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Derivable Equations and Issues Often Ignored in the Original Michaelis-Menten Mathematical Formalism

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Author's contribution

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

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ABSTRACT

Background: There has been recent shift from the core issue of Michaelian kinetics to issues regarding various kinds of quasi-steady-state assumptions. Derivable equations with which to determine reverse rate constant for the dissociation of enzyme-substrate complex (*ES*) is given less attention.
Objectives: The objectives of this research are: 1) to derive other equations from differential equations whose evaluation leads to MM equation and 2) quantify based on derived equations the kinetic parameters given less attention and duration of catalytic events.
Methods: A major theoretical research and experimentation using Bernfeld method.
Results and Discussion: The durations for *ES* dissociation (ESD) into free substrate, *S* and enzyme, *E* were much shorter than the duration of ESD into *E* and product, *P* in 3 minutes duration of assay with low [*S*]; it was the shortest and longest in 3 and 5 minutes durations respectively with high [*S*]. The durations of ESD into *E* and *P* was shortest in 3 minutes duration of assay with high [*S*]. The values of reverse rate constant, k_{-1} for ESD into *S* and *E* in 3 minutes duration of assay with high [*S*] was » the rate constant, k_2 for product formation and they are much higher than in other duration of assay.
Conclusion: The equations for the determination of the durations of various events, in a given catalytic cycle were derived. The various time regimes for each event and the rate constant for the dissociation of the *ES* can be graphically and calculationally determined as the case may be. Substrate concentration regime and duration of assay affect rate constants.

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1. INTRODUCTION

For many years Michaelian mathematical formalism describing single active site substrate binding interaction and ultimately transformation to product has attracted a lot of attention. The burning issue based on some assumptions is the validity of derived (or calculated) kinetic parameters. Several authors [1] have investigated this issue. For instance the total QSSA (tQSSA), which is valid for a broader range of parameters covering both high and low enzyme concentrations, has been introduced in the last two decades [2]. A very difficult and complex stochastic approach via chemical master equation has also been applied in the study of the applicability of quasi – steady-state approximation (QSSA) [3]. There is also interest in the application of Michaelian principle initially applicable to 'closed' to 'open' system [4]. Pioneering work of Borghan, et al. [5] clearly defined various kinds of QSSA. None of these efforts seem to be directed towards the determination of the reverse rate constant apart from the rate constant for the formation of products by Michaelian enzymes, the amylase for instance. Indeed, precluding unintended generalisation, it is also claimed that the kinetic constants in Eq. (1a) below are usually not known, whereas finding the kinetic parameters for the Michaelis-Menten (MM) approximation is a standard *in vitro* procedure in biochemistry [6].

$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} ES \xrightarrow{k_2} E + P \qquad (1a)$$

Where, k_1, k_{-1}, k_2 are the 2nd order rate constant for the formation of the *ES*, the reverse 1st order rate constant for the dissociation of *ES* into *E* and *S,* the free enzyme and substrate respectively while k_2 is the 1st order rate constant for the formation of product, *P*. Unlike the rate constant for the production of the product such as maltose in this research, there seems to be less concern for the rate constant for the dissociation of enzyme-substrate complex (*ES*) into free enzyme and substrate. This is regardless of the QSSA under which the assay was carried out. An interesting observation is that MM equation under condition for the validity of reverse QSSA (rQSSA) and standard QSSA (sQSSA) takes the same mathematical form given respectively as: $v = \frac{v_{\max}[S_0]}{K_S+[S_0]}$ and $v = \frac{V_{\max}[S_0]}{K_M+[S_0]}$. Both equations contain the same symbol for maximum velocity, v_{max}. Other symbols, v, $[S_0]$, K_s, and K_M are the velocity of catalysed amylolysis, concentration of substrate, *ES* dissociation constant, and MM constant respectively. This parameter, v_{max} may be quantitatively different for results obtained under rQSSA and sQSSA. The objectives of this research are: 1) to derive other equations from differential equations whose evaluation leads to MM equation and 2) quantify the kinetic parameters given less attention in literature and duration of catalytic events.

2. THEORY

An attempt to derive equations for the determination of the reverse rate constant and different durations of event within a given catalytic cycle needs to take into cognisance the *ES* dissociation constant and Michaelis-Menten (MM) constant; this is why relevant QSSA should be specified. If a plot of v versus $[S]$ shows hyperbolic curve the realm of sQSSA should be inferred. On the other hand, if linearity is shown for the substrate concentration range used, then rQSSA ($[S] \ll K_M$) [7] may be the case.

$$k \neq \frac{k_2[E_o]}{K_M} \qquad (1b)$$

Equation (1b) requires the introduction of molar mass; in this regard the mistake lies in the use of molar mass of the substrate. This issue is to be addressed latter in the text. Meanwhile,

$$k = \frac{k_2^x[E_o]}{K_S} \qquad (2)$$

Where $k_2^x < k_2$: This is the case, because for a total enzyme concentration, the velocity of hydrolysis is $< v_{\max}$, if $[S] < K_M$. Whichever be the case, $v_{\max}$ or $v_{\max}^x$,

$$K_S = \frac{v_{\max}}{k} \qquad (3)$$

$$K_S = \frac{v_{\max}^x}{k} \qquad (4)$$

Where the superscript x means value of rate constant and maximum velocity of hydrolysis different from k_2 and $v_{\max}$. Recall however, that Eq. (3) and Eq. (4) take their origin from $v = -\partial$

