi-steady state (rQSSA)' in which S is in a quasi-steady state with respect to enzyme-substrate complex (*ES*)?" [1]. The question implies that there is unarguably the opposite assumption, *i.e.,* the standard QSSA (sQSSA), which was originally credited to Briggs and Haldane [2] and Savageau [3]. The rQSSA is also regarded as an alternative definition of quasi-steady state, where $d[S_0]/dt \approx 0$. This was originally attributed to Segel and Slemrod. Within this approximation, at a high enzyme concentration, $[E_0]$ the conditions [4], under which $[E_0]$ is much greater than $[S_0]$ (and *K* as well), were used to derive appropriate equations. *K* is the Van Slyke–Cullen constant, equal to k_{cat}/k_1, where k_{cat} is the catalytic rate constant for product formation and k_1 is the second-order rate constant for the formation of ES. These equations were originally attributed to Schnell and Maini [6]. This notwithstanding, there is a view that the condition whereby $[S_0] \gg [E_0]$ is unnecessarily restrictive [7]. As such, the Michaelis–Menten equation can be used even when $[S_0] \approx [E_0]$ as long as the Michaelis–Menten constant, $K_M \gg 1$ or $[E_0] \ll K_M$ [7]. Consequently, sQSSA can also be valid without such restrictions. The argument in this research is that despite the conditions that minimize the restriction on the parameter domains for which sQSSA and the Michaelis-Menten equation remain valid, there is, after all, a limit to such domains.

In light of the facts and principles mentioned above [8], the idea that the velocity (initial rates, v_i) equations of the catalytic reaction have been used to determine kinetic parameters outside of the conditions for which they are valid must be supported. This suggests that certain kinetic parameters, such as the specificity constants, may not meet the requirements for classification as sQSSA, rQSSA, *etc*. As in an *in vivo* scenario, $[E_0]$ may be $\gg [S_0]$, or the former may be approximately of the same order of magnitude as its substrate concentration [6]. The former scenario ($[E_0] \gg [S_0]$) should be in line with rQSSA, while the latter ($[E_0] \approx [S_0]$) scenario may be partially consistent with sQSSA.

The goal of the study is to demonstrate the applicability of the rQSSA where the initial rates are directly proportional to the substrate concentration, aside from the usual requirement that $[E_0]$ be much greater than $[S_0]$. Under these conditions, one can demonstrate that the molar mass of a substrate, such as starch, can be determined. This reveals that, when initial rates are directly proportional to substrate concentrations for substrate molar mass determination, a zero-order specificity constant is inappropriate. The goal can be accomplished with the following objectives: 1) to derive an equation that invalidates the assumption that whenever $[S_0] \ll K_M$ (and in particular, when $[E_0] \gg [S_0]$), v_i is always = $V_{max}[S_0]/K_M$; 2) to derive an equation that shows that the ES equilibrium dissociation constant is strictly proportional to $[E_0]$; 3) to calculate, based on the derived equation, the K_d value compared with a graphical value; 4) to apply the rQSSA-based derived equation in the calculation of the molar mass of the polymer substrate; and 5) to illustrate graphically and mathematically, a limit to the extent of the parameter domain in which any other QSSA and Michaelian equation can be valid.

1.1 Significance

For the first time, the hidden non-Michaelian kinetics that affects the accuracy of Michaelian kinetic parameters has been elucidated. The error arises because the initial rates may correlate directly with substrate concentration, producing negative intercepts in double reciprocal plots and leading to less accurate kinetic parameter values *via* various analytical methods. The derivations in this study allowed the ES dissociation constant and the molar mass of starch to be determined based on a kinetic model.

2. Theory

If the initial rates are directly proportional to the concentrations of the substrate, the coefficient of determination is very likely to be ≥ 0.999 (it could be =1); being < 0.999 may be as a result of error in measurement of initial rates, initial substrate concentration, timing, *etc.*, leading to "outliers" as often referred to in the old literature [9, 10] and in papers [9, 11-13] devoted to how best to produce accurate initial rates (v_i) or rather kinetic parameters following the assay of an enzyme. The notion that v_i is directly proportional to $[S_0]$, where the proportionality constant is the ratio of the maximum velocity of enzymatic action to the Michaelis-Menten constant (or the zero-order specificity constant), can be found in many standard undergraduate text books and in high-ranking journals containing views about the *in vivo* concentration of the enzyme compared with the substrate [6, 8].

$$v_i = V_{max}[S_0]/K_M \quad (1)$$

Where V_{max} and K_M are the maximum velocity of catalytic action and Michaelis-Menten constant respectively. Equation (1) stems from the fact that, in certain situations, the concentrations of the substrate are $\ll K_M$ and the concentration of the enzyme $[E_0]$ could be $\gg [S_0]$ as is the case in an *in vivo* scenario [6, 8]. "One question that needs an answer is: does it mean that after the consumption of 4-6 slices of bread, the concentration of the enzyme in the small intestine is > the overall concentration of a carbohydrate-rich diet"? Equation (1) originates from the Michaelis-Menten equation given below:

$$v_i = \frac{V_{max}[S_0]}{K_M + [S_0]} \quad (2)$$

Thus, if, by conceptual and operational arguments, the enzyme-catalyzed reaction cannot attain half its maximum rate of catalysis until substrate concentration equal to the K_M is available, it should be inappropriate to convert Eq. (2) to Eq. (1).

It falls within the realm of common sense to observe that if $[E_0](1)$ is > $[E_0](2)$, K_M for the former should be proportionately > the K_M for the latter. It has been observed in the literature that a high-ranking biochemist [14], whose

authority in the field is almost the kind no one dares question, has consistently called for the direct measurement of specificity constant (V_{max}/K_M); thus, dividing the latter obtained from the plot of v_i versus $[S_0]$ by $[E_0]$ should translate into the direct measurement of specificity constant (SC), even if $[S_0]$ is < $[E_0]$. This is definitely inappropriate. Arguments about the appropriateness of SC as defined in Eq. (1) will be subject to some aspects of the quasi-steady-state assumption in due course.