$[S]/\partial t = k_2[E_0][S]/K_M = k[S]$. In these cases, Eq. (3) and Eq. (4), the unit of K_S is mol/L. But in the relation, $K_S = [E]_F[S_0]/[ES]$ the unit of K_S is g/L if $[S_0]$ is the mass concentration of the substrate. A circumspective view of the latter and Eq. (4) shows that if the molar mass (M_S) of the substrate (note that the molar mass of insoluble potato starch may be »1000 kg/mol) is taken into account,

$$\frac{v_{max}^{x}}{k} \neq [E]_F[S_0]/[ES]\,M_S \quad (5)$$

Another issue is that K_S is in g/L as expected when direct (or alternative direct) linear plot [8,9] and /or the conventional linear transformations the Lineweaver-Burk [10] approach in particular, using mass concentration of substrate as the independent variable is carried out. If this is the case, one may wish to know how to convert Eq. (5) into a mass-mass relationship. Two ways may be applicable; 1st is the conversion of v_{max}^{x} (maximum molar concentration of the reducing sugar yielded per mL of the enzyme per min.) to mass concentration; $v_{max}^{x(mc)} = v_{max}^{x} M_{alt}$. Therefore, the equilibrium constant in mass concentration is given as

$$[K_S^{mc}] = \frac{v_{max}^{x} M_{alt}}{k} = [E]_F[S_0]^x/[ES] \quad (6)$$

Where, once again, the superscript x means values of kinetic parameters where $[S_0] < K_M$. Equation (6) can be re-written as

$$\frac{v_{max}^{x} M_{alt}}{k} = [E]_F\,[S_0]^x/[ES] = \frac{v_{max}^{x} - v}{v}[S_0]^x \quad (7a)$$

Further rearrangement of Eq. (7a) gives

$$\frac{v_{max}^{x} M_{alt}}{k\,[S_0]^x} = \frac{v_{max}^{x}}{v} - 1 \quad (7b)$$

Equation (7b) can be translated into

$$\frac{1}{v} = \frac{M_{alt}}{k[S_0]^x} + \frac{1}{v_{max}^{x}} \quad (8)$$

Equation (8) represents another type of linear transformation similar to the transformation of MM equation.

It is not in doubt that MM constant is not single equation equilibrium constant. This is to say that it is given as $K_M = \frac{k_{-1}}{k_1} + \frac{k_2}{k_1}$; this implies that

$$S + E \rightleftharpoons ES \rightleftharpoons P + S_{FR} + E \quad (9a)$$

The variable, S_{FR} as explained elsewhere [11], is the fragment of the polysaccharide left after a given catalytic cycle; no single polysaccharide is totally hydrolysed by an appropriate hydrolase. Equilibrium constant is determined by two rate constants. An equilibrium equation such as $S + E \rightleftharpoons ES$ may be very likely. However, equilibrium such as $ES \rightleftharpoons P + S_{FR} + E$ is subject to reexamination because amylase is not known as a hydrolase and a synthase in any classification. The implication of the equation is that the enzyme amylase can catalyse the formation of glycosidic bond between the reducing sugar and the polysaccharide fragment [12]. This presupposes a possibility of the anabolic activity of amylase such that reversibility may be likely.

Meanwhile, the well known original MM equation is derived from the well known subsequent equations given as

$$\frac{d[ES]}{dt} = k_1[E]_f[S]_f \quad (9b)$$

$$v_1 = v_{-1} + V_2 \quad (10)$$

$$v_{-1} = k_{-1}[ES] \quad (11)$$

$$v_2 = k_2[ES] \quad (12)$$

The velocity of dissociation of $[ES]$ in the backward direction is v_{-1} and in the forward direction it is v_2. What seems to be plausible is that in the reaction mixture some ES may be breaking-up in a forward direction yielding product, free enzyme, and fragments if applicable, while some may be breaking-up in a backward direction yielding free enzyme and substrate; the same complex cannot be breaking-up in both directions. What must be made clear is that in a time-course assay of the enzyme v_2, v_{-1} and ultimately v_1 should be decreasing with a fixed substrate concentration due to depletion if product inhibition is precluded. However, the variables may be increasing with increasing concentration of the substrate at the initial transient. Thus it is very clear why the negative sign should appear in the equation [13] below if assay of the enzyme is carried out at a fixed duration and varying concentration of the substrate which is in line with earlier MM experiment. The breakdown of the *ES* either to the product and free enzyme or to the substrate and free enzyme leads to its decreasing concentration which tantamount to an increase in the free enzyme.

$$-\frac{d[ES]}{dt} = (k_{-1} + k_2)[ES] \quad (13)$$

The equation is a first order equation if $k_{-1} + k_2$ is taken as $k_{-1,2}$ and the implication can be elucidated given that

$$[E_0] = [E_f]_{(t_0)} + [ES]_{(t_0)} \quad (14)$$

Equation (14) shows that *ES* does not possess zero hour existence; it may take time t_0 to form and extra time, τ to break down. Meanwhile,

$$[ES]_{(t_0)} = [E_0] - [E_f]_{(t_0)} \quad (15)$$

To integrate Eq. (13), there is need to note that $-\frac{d[ES]}{dt} = \frac{d[E_F]}{dt}$. Therefore,

$$\frac{d[E_F]}{dt} = k_{-1,2}([E_0] - [E_F]) \quad (16)$$

Therefore, if integration of Eq. (13) gives $\ln \frac{[ES]_{(t_0)}}{[ES]_{(t_0+\tau)}} = k_{-1,2}\,\Delta t = k_{-1,2}\tau$ $\left(\text{note that } [ES]_{(t_0+\tau)} < [ES]_{(t_0)}\right)$ then as long as $[ES]_{(t_0)} = [E_0] - [E_F]_{(t_0)}$ and $[ES]_{(t_0+\tau)} = [E_0] - [E_F]_{(t_0+\tau)}$, after time $t_0 + \tau$, the free enzyme should be > the free enzyme after time t_0. So, $[E_F]_{(t_0+\tau)} > [E_F]_{(t_0)}$. What this analysis seems to show is that there is always *ES* between t_0 and $t_0 + \tau$ because complex formation does cease to occur as some dissociate into product and free enzyme. At a given duration of an assay « ∞ (say between 1-3 min), there may be a vast number of molecules of *ES* such that when the assay is terminated, there may be some *ES* molecules left; it is immaterial whether they break done to free substrate and free enzyme due to the reducing agent added to terminate the reaction since no product results from such. Therefore, result of integration of Eq. (13) can be written as

$$\ln \frac{[ES]_{(t_0)}}{[E_0] - [E_F]_{(t_0+\tau)}} = (k_{-1} + k_2)\tau \quad (17)$$