If, indeed, v_i is directly proportional to $[S_0]$, then the following relationship should hold.

$$\frac{[S_0]}{M_3\left(\frac{[S_0]}{M_3}+\frac{[E_0]}{M_2}\right)} = \frac{v_i}{v_i + V_{\max}^{prs}} \tag{3a}$$

Where, M_3, M_2 are the pre-steady-state maximum velocity (PRSV), molar mass of substrate, and molar mass of the enzyme, respectively. Issues regarding PRSV have been investigated elsewhere [15]. However, the choice of PRSV is purely a coincidence; otherwise, the equations being derived are equally applicable to any linear phase, including early steady-state (SS) [16].

$$\frac{[S_0]}{\left([S_0]+\frac{M_3\,[E_0]}{M_2}\right)} = \frac{v_i}{v_i + V_{max}^{prs}} \tag{3b}$$

Expanding Eq. (3b) gives:

$$v_i\left(\frac{M_3\,[E_0]}{M_2}\right) + v_i[S_0] = [S_0]\left(v_i + V_{\max}^{prs}\right) \tag{3c}$$

$$v_i\left(\frac{M_3\,[E_0]}{M_2}\right) = V_{\max}^{prs}[S_0] \tag{4}$$

$$M_3 = \frac{V_{\max}^{prs}[S_0]M_2}{[E_0]v_i} \tag{5}$$

An unbiased critical examination of Eq. (5) shows that, the equation can only be valid if v_i is totally and directly proportional to $[S_0]$ and, expectedly, there should be a constant for a given concentration of the enzyme, which in turn influences the magnitude of v_i. The coefficient of determination (R^2) could be = 1. Thus, Eq. (5) falls outside the realm of Michaelian kinetics or the standard quasi-steady-state approximation (sQSSA). It should be applicable to reverse QSSA (rQSSA). This is the core reason why the PRSV or its steady-state velocity (SSV) counterpart is a better choice because it is much lower than the zero-order (asymptotic) values of the actual maximum velocity.

The second equation is:

$$v_i = \frac{V_{\max}^{prs}[S_0]M_2}{[E_0]M_3} \tag{6}$$

Equation (6) clearly shows that the proportionality constant, φ (or slope) is given as:

$$\varphi = \frac{V_{\max}^{prs}M_2}{[E_0]M_3} \tag{7}$$

Either from Eq. (6) or Eq. (7), the most important observation is that, the reciprocal of the equilibrium dissociation constant (K_d), otherwise called the association constant (K_a), is given as:

$$\frac{1}{K_d} = \frac{M_2}{[E_0]M_3} \tag{8}$$

Based on Eq. (8), one can convincingly opine that like K_M, K_d is directly proportional to $[E_0]$. However, the enabling scenarios differ for K_M and K_d; while the $[S_0]$ range for the former must fall between values < the K_M and values ≫ K_M, the $[S_0]$ range for the latter must be ≪ K_M if known *a priori*. Most importantly, the $[E_0]$ value suitable for K_M must be ≪ $[S_0]$ in line with expectations of sQSSA, while, in addition, $[E_0]$ must be ≫ $[S_0]$ for the determination of K_d as expected in a rQSSA scenario. It follows from Eq. (8) that if the values of M_2 and M_3 are known, the K_d for any enzyme of known concentration can be calculated and used to estimate the $[S_0]$ range that falls between values < K_d and values ≪ K_M.

With this background theory, it is clear that there are cogent reasons to rewrite Eq. (1), which becomes:

$$v_i \neq V_{max}[S_0]/K_M \tag{9}$$

Thus, in place of Eqs (1) and (9), the following equation applies:

$$v_i = \frac{V_{\max}^{prs}[S_0]}{K_d} \tag{10}$$

There is hardly any one-substrate-one-enzyme reaction in which the reverse reaction and forward reaction may not occur. The difference lies in the magnitude of V_{max}, which may be high if $[E_T]$ is high with either the correspondingly much higher values of $[S_T]$ for the sQSSA case or much lower $[S_T]$ values for the rQSSA case; thus the following may hold:

$$K_d + V_{max}/[E_0] = k_1K_M \tag{11}$$

Equation (11) is therefore, strictly speaking, not applicable to rQSSA, but rather it is applicable to sQSSA. Equation (11) is despite the view that where $[E_0]$ ≫ $[S_T]$, the following equation, which is very similar to the Michaelis-Menten equation, is applicable.

$$v_i = \frac{V_{\max}^{prs}[S_0]}{K_d + [S_0]} \tag{12}$$

In this research, however, Eq. (12) is redesignated as one that is appropriate for a situation in which $[E_0]$ is ≈ $[S_0]$. This is to imply that any plot of v_i versus $[S_0]$ may not be far from Michaelian kinetics, but the zero-order (or asymptotic) value of the maximum velocity is not attainable under such a situation. This is in line with the view elsewhere [17] that "when both the sQSSA and rQSSA are invalid, the initial enzyme and substrate concentrations are comparable". A double reciprocal linearization of Eq. (12) gives the slope as: V_{max}^{prs}/K_d, yet, V_{max}^{prs} is < the magnitude appropriate for $[E_0]$ if assayed with a substrate concentration range that does not include saturating concentrations of the substrate. Because Eq. (11) is more relevant to the Michaelian equation, Eq. (12) can be intuitively related to Eq. (10) as follows:

$$K_d + V_{\max}^{prs} / [E_0] = k_1^{prs}K_M^{prs} \tag{13}$$

Equation (13) is born out of a reasonable postulation to the effect that:

$$K_d + k_{\text{cat}}^{prs} \cong k_1^{prs} K_M^{prs} \quad (14)$$

Where k_1^{prs} and K_M^{prs} are respectively 2nd order rate constant which is > pre-steady-state value but < zero-order value and Michaelis-Menten-like constant which is > than K_d but < K_M. This simply means that K_d in Eq. (12) may be replaced by K_M^{prs}, a parameter which is therefore, neither the true K_d or the true K_M. Again this implies that, it is only a situation where the enzyme attains total saturation, that guarantees the true value of a K_d which may be equal to the value as defined by Eq. (8). Also, given different values of substrate concentration range, for the same concentration of the enzyme, different values of K_d are expected.