Meanwhile, recall that $[E]_f = [E_0] - [ES]$ and at *t* = 0, $[ES]_{(t=0)} = 0$ and substitution of the former into Eq. (9b) gives after integration and rearrangement,

$$\ln \frac{[E_0]}{[E_0] - [ES]_{(t)}} = k_1[S_0]\Delta t \quad (18)$$

A plot of $\frac{1}{\Delta t} \ln \frac{[E_0]}{[E_0] - [ES]_{(t)}}$ versus $[S_0]$ should give a 2nd order rate constant which may be large if the substrate has a very large molar mass, if known: But intuitively, Δt should be « 1. Restating the following equations enables further examination of certain issues.

$$v = -\frac{d[S]}{dt} = k_1[E_0][S] \quad (19)$$

$$v = -\frac{d[S]}{dt} = \frac{k_2}{K_M}[E_0][S] \quad (20)$$

It should be emphasised that Eq. (19) and Eq. (20) are based on the assumption that $[S] < K_M$. This is unlike Eq. (17) and Eq. (18). But if this was to be the case, then instead of K_M, K_s should be applicable. The implication of Eq. (19) and Eq. (20) is that $\frac{k_2}{K_M} = k_1$ or more appropriately, $\frac{k_2}{K_s} = k_1$ based on the demand for the condition for the validity of rQSSA ($[E_0]/(K_S + [S_0]) > 1$) [7]. This is such that $[E_f] \approx [E_0]$. Integration of both equations gives

$$\ln \frac{[S_0]}{[S]_{(t)}} = [E_0]k_1\Delta t \quad (21)$$

The immediate preceding equations may appear very familiar and very simple but they cannot be trivialised because their misapplication has far reaching negative consequences. Meanwhile, $\frac{1}{\Delta t} \ln \frac{[S_0]}{[S]_{(t)}}$ plotted versus the $[E_0]$ gives a 2nd order rate constant if the assay is carried out at a fixed $[S]$ and Δt (in this case, duration of assay >1s) with varied concentration of the *E*. But if the concentration of the enzyme and substrate are fixed, a plot of $\ln \frac{[S_0]}{[S]_{(t)}}$ versus Δt gives a pseudo-first order rate constant $= [E_0]k_1$. Division of such 1st order rate constant by $[E_0]$ gives the 2nd order rate constant. Since the molar masses of enzymes are known, the unit of k_1 should always be L/mol/s. There should be a way of calculating k_1. This is unlike Eq. (18) in which, the molar mass of the substrate needs to be known. But it appears that the value of the product of k_M and k_1 may be « $k_{-1} + k_2$ if k_1 is not very large. The solution lies in the recognition for the use of the molar mass of product, maltose for instance. This should justify, if introduced, the presence of the molar mass of maltose in Eq. (19) and Eq. (20). Meanwhile, in literature the assumption of steady state, presupposes as stated earlier that, $\frac{dC}{dt} \approx 0$ or rather $\frac{d[ES]}{dt} \cong 0$; this implies that before stead state, the equation [7,14] below describes the net rate of formation of *ES*.

$$\frac{d[ES]}{dt} = k_1[E][S] - (k_{-1} + k_2)[ES] \quad (22)$$

Where $[E]$ is the concentration of free enzyme ($[E_0] - [ES]$) and $[S]$ is the concentration of the substrate taken to be $[S_0]$ the initial concentration even when the time, $t > 0$ but « ∞ in line with

MM formalism. Equation (22) is given below in an unclear rearranged form.

$$\frac{d\,[ES]}{\mathrm{d}\,t}/(k_1[S_0]+k_{-1}+k_2)+[ES]=\frac{k_1[E_0][S_0]}{k_1[S_0]+k_{-1}+k_2} \quad (23)$$

The equation, Eq. (23), is said to be valid when [Chaplin]

$$\frac{d\,[ES]}{\mathrm{d}\,t}/(k_1[S_0]+k_{-1}+k_2)\text{«}[ES] \quad (24)$$

The differential Eq. (23) has been described as a difficult equation to handle, but may be greatly simplified if it can be assumed that the left hand side is equal to [*ES*] alone [14]. But this can be achieved if simple approach can be applied in the derivational process. Taking $[E]$ as $[E_0]-[ES]$ and substituting same into Eq. (22) yields after the 1st rearrangement the following.

$$\frac{\mathrm{d}\,[ES]}{\mathrm{d}\,t}=k_1[E_0][S_0]-\big(k_1[S_0]+(k_{-1}+k_2)\big)[ES] \quad (25)$$

Division by $k_1[S_0]$ followed by rearrangement gives

$$\frac{\mathrm{d}[ES]}{[E_0]-\{1+(k_{-1}+k_2)/k_1[S_0]\}[ES]}=k_1[S_0]\mathrm{d}t \quad (26)$$

On the assumption that $[S_0]$ remains ≈ constant within a very short duration of assay, Eq. (26) represents a simple 1st order differential equation. Thus,

$$\int_{t=0}^{t>0}\frac{\mathrm{d}\,[ES]}{[E_0]-\{1+(k_{-1}+k_2)/k_1[S_0]\}[ES]}=k_1[S_0]\int \mathrm{d}\,t \quad (27)$$

$$\frac{-1}{1+\frac{k_{-1}+k_2}{k_1[S_0]}}\mathrm{I\,n}([E_0-[ES])=k_1[S_0]t+c(\text{a constant}) \quad (28)$$

When time $t=0, [ES]=0$. Thus with *c* given as $\frac{-\mathrm{I\,n}\,[E_0]}{1+\frac{k_{-1}+k_2}{k_1[S_0]}}$, and after rearrangement, the final equation can be given as

$$\frac{1}{t}\mathrm{I\,n}\frac{[E_0]}{[E_0]-[ES]_{(t)}}=k_1[S_0]+(k_{-1}+k_2) \quad (29a)$$

Incidentally, $k_1=\frac{(k_{-1}+k_2)}{K_\mathrm{M}}$. Therefore, Eq. (29a) can be written as

$$\frac{1}{t}\mathrm{I\,n}\frac{[E_0]}{[E_0]-[ES]_{(t)}}=\frac{(k_{-1}+k_2)}{K_\mathrm{M}}[S_0]+(k_{-1}+k_2) \quad (29b)$$

A critical view of Eq. (29b) reveals that the left hand side (LHS) plotted against $[S_0]$ cannot yield intercept >1. As long as $(k_{-1}+k_2)$ » 1, the time *t* « 1. It is hereby postulated that $1/t$ is a constant parameter $(>k_2)$: The argument now is that if MM equation can be stated in a form according to Cornish – Bowden [15] as follows, can the same be applicable to what seems to be a general equation such as Eq. (29b). The equation is $v=v_{\max}([S_0]-[P])/(K_\mathrm{M}+[S_0]-[P])$. If permissible, Eq. (29b) can be restated as

$$\frac{1}{t}\mathrm{I\,n}\frac{[E_0]}{[E_0]-[ES]_{(t)}}=\frac{(k_{-1}+k_2)}{K_\mathrm{M}}([S_0]-[P])+(k_{-1}+k_2) \quad (29c)$$

To obtain positive value of $k_{-1}+k_2$, the intercept obtained from the plot of $\frac{1}{t}\mathrm{I\,n}\frac{[E_0]}{[E_0]-[ES]_{(t)}}$ versus $[S_0]-[P]$ needs to be positive and > 1. The equation is therefore, validly applicable in terms of yielding $k_{-1}+k_2 > k_2$ as $[ES]_{(t)}\rightarrow[E_0]$. Meanwhile, Eq. (29a) can be re-written as (given that $k_{-1}+k_2=k_1K_\mathrm{M}$)

$$\frac{1}{t}\mathrm{I\,n}\frac{[E_0]}{[E_0]-[ES]_{(t)}}=k_1[S_0]+k_1K_\mathrm{M}=k_1([S_0]+K_\mathrm{M}) \quad (30a)$$

Therefore, given that $[S_0]+K_\mathrm{M}=v_{\max}[S_0]/v$ in line with Michaelian principle, Eq. (30a) can be written 1st as

$$\frac{1}{k_1 t}\mathrm{I\,n}\frac{[E_0]}{[E_0]-[ES]_{(t)}}=[S_0]+K_\mathrm{M} \quad (30b)$$

However, for reason that will be unfolded latter in the text, one can divide $[S_0]+K_\mathrm{M}=v_{\max}[S_0]/v$ by the molar mass of maltose to give the number of moles of maltose per unit volume in any mass concentration of the substrate, S and K_M or K_S. Thus, substitution of $v_{\max}[S_0]/vM_{\mathrm{alt}}$ into Eq. (30b) and rearrangement gives

$$\frac{1}{v}=\frac{M_{\mathrm{alt}}}{v_{\max}\,[S_0]\,k_1 t}\mathrm{I\,n}\frac{[E_0]}{[E_0]-[ES]_{(t)}} \quad (30c)$$

Besides Eq. (30c) which contains 2nd order rate constant given as $k/[E_0]$ can be used to determine t by graphical method. Hence a plot of left hand side (LHS) versus right hand side (RHS) can purposefully give a slope (S_{lope}) which enables the determination of *t*.

The 2nd order rate constant can be re-introduced into Eq. (30c) as $\frac{(k_{-1}+k_2)}{K_\mathrm{M}}M_{\mathrm{alt}}$ to give

$$\frac{1}{v}=\frac{K_\mathrm{M}}{v_{\max}\,[S_0]\,(k_{-1}+k_2)t}\mathrm{I\,n}\frac{[E_0]}{[E_0]-[ES]_{(t)}} \quad (31a)$$

Equation (31a) results from a clear cancellation of M_{alt} which appears as denominator and numerator. Rearrangement of Eq. (31a) gives

$$\frac{1}{\mathrm{I\,n}\frac{[E_0]}{[E_0]-[ES]_{(t)}}} = \frac{vK_{\mathrm{M}}}{v_{\max}\,[S_0]\,(k_{-1}+k_2)t} \quad (31b)$$

The MM constant and $[S_0]$ are mass concentrations of the same chemical species whose molar mass may not be known as applicable to potato starch in this research. But as a ratio of one to the other, information about the molar mass may not be necessary. However, taking number of moles of maltose in the substrate from where the product is obtained ensures dimensional consistency and mass conservation. The issue that needs to be considered in Eq. (31b) is that $\frac{1}{t} > k_2$ because the event of *ES* formation takes time < the total time needed for the binding of *E* to *S*, bond breaking and making, and product release [16]. The proposition or rather postulation in this research is that $k_{-1} + k_2$ under steady state condition is $\neq$ to value under pre-steady condition. This postulation requires that *t* the duration of breaking of ES to both E and S and E and P needs to be determined.

The differential equation below is simple and straight forward but an important fundamental issue is often ignored. The equation is

$$\frac{\mathrm{d}\,[ES]}{\mathrm{d}\,t} = k_1[E]_{\mathrm{f}}[S]_{\mathrm{f}} \quad (32)$$

Where as usual, [*ES*], [*E*]$_{\mathrm{f}}$, and [*S*]$_{\mathrm{f}}$ are the enzyme-substrate complex, free enzyme, and free substrate concentrations. The unit of the 2$^{\mathrm{nd}}$ order rate constant, k_1 may be L/g min if the unit of $[S]_{\mathrm{f}}$ is g/L. However, recall that $ES = v/k_2$; hence $\frac{\mathrm{d}[ES]}{\mathrm{d}\,t} = \frac{\mathrm{d}\,v}{k_2\mathrm{d}\,t}$. Where as usual v is the velocity of product (reducing sugar) formation if alpha-amylase (1, 4-alpha-D-glucan glucanohydrolase-(EC 3.2.1.1)) for instance is the case. Most often than not, the unit of v is (mol/L)/mL.min. Thus if mass concentration of the product is preferred, the latter is multiplied by the molar mass (M_{alt}) of maltose as the product. So,

$$[S]_{\mathrm{f}} = [S_0] - v\,tM_{\mathrm{alt}} \quad (33)$$

Division by M_{alt} gives the number of moles of maltose per liter of the free substrate just as twice the molar mass of substrate divided by M_{alt} is $\approx$ the degree of polymerisation: For the purpose of emphasis, such division merely gives an approximation of the real value. Recall too that $v = k\,[S_0]$ but $[S_0]$ should be in moles/L such that $[S_0]/M_{\mathrm{s}}$ (where M_{s} is the molar mass of substrate, a polysaccharide) may give value of k » 1/min unlike division by M_{alt}; the latter may give value similar to, In $([S_0]/[S]_{\mathrm{f}})/t$ (in this case t = chosen duration of assay). Therefore, Eq. (32) becomes better if re-written as:

$$\frac{\mathrm{d}\,[ES]}{\mathrm{d}\,t} = k_1[E]_{\mathrm{f}}[S]_{\mathrm{f}}/M_{\mathrm{alt}} \quad (34)$$

The advantage and relevance of introducing M_{alt} is to be seen shortly.