2.1 Validity of various QSSA *vis-à-vis* appropriate definitions and values of K_d and K_M

This section examines what validates various QSSA and relates them to the values of K_M and K_d, thereby enhancing the accuracy of kinetic constants. The principles and facts derived from Schnell & Maini's [17] research support this analysis. Beginning from the idea of a general equation of initial velocity [17], one writes:

$$v_i = \frac{V_{\max}[S_0]}{\phi + [S_0]} \quad (15)$$

Where, ϕ is given as:

$$\phi = K_d + \frac{K}{1 + d[C]/d[S]} \quad (16)$$

First, one considers the condition that the sum of initial substrate concentration ($[S_0]$) and K_M greatly exceeds the initial enzyme concentration ($[E_0]$), that is (but $[S_0]$ alone could be $\gg [E_0]$),

$$\frac{[E_0]}{K_M + [S_0]} \ll 1 \quad (17)$$

Setting $d[ES]/dt \approx 0$ implies that $d[ES]/d[S] \rightarrow 0$ and $\phi = K_M$ in sQSSA velocity equation. This investigation presents equation and the possibility that are not in compliance with the conditions that validates sQSSA. Therefore, Eqs (8) and (10) bear no iota of conceptual relevance to the equation based on sQSSA. This is apparently the reason why Borghans *et al.* [18] admitted that inequality, In-Eq. (17) cannot hold having observed that with very high concentration of the enzyme, "K_M" is small; reference to K_M is only as usual (as was the case in a very recent paper [15], though the general concept developed in this study remains relevant) otherwise it is appropriately the K_d (or under an exceptional circumstance to be looked into shortly, it may be considered as a special K_M different from the usual K_M.).

When K_d is the case, $[E_0]$ is $\gg [S_0]$ ($[E_0]/[S_0] \gg 1$) and the appropriate assumption is the rQSSA otherwise known as equilibrium approximation given as, $d[S_0]/dt \approx 0$; the latter presupposes that ϕ is = K_d. The view by Schnell and Maini [17] that "when both the sQSSA and rQSSA are invalid, the initial enzyme and substrate concentrations are comparable" is very instructive in that it goes to show that the equation derived in this study is very appropriate, being a product in which $[E_0]/[S_0] > 1$ and not when $[E_0]$ is $\approx [S_0]$ or of comparable magnitude; this is with reference to Eqs (3a) through (10). On the other hand, there is no way zero-order kinetics (a saturating phenomenon) could be the case, even if sQSSA could have been valid if $[E_0]$ is $\approx [S_0]$ even though current opinion seems to suggest something on the contrary [7] in support of the notion that with total substrate concentration ($[\acute{S}] = [S] + [ES]$), the parameter domain for which it is permissible to employ the classical assumption ($d[ES]/dt \approx 0$) can be extended. Much earlier view is that sQSSA can provide a good approximation even when $[S_0] \approx [E_0]$ (but this cannot be for all concentrations of S, otherwise, $0.5V_{\max}$ cannot be attained) as long as $[E_0]$ is small compared to K_M [1].

To achieve the goal, total QSSA (tQSSA) based on the concept of total substrate concentration is adopted in addition to an unfamiliar singular perturbation method for aggregated variable; this enables the derivation of velocity equations of substrate hydrolysis (*e.g.* amylolysis where applicable) and product formation [7]. For the purpose of comprehension, the total substrate concentration ($[\acute{S}]$) is an aggregated or lumped variable [7]. Again the equations are to enable core biochemists to determine kinetic parameters under conditions in which neither the sQSSA nor the rQSSA are valid [7]. The position taken in this study is that regardless of the criteria adopted that validates any of the QSSA, the foundation upon which Michaelian concept rests cannot be jettisoned. One needs to be circumspect in ensuring that where $[S_0]$ needs to be $\gg [E_0]$, the appropriate assumption must be inferred just as when $[S_0] \ll [E_0]$; in other words it is either sQSSA ($d[ES]/dt \approx 0$) or as in this study, rQSSA ($d[S]/dt \approx 0$). It may not be impossible to encounter a situation in which $d[ES]/d[S]$ is at least ≈ 1; such needs to be investigated.

Further to the problem of validity, one needs to analyze the basis of the claim in this study that the parameter domain in which any QSSA and Michaelian equation are valid needs not be *ad infinitum* in favour of rQSSA. If v_i is strictly proportional to $[S_0]$ for the first 2-3 data-points, a double reciprocal plot can yield a small negative intercept and a larger slope. This can be illustrated in the result and discussion section; kinetic parameters obtained in such situation cannot be valid and where the 2-3 data points are part of the broader range of data points, the results-the kinetic parameters-may be less accurate. As explained in a pre-print [19], $[S_0]_n v_{n-1} - [S_0]_{n-1} v_n$ is = zero (n is the number of assays according to different number of substrate concentrations) and consequently the equation below is expected to give invalid result (*i.e.* infinite maximum velocity and infinite Michaelis-Menten constant).

$$V_{\max} = \frac{v_n v_{n-1}([S_0]_n - [S_0]_{n-1})}{[S_0]_n v_{n-1} - [S_0]_{n-1} v_n} \quad (18)$$

The equation for the Michaelis-Menten counterpart is:

$$K_M = \frac{[S_0]_n [S_0]_{n-1}(v_n - v_{n-1})}{[S_0]_n v_{n-1} - [S_0]_{n-1} v_n} \quad (19)$$