Meanwhile, $[S]_{\mathrm{f}} = [S_0]/e^{k\,t}$ and substitution and integration follow shortly. Meanwhile $[E]_{\mathrm{f}} = [E_0] - [ES]$; the latter is to be substituted into Eq. (34).

Thus,

$$\frac{\mathrm{d}\,[ES]}{\mathrm{d}\,t} = k_1([E_0] - [ES])[S_0]/e^{k\,t}M_{\mathrm{alt}} \quad (35)$$

In this case, t «1 because it is the time taken to form the *ES*.

$$\int_0^t \frac{\mathrm{d}\,[ES]}{[E_0]-[ES]} = \frac{1}{M_{\mathrm{alt}}}\int_0^t \frac{k_1[S_0]\mathrm{d}\,t}{e^{k\,t}} \quad (36a)$$

$$-\,\mathrm{I\,n}\,([E_0] - [ES]) = \frac{-\,k_1[S_0]e^{-k\,t}}{k\,M_{\mathrm{alt}}} + c \quad (36b)$$

When $t = 0, c = -\,\mathrm{I\,n}\,[E_0] + \frac{k_1[S_0]}{kM_{\mathrm{alt}}}$. Substitution of this into Eq. (36b) gives

$$-\,\mathrm{In}\,([E_0] - [ES]) = \frac{-\,k_1[S_0]\,e^{-k\,t}}{k\,M_{\mathrm{alt}}} - \mathrm{I\,n}[E_0] + \frac{k_1[S_0]}{k\,M_{\mathrm{alt}}} \quad (36c)$$

Rearrangement gives.

$$\mathrm{I\,n}\frac{[E_0]}{[E_0]-[ES]} = \frac{k_1[S_0]}{k\,M_{\mathrm{alt}}}(1 - e^{-k\,t}) \quad (37a)$$

A close view of Eq. (37a) shows that $[S_0](1 - e^{-k\,t})$ is part of the amount of substrate forming *ES* and, in line with mass conservation principle it is equivalent to the mass concentration of product such that division by M_{alt} is $\approx$ the molar concentration of maltose, the product, thereby justifying the equations where M_{alt} appeared.

Since $k_1 = k/[E_0]$ Eq. (37a) is re-written as:

$$\mathrm{I\,n}\frac{[E_0]}{[E_0]-[ES]} = \frac{[S_0](1-e^{-k\,t})}{[E_0]M_{\mathrm{alt}}} \quad (37b)$$

Equation (37b) can be rearranged to give an operationally useful equation for the determination of t. Thus,

$$\mathrm{I\,n}\frac{1}{1-\frac{[E_0]M_{\mathrm{alt}}}{[S_0]}\mathrm{I\,n}\frac{[E_0]}{[E_0]-[ES]}} = k\,t \qquad (38)$$

The left hand side is to be plotted versus calculated values of k to yield a slope, t as required. Meanwhile additional issue arising from Eq. (37a) is the possibility of deriving $k_{-1}+k_2$ given that $k_1 = (k_{-1} + k_2)/K_M$ and substituting same into the former, and with the realisation that K_M is in moles of maltose per litre (this implies that the product of K_M and M_{alt} becomes the mass concentration in the equation-Eq. (37a)) gives

$$\mathrm{I\,n}\frac{[E_0]}{[E_0]-[ES]} = \frac{(k_{-1}+k_2)[S_0]}{K_{\mathrm{M}}k}(1 - e^{-k\,t}) \qquad (39)$$

Thus, having obtained t from the plot against k (Eq. (38)), another plot of the left hand side of Eq. (39) versus $[S_0]\,(1 - e^{-k\,t})/k$ gives a slope $= \frac{(k_{-1}+k_2)}{K_{\mathrm{M}}}$ from where k_{-1} can be calculated. There is need to point out the fact that any calculation whose result is < k_2 may not correctly represent $k_{-1} + k_2$.

At this juncture there is need to recall that, dissociation into the free enzyme and substrate and dissociation into the free enzyme and products are independent processes, and, as such the following equations need to be derived. First is the notion that, $\frac{\mathrm{d}\,[ES]}{\mathrm{d}\,t} = -k_{-1/2}[ES] = \frac{\mathrm{d}\,[E]}{\mathrm{d}\,t}$ where $k_{-1/2}$ means rate constant for dissociation into the free enzyme and substrate or free enzyme and product.

$$\frac{\mathrm{d}\,[ES]}{\mathrm{d}\,t} = -k_{-1/2}[ES] = k_{-1/2}([E_0] - [E]_{\mathrm{f}}). \qquad (40a)$$

However, one may realise that $[ES] = [E]_f[S]_f/K_M$ and substitution into Eq. (40a) gives

$$\frac{\mathrm{d}\,[ES]}{\mathrm{d}\,t} = -k_{-1/2}\frac{[E]_{\mathrm{f}}[S]_{\mathrm{f}}}{K_{\mathrm{M}}} \qquad (40b)$$

The parameter, $[E]_f$ being $[E_0] - [ES]$ changes Eq. (40b) into

$$\frac{\mathrm{d}\,[ES]}{\mathrm{d}\,t} = -k_{-1/2}\frac{([E_0]-[ES])[S_0]}{\exp(k\,t).K_{\mathrm{M}}} \qquad (40c)$$

Where, $[S]_f$ is replaced by $[S_0]/\exp(k\,t)$. Next is the integration of Eq. (40c) as follows.

$$\int_i^t \frac{\mathrm{d}[ES]}{[E_0-[ES]]} = \frac{-k_{-1/2}[S_0]}{K_{\mathrm{M}}}\int_i^t \exp(-k\,t)\,\mathrm{d}t \qquad (41a)$$

Proceeding further requires the understanding that only extant *ES* dissociates. It is either to consider its zero concentration where $t = 0$ or concentration > 0 where $t = i$ ($i > 0$). The latter is preferred for now. Since it takes time to form *ES*, $i = t - \tau$ where τ is the time it takes for the dissociation of *ES* into free enzyme and product as intended subsequently in this section. Therefore, instead of time = 0, $i = t - \tau$ during which *ES* exists before dissociation proceeds. The outcome of integration is

$$-\mathrm{I\,n}\,([E_0] - [ES]) = -\frac{k_2[S_0](-\exp(-k\,i))}{K_{\mathrm{M}}k} + c \qquad (41b)$$

Where, as usual, c is an arbitrary constant. Therefore, when the time is i (= $t - \tau$)

$$c = -\mathrm{I\,n}\,([E_0] - [ES]) - \frac{k_2[S_0]e^{-k\,(t-\tau)}}{K_{\mathrm{M}}k} \qquad (41c)$$

$$-\mathrm{I\,n}\left([E_0] - [ES]_{(t)}\right) = \frac{k_2[S_0]e^{-k\,t}}{K_{\mathrm{M}}k} - \left(\mathrm{I\,n}\left([E_0] - [ES]_{(t-\tau)}\right) + \frac{k_2[S_0]\,e^{-k\,(t-\tau)}}{K_{\mathrm{M}}k}\right) \qquad (42a)$$

Rearrangement of Eq. (42a)

$$\mathrm{I\,n}\frac{[E_0]-[ES]_{(t-\tau)}}{[E_0]-[ES]_{(t)}} = \frac{k_2[S_0]e^{-k\,t}}{K_{\mathrm{M}}k}(1 - e^{k\,\tau}) \qquad (42b)$$

Further rearrangement which takes into account the fact that with time, $[ES]_{(t)} = 0$ (as $(t - \tau) \rightarrow t$; $t > t-\tau$) gives

$$\mathrm{I\,n}\frac{[E_0]-[ES]_{(t)}}{[E_0]-[ES]_{(t-\tau)}} = \frac{k_2[S_0]e^{-k\,t}}{K_{\mathrm{M}}k}(e^{k\,\tau} - 1) \qquad (42c)$$

Equation (42c) is rearranged to give

$$\frac{K_{\mathrm{M}}k}{k_2[S_0]e^{-k\,t}}\mathrm{I\,n}\frac{[E_0]}{[E_0]-[ES]_{(t-\tau)}} + 1 = e^{k\,\tau} \qquad (43)$$

Taking the natural logarithm of Eq. (43) gives

$$\mathrm{I\,n}\left(\frac{K_{\mathrm{M}}k}{k_2[S_0]e^{-k\,t}}\mathrm{I\,n}\frac{[E_0]}{[E_0]-[ES]_{(t-\tau)}} + 1\right) = k\,\tau \qquad (44)$$

A plot of the left hand side of Eq. (44) versus k (k is determined as described in method section) enables the determination of τ as slope. The slopes from Eq. (31b) and Eq. (30c) can be equated to each other to give

$$\frac{1}{t\,v_{\max}}\frac{K_{\mathrm{M/S}}}{k_{-1}+k_2} = \frac{M_{\mathrm{alt}}}{tk_1v_{\max}} \qquad (45)$$

Where, the symbol $K_{M/S}$ could be either the MM constant or the *ES* dissociation constant. Finally,

$$k_{-1} + k_2 = \frac{K_{\mathrm{M}}K_1}{M_{\mathrm{alt}}} \qquad (46)$$

3. MATERIALS AND METHODS

3.1 Materials

3.1.1 Chemicals

Aspergillus oryzea 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 Mumbai, India. Hydrochloric acid, sodium hydroxide, and sodium chloride were purchased from BDH Chemical Ltd, Poole England. Distilled water was purchased from local market. The molar mass of the enzyme is ~ 52 k Da [17,18].

3.1.2 Equipment

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

3.2 Methods

The method reported here is as previously adopted but restated here for quick reference [11]. The enzyme was assayed according to Bernfeld method [19] using gelatinised potato starch whose concentration range was 10-20 g/L. Reducing sugar produced upon hydrolysis of the substrate at room temperature using maltose as standard was determined at 540 nm with extinction coefficient equal to ~ 181 L/mol.cm. The duration of assay ranges from 1-5 min. A mass concentration = 2 mg/L of *Aspergillus oryzea* alpha-amylase was prepared in Tris HCl buffer at *p*H = 6.

3.2.1 Determination of pseudo-first order rate constant, *k*.

It is imperative to disclose that k needs to be determined by substitution of v_{max}, slope from the plot of v versus $[S_0]/[E]_f$, and $[S_0]$ into a quadratic equation (Eq. (47)) derived in a submitted manuscript. It seems reasonable considering that k_2 is given as $v_{max}/[E_0]$.

$$k = M_{\text{alt}}\left(\frac{v_{\max} \pm \sqrt[2]{v_{\max}^2 - 4\,S_{\text{lope}-1}[S_0]}}{2[S_0]}\right) \qquad (47)$$

3.2.2 Determination of rate constants and different time scales

The determination of the rate constant for the disappearance of the substrate as function of [*S*] and v_{max} was carried out according to Eq. (47). The duration of the *ES* formation before the onset of product formation is according to Eq. (38); time taken for the dissociation of the *ES* to product and free enzyme is according to Eq. (44); the same is applicable to the dissociation of the *ES* to *E* and *S* where k_{-1} is applicable. Equation (46) was used to determine $k_{-1}+k_2$ given k_1 according to Eq. (37a) or directly with Eq. (39). In order to eliminate outliers, the v_{max}, K_M and [*S*] were substituted into the MM equation for the calculation of all velocities of amylolysis used for graphical determination of relevant parameters. Microsoft Excel was used to plot graphs including alternative direct linear plot for the determination of kinetic parameters as previously described [8,20].