Therefore, if $[S_0]_n v_{n-1} - [S_0]_{n-1} v_n$ is = zero, the separate infinite values of $V_{\max}$ and K_M are summarily invalid, yet the specificity constant, SC, defined as $V_{\max}/K_M$ given below seem valid-the absence of infinity clause.

$$\frac{V_{\max}}{K_M} = \frac{\nu_n \nu_{n-1}([S_0]_n - [S_0]_{n-1})}{[S_0]_n [S_0]_{n-1}(\nu_n - \nu_{n-1})} \quad (20)$$

It needs to be made clear that, Eq. (20) is characteristically a general one because it is error sensitivity invariant. This is despite the fact that in the separate occurrence of the respective equations, V_{max} and K_M may not be valid thereby partially justifying the proposition by an imminent biochemist [14], that SC should be seen as a unique and singular kinetic parameter; it is however, very necessary to specify, the QSSA that is validly relevant to the SC generated with the assurance that substrate concentration regime (or range) matches the concentration of the enzyme in terms of either being approximately equal to, a little less than, much less than or much greater than $[E_0]$; note that, the choice of substrate concentration range and the $[E_0]$ that validates sQSSA and Michaelian equation, does not necessarily imply that the zero-order kinetic parameters, K_M, k_{cat} or preferably, SC were attained. So, if v_n is = $2v_{n-1}$ and correspondingly, $[S_0]_n$ is $2[S_0]_{n-1}$, Eq. (20) transforms into:

$$SC = v_n/[S_0]_n \text{ (or } v_{n-1}/[S_0]_{n-1}) \quad (21)$$

Hence, going by the definition of V_{max} and K_M, it stands out clearly that, V_{max}/K_M is not equal to the ratio of initial rate to the corresponding concentration of substrate, which could have been a characteristic of a single-turnover event. Hence in circumstance in which the initial rate is consistently proportional to the concentration of the substrate (with the possibility that the coefficient of determination is ≥ 0.999), Eq. (21) represents SC for a scenario where rQSSA is valid (QED). Another equation in the literature [20] which can redefine the limit of the parameter domain for which sQSSA and rQSSA is valid is given as follows:

$$\ln \frac{[E_0]}{[E_0]-[ES]} = \frac{(k_{-1}+k_{cat})[S_0]}{K_M k}(1 - e^{-k t}) \quad (22)$$

Where k_{-1}, k_{cat}, k, and t are the reverse first-order rate constant for the dissociation of ES into free E and S, catalytic first-order rate constant, pseudo-first order rate constant for the utilization of the substrate, and the duration of ES formation (or the life span) of ES.

While Eq. (22) represents a general principle in terms of what it represents, K_M in the equation may not be the actual K_M if according to recommendation in the literature [8] that the agreement between the sQSSA solution and the numerical solution is quite good when $[E_0] \leq 0.01[S_0]$; the simple issue is that some of the concentrations of the substrates must be about 40 to 100-fold higher than $[E_0]$. Any substrate concentration range < say 40 to 100-fold < [E0] may not be in good agreement with "zero-order level" sQSSA. In such scenario, rQSSA may be relevant and Eq. (22) may be applicable because in the equation given below (Eq. (23)), where k_{-1}^{prs}, k_{cat}^{prs}, and S_{lope}, are the pre-steady-state-like reverse first-order rate constant for the dissociation of ES into free E and S, catalytic first-order rate constant, and a slope from the plot of left hand side of Eq. (22) versus $[S_0](1 - e^{-k t})/k$, k_{cat}^{prs} must be < $K_M^{prs} S_{lope}$ otherwise, the originating initial rates are only likely to be relevant where rQSSA is valid-$k_{cat} > K_M S_{lope}$.

$$\left(k_{-1}^{prs} + k_{cat}^{prs}\right) = K_M^{prs} S_{lope} \quad (23)$$

Note however, that the slope is actually the second-order rate constant, k_1^{prs} for the formation of ES. The result section gives better insight to the issues.

3. Materials & Methods

3.1Materials

3.1.1 Chemicals

Aspergillus oryzae alpha-amylase (EC 3.2.1.1) and potato starch were purchased from Sigma-Aldrich, USA. Tris 3, 5—dinitrosalicylic acid, maltose, and sodium potassium tartrate tetrahydrate were purchased from Kem Light Laboratories in Mumbai, India. Hydrochloric acid, sodium hydroxide, and sodium chloride were purchased from BDH Chemical Ltd., Poole, England. Distilled water was purchased from the local market. The molar mass of the enzyme is ~ 52 k Da [21, 22].

3.1.2 Equipment

An electronic weighing machine was purchased from Wensar Weighing Scale Limited, and a 721/722 visible spectrophotometer was purchased from Spectrum Instruments, China; a *p*H metre was purchased from Hanna Instruments, Italy.

3.2 Methods

The enzyme was assayed according to the Bernfeld method [23] using gelatinised potato starch, whose concentration range was 0.3–3 g/L. Reducing sugar produced upon hydrolysis of the substrate at room temperature using maltose as a standard was determined at 540 nm with an extinction coefficient equal to 181 L/mol.cm. The duration of the assay was 3 minutes. A mass concentration of 2 mg/L of *Aspergillus oryzae* alpha-amylase was prepared in Tris-HCl buffer at *p*H = 7.

3.2.1 Determination of pseudo-first order rate constant, *k* and second order rate constant, k_1

The determination of the pseudo-first order constant, *k,* for the utilization of the substrate is as described elsewhere [24], with modification as follows: The result of the integration of a polynomial equation from the plot of initial rates v_i versus $[S_0]$ was fitted to the values of the former to give substrate concentrations that were < both the initial concentrations of the substrate and either K_d or K_M. The new, but lower substrate concentrations were substituted into the polynomial equation to generate the corresponding lower velocities that were then used, as described in the literature [24], for the calculation of different values of *k*. The *k* values were then substituted into an equation as described in the literature [20] for the determination of the life span (*t*) of ES. Again, the value of *t* is substituted into Eq. (22) for the calculation of k_1 as described in the literature [20].

3.2.2 Determination of ES dissociation constant, K_d and molar mass, M_3 of potato starch

Equation (8) was applied in the determination of K_d. The 2nd equation for the determination of M_3 is dependent on a reverse first-order rate constant for the dissociation of ES into free S and E. This is where Eq. (23) is relevant, and by being written as:

$$\frac{k_{-1}^{prs}}{k_1^{prs}} = K_M^{prs} - \frac{k_{cat}^{prs}}{k_1^{prs}} \quad (24a)$$

Where k_{-1}^{prs}/k_1^{prs} is = K_d.

$$k_{-1}^{\text{prs}} = \left(K_{\text{M}}^{\text{prs}} - \frac{k_{\text{cat}}^{\text{prs}}}{k_1^{\text{prs}}}\right) k_1^{\text{prs}} \quad (24b)$$

3.3 Statistical Analysis

Assays were conducted in duplicate. The arithmetic mean of each initial rate was used to carry out double reciprocal plots and other plots. Micro-Soft Excel was explored for the determination of standard deviation (SD) where necessary.

4. Results and Discussion

To begin with, it is imperative to note that whenever a plot of initial rates versus different substrate concentrations gives a negative coefficient of the leading term in a resulting polynomial, Michaelian kinetic characteristics are implicated; it may not be enough to guarantee the attainment of actual K_{M} and $V_{\max}$ at the asymptotic level if $[S_0]$ is not $\gg$ $[E_0]$. The derived equation showed that if fitted to initial rates and plotted versus $[S_0]$ for an enzyme concentration of 2 mg/L, it gives a value that is < zero-order SC (Table 1). As Eq. (8) shows, the ES dissociation constant is directly proportional to the mass concentration, or molar concentration, if the molar mass of the enzyme is known, and to the molar mass of the substrate if it is known accurately. Thus, K_{d} is *ipso facto*, an established parameter. It is also amenable to experimental determination. The experimental values, obtained by a graphical approach (double reciprocal plot) based on fitting a modified Michaelian equation (Eqs (18) and (19)) to initial rates at different $[S_0]$ and by calculation using Eq. (8), are, respectively, $K_{\text{d}} \approx 2.396$ g/L and V_{max}^{prs} =202.253 μM/min; $K_{\text{d}} \approx 2.407$ g/L and V_{max}^{prs} = 202.618 mM/min; and $K_{\text{d}} = 2.48231$ g/L.

The polynomial equation, generated from the plot of the initial rate versus $[S_0]$, is given as:

$$Y\,(i.e.\,v_i) = -08606\,x^2 + 6.1207x\,(i.e.\,[S_0]) + 0.6053;\, R^2 = 0.9948 \quad (25)$$

Equation (25) expresses a trend towards Michaelian kinetics due to the occurrence of a negative leading coefficient. This sQSSA relic contrasts with an almost perfect linear (R^2 = 0.9993) relationship between calculated values of rate and calculated $[S_0]$ as described in Fig 1. This case is more characteristic of rQSSA. Further to this is the consideration of a situation in which the initial rate for $[S_0]_{\text{n}}$ is twice the initial rate for $[S_0]_{\text{n}-1}$; thus, with $[S_0]_1 = 0.3$ g/L and $v_1 = 2.251359$ exp. (−5) M/min; $[S_0]_2 = 0.6$ g/L and $v_2 = 2$ (2.251359) exp. (−5) M/min; $[S_0]_3 = 0.9$ g/L and $v_3 = 3$ (2.251359) M/min exp. (−5) covering the first three data used for a plot, the unfolding result shows as expected, an equation of linear regression given as:

$$v_i = 7.5045 \text{ exp.}(-5)\,[S_0]\;(R^2 = 1) \quad (26)$$

However, it is important to note that only the first initial rate is directly experimental while the other two are calculated by multiplying the first rate by $[S_{\text{n}}]/[S_{\text{n}-1}]$ for the purpose of illustration, a process not too different from simulation as applicable to well-known "high-reputation advanced publishers, FEBs, Elsevier, publisher of PNAS, Beilstein Journal, Biochemistry Journal (Oxford/Jn.), *etc.*; this research should not be an exception given that it is more of an experimental study with substantial theory. Dividing the slope by the molar concentration of the enzyme gives an SC-like value of 1951.17 L/g. min, which is < 2194.732 L/g.min and ≈ 2188.645 L/g. min calculated from the table of values of $V_{\max}^{prs}$ and K_{d} (rewritten as: K_{M}^{prs}); these values are, however, > the value (1298.414/g. min) obtained from the slope in Fig 1, a typical "rQSSA plot".

As shown in this study, the K_{d} calculated on the basis of Eq. (24b) is different from the definite value obtained based on Eq. (8); this implies that it is not unlikely that different values of the experimental K_{d} can be obtained given different substrate concentration ranges for the same enzyme under the same assay condition. This is as long as the concentration range is < than the putative K_{M} value of the enzyme, and better still, it should be $\ll [E_0]$ [6, 18]. A very important observation is that the 2nd order rate constant k_1 for the formation of the ES was determined and applied in the determination of the first-order rate constant for the dissociation of the ES into free E and S; different values (Table 1) are as a result of different values of K_{M} explored. The most important deductions are, however, the observation that the zero-order SC cannot be inferred from data points—the initial rates in particular—that either validate only rQSSA or partially validate sQSSA or by extension of the parameter domain that validates both rQSSA and sQSSA [25, 26]. The value of $V_{\max}^{prs}$ (≈124.198 mM/min) is based on the slope in Fig 1 and Eq. (10). This is a typical result that shows that the zero-order maximum velocity often inferred from Eq. (10) is inappropriate.