3.3 Statistical Analysis

Values of velocities of hydrolysis of starch are expressed as mean ± SD; sample size, *n*, is equal to 4. A method described by Hozo, et al. [21] was used to determine the SD. The mean values of experimental velocities from different duration of assay were used for the determination of v_{max} (including k_2) and K_M.

4. RESULTS AND DISCUSSION

While this research is primarily a major theoretical exposition, nevertheless, there is a need to create data with which to test the practicability and possible application of some of the equations derived. To achieve the goals assay of the enzyme using two substrate concentration regimes-lower concentration range (2-4 g/L) and higher concentration range (10-20 g/L). The results shown in Table 1a (higher substrate concentration regime) and 1b (lower substrate concentration regime) are the velocities of amylolysis; the maximum velocities and Michaelian constant/dissociation constant are shown in Table 1c. As in previous publication [20], the kinetic parameters generated within different durations of assay were different, with the exception of the observation in 3 minutes duration of assay in this research which showed a departure from general trend - a decreasing trend in previous publication [20]. Although these issues are important, the main issues are addressed in Table 2.

According to Eilertsen, et al. [22] an enzyme catalysed reactions typically consist of multiple regimes; each regime marks a domain over which certain kinetic behavior and corresponding rate laws can be assumed to be valid. They identified two distinct timescales, the "characteristic and matching" times scales in each kinetic regime of enzyme catalysed reaction. The characteristic time scale provides a rough estimate of the duration of a particular kinetic regime while matching timescale determines the temporal boundary of the corresponding kinetic regime. Whatever be the case, the unclear technical issues raised by the authors seem to confirm or support the proposition that each event, the *ES* formation, dissociation to either free substrate or product or both and free enzyme occur separately in different periods.

The extrapolated results (different durations of (pre-) catalytic events and various rate constants) for different duration of assay are shown in Table 2. It requires time for the enzyme to bind effectively at the active site [23]. There may be binding with site other than active site that has no catalytic effect. It is the extant *ES* that either dissociates into free enzyme and product/substrate. Proceeding further requires that one recalls that time regime is in focus. Before the onset of steady state, there is initial product formation and release and failure of product formation and consequently dissociation into free substrate all of which are time dependent events. In literature may be found related issues in a paper concerned with space-time and entropic characterisation of *Aspergillus oryzea* alpha-amylase [24]. Table 2, in this research, contains results exemplifying and summarising the claim enunciated earlier. In all duration of assay, the durations of the *ES* formation were different; it was much longer in 3 minutes duration of assay than other durations except in 5 minutes duration of assay. There was increasing trend in the duration of *ES* dissociation into free enzyme and product as applicable to 1, 2, and 5 minute's duration of assay. The duration in 3 minutes with low [*S*] is much longer than any other duration of assay; the shortest is in 3 minutes duration of assay with higher [*S*]. If $k_{-1} > k_2$ the time taken for the process $ES \rightarrow E + S$ should be shorter. Hence the time taken within 1, 2, and 3 minutes duration of assay is in the following order: 1<2<5 minutes; while the time taken for the same process in 3 minutes duration of assay with low [*S*] falls within the range, 8.4-78790 μmin (Table 2). The duration of the same process in 3 minutes duration of assay with high [*S*] is much shorter.

As stated earlier in the text, the turnover number often referred to as rate constant for the formation of product, has been of interest to researchers for the purpose of kinetic and thermodynamic characterisation of some enzymes [16,24-27]. The most commonly studied are hydrolases, amylases in particular because of their industrial uses [28]. Recently, nucleoside ribohydrolase was studied enabling the comparison of the rate between enzymatic and non-enzymatic hydrolysis [29].

Stopped-flow analysis is one of the methods [30] needed to study kinetics and thermodynamics of enzyme catalysed reactions. Looking inwards, various equations pertaining to different stages of enzyme catalysed reactions indicated as headings in Table 2 were formulated. Thus, in this research, the effect of time

Table 1a. Velocities of hydrolysis of higher concentration range of gelatinised potato starch in different duration of assay

[*S*]/g/L	DUR/min	DUR/min	DUR/min	DUR/min
	1	2	3	5
	v/μM/mL.min	*v*/μM/mL.min	*v*/μM/mL.min	*v*/μM/mL.min
10	933.5±90	752.8±18.2	624.2±3.5	514.1±4.3
12	945.80±10	915.0±7.2	695.1±3.3	553.8±4.4
14	995.8±11	929.5±8.8	765.0±3.2	560.6±2.9
15	1019.5±10	946.8±11	823.68±3.3	565.3±7.9
16	1087.0±12	975.3±6.6	885.0±3.4	595.8±3.4
17	1178.0±10	985.6±6.6	915.2±2.0	645.9±23.6
18	1278.0±10	999.4±7.5	925.8±2.0	651.9±3.4
20	1345.0±32	1058.0±4.4	1085.2±27.7	656.9±8.9

The parameters, v and [S] are the velocity of hydrolysis with the enzyme and mass concentration of the gelatinised potato starch respectively; DUR denotes the duration of assay

Table 1b. Velocities/U/mL in 3 minutes assay with lower [*S*] range

[*S*]/g/L	[*S*]/g/L	[*S*]/g/L	[*S*]/g/L	[*S*]/g/L	[*S*]/g/L	[*S*]/g/L	[*S*]/g/L
2	2.4	2.8	3	3.2	3.4	3.6	4
v (μM/mL.min)	*V* (μM/mL.min)	*V* (μM/mL.min)	*V* (μM/mL.min)	*V* (μM/mL.min)	*V* (μM/mL.min)	*V* (μM/mL.min)	*V* (μM/mL.min)
92.6±2.5	~ 118.9±0.9	133.3±1.1	140.0±1.1	150.3±1.2	151.9±6.4	160.1±1.3	160.1±5.8

The parameters, v and [S] are the velocity of hydrolysis with the enzyme and mass concentration of the gelatinised potato starch respectively

Table 1c. Michaelis-Menten constant, K_M or *ES* dissociation constant, K_S and maximum velocity of hydrolysis