Table 1: Experimental data-Independent and dependent variables

$[S_0]$/g/L	0.3	0.6	0.9	1.2	1.5	1.8	2.0	3.0
v_i/μM/min	22.514	40.5	55.2	67.4	77.8	86.7	91.9	113.3
K_{M}^{prs} (LWB)/g/L	2.396							
K_{M}^{prs} (Eqs (18 &19))/g/L	2.407±0.08 (n=7)							
$V_{\max}^{prs}$ (LWB) /μM/min	202.253							
k_{cat}^{prs} (LWB)/*exp.*(+3)min	≈ 5.259							
$V_{\max}^{prs}$ /μM/min (Eqs (18/19))	202.618 ±3.2 (n=7)							
k_{cat}^{prs}/*exp.*(+3)min Eqs.(18&19)	≈5.268±0.083							
K_{d} (Eq. (8))/g/L	2.482							
k_1/*exp.*(+4)L/g. min	4.88							
k_{-1}(LWB)/ *exp.* (+5) min (Eq. (24b))	≈1.0975							
k_{-1}/ *exp.* (+5) min (Eqs (18&19))	≈1.122							
k_{-1}/ *exp.* (+5) min (Eq.(8))	≈1.2112							
M_3 (LWB)/*exp.* (6) g/mol.	62.296							
M_3 (Eqs (18 &19))/*exp.* (6) g/mol.	63.582							

$[S_0]$, K_{d}, and $V_{\max}^{prs}$ are the substrate concentration, ES dissociation constant, and pre-steady-state maximum velocity respectively; k_{cat}^{prs}, K_{M}^{prs} and M_3 are the pre-steady-state-like catalytic rate, pre-steady-state-like Michaelis-Menten constant, and molar mass of the substrate respectively. Based on Eq. (24a), K_{b} is ≈ 2.288 g/L using K_{M}-like (K_{M}^{prs}) result from Lineweaver-Burk (LWB) plot [27]; the value is ≈ 2.299 g/L using K_{M}^{prs} based on Eqs (18&19).

While noting a situation in which $[S_0]_{\text{n}}v_{\text{n}-1} - [S_0]_{\text{n}-1}v_{\text{n}}$ is = 0, fitting a double reciprocal equation to such data gives a perfect straight line whose intercept is a small negative number while the slope is large; such a negative intercept does not show up if the three data points are part of the remaining five as shown below (Eq. (27d)). Table 1 shows the primary experimental data, the initial rates (average of

duplicate studies, n = 2), and the corresponding concentrations of the substrate.

$$y = 13327\,x - 3.0242 \text{ (Expectedly, } R^2\text{=1 for S/N, 1-3)} \quad (27a)$$

$$y = 13301\,x + 4185.9 \ (R^2\text{= 0.9999for S/N, 6-8}) \quad (27b)$$

$$y = 11847\,x + 4944.3 \ (R^2\text{=0.995 for S/N, 1-8}) \quad (27c)$$

$$y = 11684\,x + 4180.8 \ (R^2\text{=0.9765 for S/N, 1-8}) \quad (27d)$$

Equation (27d) is the outcome of the inclusion of the non-Michaelian initial rates (2 rates) that contributed to Eq. (26) in the double reciprocal plot. All plots, direct linear (or its alternative variant), and nonlinear regression, seem to mask the place and the role of the error introduced where the initial rates exhibit both rQSSA and a little bit of sQSSA validating attributes. In other words, where initial rates reproduce Eq. (26) and Fig 1 in any assay, nothing should be mentioned about sQSSA. Furthermore, Eqs (26) and (27a) are generally applicable where a single turnover event is desired, giving the impression that, in such a scenario, rQSSA is validly relevant because $[E_0]$ is ≫ the highest $[S_0]$ in the substrate concentration range chosen.

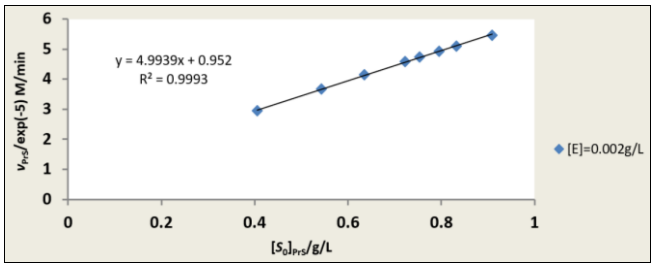


Fig 1: Experimental illustration of non-Michaelian linearised relation between calculated rates (v_{prs}) from fitting polynomial equation to calculated $[S_0]_{prs}$ based on $[S_0]_{prs} = \sqrt[2]{\beta M_{alt} v t_d/\alpha}$ where M_{alt}, v, t_d, α, and β are the molar mass of maltose, the product, velocity of hydrolysis, duration of assay, coefficient of the leading term, and coefficient of the term with unit power or exponent in the polynomial equation generated from the plot of initial rate versus $[S_0]$ (Eq. (24))

In the interest of biochemists, biophysicists, biochemical engineers, *etc*. the individual events at the active site are easily isolated and studied without catalytic cycling, if single-turnover conditions are adopted [28]. In such a scenario, the substrate is saturated with enzyme ($[E_0] \gg [S_0]$) so that all of the substrate will participate in the 'single turnover' [28]. This is the reason why the initial rate should always be directly proportional to $[S_0]$, as orchestrated by Eq. (10), and the demonstration of the implication of Eq. (10), represented by Eq. (26). The obvious is that in a true single turnover assay, the next higher initial rate will always be $([S_0]_n/[S_0]_{n-1})$-fold > than v_{n-1}; this is the reason why in a real situation, apart from timing error and substrate depletion at the lower end of the substrate concentration range, there will be a small negative intercept (Eq. (27a)). This is clearly an expression of both Michaelian and sQSSA invalidity. This study used $[S_0]$ values, which are not very high compared to the $[E_0]$, though the latter is > all except 3 g/L of the insoluble gelatinized starch if the literature value of the molar mass of the insoluble potato starch is taken to be correct in the face of other values [29-31].