DUR(Low [S])/min	DUR/min	DUR/min	DUR/min	DUR/min
3	**1**	**2**	**3**	**5**
K_M (or K_S)	K_M (or K_S)	K_M (or K_S)	K_M (or K_S)	K_M (or K_S)
~ 5.55	14.79	~ 11.11	~ 41.87	~ 8.08
v_{max}/μM/mL.min	v_{max}/(μM/mL.min	v_{max}/μM/mL.min	v_{max}/μM/mL.min	v_{max}/μM/mL.min
398.60	2174.00	1650.00	3164.00	914.91

DUR denotes the duration of assay; K_M and K_S are the Michaelis-Menten and enzyme-substrate, ES dissociation constant into free enzyme and substrate respectively: The alphabet, v is the velocity of hydrolysis of starch. Accept otherwise stated, the Pseudo-first order rate constants (k) as a function of [S] is determined in1-5 minutes duration of assay using higher substrate concentration range-10-20 g/L

Table 2. Duration of different events within the active site of alpha-amylase during different duration of assay and rate constants

DA/min	DESF/min	DESD→ *P* & *E* / *min*	DESD→ *S* & *E* / *min*	k_2 / min	(k_{-1}+k_2) / min	k_{-1} / min	k_1/L /mol.min
3-low [*S*]	10.48×10^{-6}	89.98×10^{-6}	18.74×10^{-6}	$\sim 10.36 \times 10^{3}$	60.00×10^{3}	49.64×10^{3}	3.70×10^{6}
1	3.07×10^{-6}	15.52×10^{-6}	8.40×10^{-6}	56.52×10^{3}	161.00×10^{3}	104.48×10^{3}	3.70×10^{6}
2	5.82×10^{-6}	19.60×10^{-6}	27.4×10^{-6}	42.90×10^{3}	73.58×10^{3}	30.68×10^{3}	2.26×10^{6}
3	0.44×10^{-6}	6.03×10^{-6}	0.60×10^{-6}	82.26×10^{3}	1680×10^{3}	1597.74×10^{3}	13.70×10^{6}
5	15.14×10^{-6}	33.15×10^{-6}	78790.00×10^{-6}	23.79×10^{3}	23.80×10^{3}	$\sim 0.01 \times 10^{3}$	1.01×10^{6}

Except otherwise stated all values are approximations to 2 decimal places; DA, DESF, and DESD→ P and E denote duration of assay, duration of enzyme substrate complex (ES) formation, and duration of ES dissociation into product (P) and free enzyme (E); S, k_2, and k_{-1}, are the free substrate, rate constant for the formation of product, and the rate constant for the dissociation of ES into free substrate and free enzyme respectively

(duration of assay) and the type of QSSA are put into consideration in the characterisation of the kinetic parameters. Based on the fact that no total hyperbolic curve was observed for different duration of assay, the condition for the validity of different kinetic parameters is as required by rQSSA for lower *S* concentration regime in particular [7]. This is not to say that the Michaelis-Menten like equation $v = \frac{v_{\max[S_0]}}{K_s+[S_0]}$, a brainchild or corollary of Michaelian model is no longer valid in the derivation of the equations: In such situation, k_{-1} may be much greater than k_2. Thus the only differences lie in the kinetic parameter, k_{-1} and k_2 in particular; a plot of *v* versus [*S*] giving coefficient of determination (unavoidably omitted in this research for the sake of brevity) ≥ 0.98 as in this research, may be due to outliers resulting from imperfect assay and not due to approach to zero order kinetics that epitomises Michaelis-Menten kinetics.

One may state that, like previous research [21] using the same enzyme, different K_M (or K_S) and v_{max} (Table 1) were obtained for different durations of assay (Table 1). *Ab initio*, the magnitude of K_M (or K_S) expresses the degree of stability of *ES*. As this research shows, the reverse rate constant (k_{-1}) (Table 2) was much higher for high K_M (or K_S) than for low K_M (or K_S) values (Table 1). Nonetheless one cannot preclude substrate depletion and product inhibition or perhaps synthetic activity at the longest duration of assay in this research if the report by Kobayashi [12] is taken into account. Perhaps increasing amount of polysaccharide fragments and decreasing amount of parent polysaccharide with longer duration of assay may account for the observation (much lower parent polysaccharide concentration per unit time) including the values of k_2 that constituted a much smaller part of $k_{-1} + k_2$. The high magnitude of k_{-1} in 3 minutes duration of assay with lower [*S*] as well as in 1minute and 3 minutes durations of assay with higher [*S*] showed that there may be lower rate of forward reaction – dissociation into product and free enzyme – than the reverse reaction (Table 2).

While admitting that with sufficient data collection over a wide range of substrate concentration, and suitable graphical analysis, it is possible to determine from stopped flow measurement rate constants, k_{-1} and k_1 the authors [31] however, posit that the latter is very difficult to measure. But in this research, a Michaelian enzyme is such that allows the calculation of k_{-1} and k_1 as long as it is understood that any given polysaccharide substrate is equivalent to [*S*] /M_{alt} moles of maltose per litre given that within a very short period of assay only maltose is produced as explained earlier in the test. The values of k_1 showed increasing trend in 1, 2, and 5 minutes duration of assay; the values for 1 minute with high [*S*] and 3 minutes with low [*S*] were surprisingly equal; the value in 3 minutes with high [*S*] was much higher. Since $k_1 = k/[E_0]$, as defined in the text, Eq. (46) serves as a means for the determination of $k_{-1} + k_2$; besides, if k_2 is usually calculated, there is no justification why k_{-1} or $k_{-1} + k_2$ cannot be calculated. If K_s is the case, as expected when rQSSA is applicable [7], $K_S k_1/M_{alt} = k_{-1}$.

5. CONCLUSION

The equations for the determination of the durations of various events, the *ES* formation and dissociation into either product or substrate and free enzyme, before and during steady state in a given catalytic cycle are derivable and were indeed, derived. The various time regimes for each event and the rate constant for the dissociation of the *ES* can be graphically and calculationally determined as the case may be. Substrate concentration regime and duration of assay affects rate constants.

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COMPETING INTERESTS

Author has declared that no competing interests exist.

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