As depicted in Fig 2, there is a "far-right rQSSA" domain where it is impossible to infer any condition that validates the Michaelian equation and the associated sQSSA, as again illustrated by Eq. (26); this and Eqs (6 and 7) present the only means by which one can calculate the sub-zero-order maximum velocity, a peculiarity of a 'single turnover' catalytic activity whose conditions validate rQSSA. In this case, the molar masses of the substrate and enzyme, with known mass concentrations, must be known if the V_{max}^{prs} is to be calculated; otherwise, the slope indicated in Eq. (26) remains only a SC-like value. The value of V_{max}^{prs} is ≈186.2724 μM/min. This value is clearly < than the values (Table 1) obtained from the LWB plot and Eqs (18 and 19). As shown in Fig 1 and Eq. (26), the values of V_{max}^{prs} cannot be equal because of their different their slopes; the result from Fig 1 is ≈ 123.964 μM/min. The point that cannot be ignored is that Eq. (1) cannot be used to calculate the V_{max} if the substrate concentration range is ≪ the known K_M of the enzyme whose concentration is either ≫ all concentrations of the substrate or ≈ $[S_0]$ [6, 18]. Any claim to the contrary, that V_{max} is known *a priori* for the determination of a mixed order (steady-state plus a near-zero-order state) K_M, is invalid because all the $[S_0]$ values against which the pre-steady-state initial rates were plotted are < than the putative K_M value.

A clearer picture is obtainable considering Fig 2, where, anticlockwise from A, the condition that validates rQSSA with a higher concentration of the enzyme is the case [6, 18]. This is also a trend towards "single turnover" catalytic activity [28]. Clockwise, beginning from B, there is a higher tendency for the condition that validates both the Michaelian equation and the sQSSA. However, the view that "when both the sQSSA and rQSSA are invalid, the initial enzyme and substrate concentrations are comparable" seems to contradict the notion that $[S_0]$ needs not be ≫ $[E_0]$ for sQSSA to be valid and the claim in the literature [7] that the Michaelian equation and QSSA can still be valid if $[S_0]$≈$[E_0]$. Also contradicted is the notion of total QSSA (tQSSA), which is intended to extend the parameter domain for which both rQSSA and sQSSA could still be valid [25, 26]. In any case, what can be deduced from Fig 2 is that, as $[S_0]$ and $[E_0]$ tend towards equality, anticlockwise direction from B and clockwise direction from A make respectively the sQSSA and rQSSA less valid, but the transformation of the Michaelis-Menten equation can still be fitted to the initial rates as demonstrated with the experimentally generated equations, Eqs (27b-27d), unlike Eq. (27a).

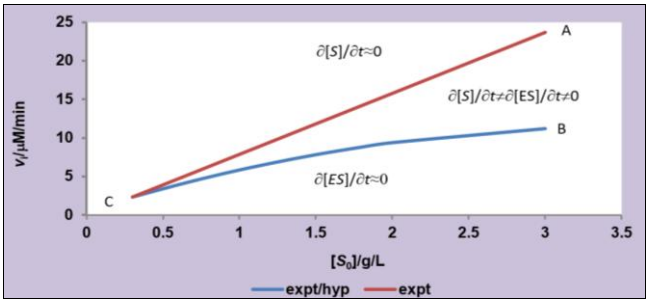


Fig 2: Graphical illustration of the domain of validity of different QSSA with the characteristic mathematical expression. Only the first data point in plot A, is experimental (expt.); the rest are hypothetical (hyp) while in plot B all the data points are experimental

The plot (Fig 2) shows mathematically that either only sQSSA and the Michaelian equation (line B and other lines that can be below it (the d[*ES*]/d*t*≈0 case)) or rQSSA (line A and any other lines above it (the d[*S*]/d*t*≈0 case)) if the first initial rate is half the next initial rate, and the corresponding concentrations of the substrate are such that the first is also half the next higher concentration of the substrate, which is peculiar to a single-turnover kinetics. Under the prevailing conditions between lines A and B, neither rQSSA nor sQSSA is fully validated; a shift of CA towards B through the middle invalidates it, while a shift of CB towards A invalidates it.

Fig 3 illustrates the importance of rQSSA in specifying assay conditions and the influence of physicochemical factors on kinetic parameters. The linear regression of the initial rate (v_i) against the sub-K_M substrate concentrations validates rQSSA. This enhances the determination of a pre-steady-state maximum velocity of approximately 135.348 mM/min based on the derived equations. The SC-like value is 1,417.65 L/g min; however, it may not directly apply to the rQSSA or sQSSA equations. Additionally, the inset depicting LWBP does not yield exact kinetic parameters for strict sQSSA because the condition $[S_0] \gg [E_0]$ is necessary to achieve true maximum velocity. The fig corroborates the claim in the literature that the Michaelian equation remains valid even when $[S_0]$ is comparable to $[E_0]$.

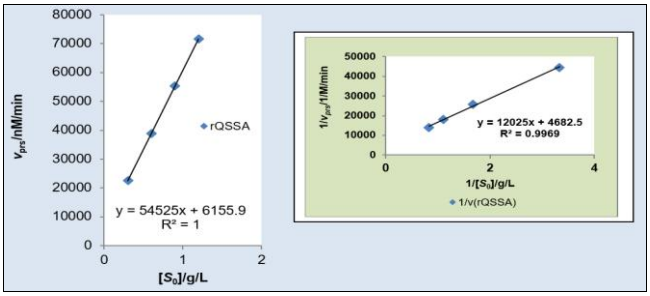


Fig 3: Plot illustrating how two set of similar initial rates can display both Michaelian kinetics (Lineweaver-Burk plot, LWBP, without negative intercept (*see the inset*) or polynomial with negative coefficient of the leading term) or sQSSA and rQSSA (a linear regression with $R^2 \geq 0.999$; in this case R^2 is = 1). The K_M-like value is ≈ 2.568 g/L; the K_d equivalent is = 2.5237 g/L and the molar mass of the starch based on Eq. (8) is ≈ 65.616 exp. (+6) g/mol

The paper by Tzafriri and Edelman [26] exemplifies a scenario in which the rQSSA is applicable if $[E_0] \gg K_M$ and if the former is also as large as $[S_0]$ the latter—the implication of such is depicted in Fig 3, but it is clear that K_M is $[E_0]$-dependent. Thus, different concentrations of the same enzyme under the same condition require different concentrations of the substrate for the attainment of maximum velocity (or for the orchestration of saturation phenomena) and consequently different values of K_M. It is therefore obvious that in this study, where $[E_0]$ is > $[S_0]$, the condition relevant to rQSSA was very much the case; however, this is not to imply that there is no relic of sQSSA given the experimentally generated polynomial with a negative coefficient of the leading term. This notwithstanding, "reverse quasi-steady-state (rQSSA)," in which S is in a quasi-steady state with respect to ES [1], characterizes the main results obtained in this study, and it represents one of the few instances where quantitative effect, as opposed to qualitative and pure mathematical analysis, is carried out.

The preceding issues are further buttressed in Fig 4 which illustrates the domain where rQSSA and sQSSA are strictly valid (or upheld), and the domain in which QSSA as either rQSSA or sQSSA may be applicable or valid and beyond which neither may be valid. Line A (blue) which illustrates the domain of rQSSA validity, can become increasing valid if the concentration of the enzyme is increased for the same concentrations of the substrate [6, 17] leading to upward adjustment of line A; the converse is the case if the substrate concentration is decreased for the same concentration of the enzyme; line C (red) which illustrates the domain of sQSSA validity, can become increasing valid if the concentration of the enzyme is decreased for the same concentrations of the substrate leading to downward adjustment of line C (note that there could be upward adjustment if the concentrations of substrate is increased while the concentration of the enzyme is either decreased or remain the same and ≪ $[S_0]$); the converse is the case if the substrate concentration is decreased for the same concentration of the enzyme; line B (green) has a dual representation of either conditions that validate rQSSA or sQSSA. Any increase in the concentration of the enzyme invalidates completely, the sQSSA, while any decrease in the concentration of the enzyme invalidates the rQSSA.

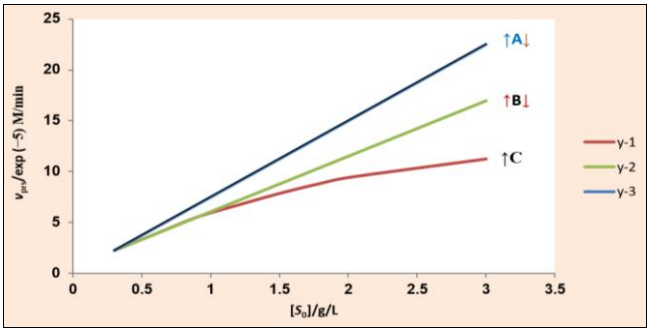


Fig 4: Experimental and simulational plots for illustrating the domain where strict rQSSA, strict sQSSA, and the domain in which QSSA as either rQSSA or sQSSA may be applicable or valid and beyond which neither may be valid

Other kinetic parameters that are indirectly determined according to Eq. (22) are a reflection of the limit of the validity domain of sQSSA in favour of rQSSA, which has cognate kinetic constants such as the second order rate, k_1, (for the formation of ES), and the reverse first-order rate, k_{-1}, determined first based on Eq. (22) for the determination of k_1, and second based on Eq. (23) for the determination of k_{-1} (see Table 1). This would have been impossible if the experimentally generated and simulated initial rates were applicable to sQSSA if the concentration of the enzyme was ≪ the concentration of the substrate. This has also made it possible to determine the molar mass of the insoluble potato starch if the label on the plastic container of starch purchased from Sigma is not faked by the distributor in the local major market. The molar mass is determinable given the following values (Table1): 62.296 *exp*. (+6) derived from LWB and 63.582 *exp*. (+6) g/mol. The calculated value based on Fig 3 (inset-LWB plot) is 65.616 *exp*. (+6) g/mol. The values compare with the cited literature values of 64.54 *exp*. (6) g/mol. [29]; however, a higher value of 77.3 *exp*. (+6) g/L [30] was also reported by the same author [31].

5. Conclusion

The work established that the equilibrium dissociation constant, K_d, is proportional to $[E_0]$; developed equations that challenge the assumption that $v_i = V_{max}[S_0]/K_M$ for $[S_0] \ll K_M$; and showed that K_d computed from an rQSSA-derived equation can be greater than that obtained graphically. The graph and mathematical equation showed the limits of validity for both sQSSA and rQSSA, and the generated equation could be used to compute the molar mass of starch. When substrate concentration is $\ll K_M$, a K_M-like value ($< K_d$) can be achieved; nevertheless, under certain conditions, a K_M-like value (≈2.569 g/L) exceeds the putative K_d (2.482 g/L). The molar mass of insoluble potato starch is estimated between 62.296 and 65.616 *exp*. (+6) g/mol. In contrast to existing literature, this study utilizes velocity, initial rate, and catalytic reaction equations under valid QSSA conditions, addressing the invalidity of sQSSA when $k_{cat} > K_M k_1$. Future research aims to derive a Lineweaver-Burk-like equation for estimating the molar mass of polymers, such as starch.

6. Ethical Approval

NA.

7. Acknowledgement

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8. Source of Funding

None.

9. Conflict of Interest

No conflicts of interest declared.

10. Author (s) Contribution

The sole author designed, analyzed, interpreted and prepared the manuscript.

11. Dedication

I dedicate this study to Brigadier General Samuel Osaigbovo Ogbemudia. During his time as military governor of the now-defunct Mid-West State (later renamed Bendel State and finally split into Edo and Delta states), General Ogbemudia managed and increased significant welfare programs for teaching staff. One of these packages was the approval and unprecedented implementation of auto and refurbishment loans. Distorted political considerations did not influence the outcome; rather, it was aimed at benefiting all without discrimination. *Retirees where exceptionally comfortable!*

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