

1 **Title: A Two-part Approach to the Determination of**
2 **Intrinsic Rate Constants of an Alpha-amylase Catalysed**
3 **Reaction.**

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7 **ABSTRACT**

Background: There is a need for equations with which to calculate the intrinsic rate constants that can further characterise enzyme catalysed reactions despite what seems to be conventional differences in methodology in the literature.

Methods: Theoretical, experimental (Bernfeld method), and computational methods.

Objectives: 1) To derive an alternative intrinsic rate constants equations consistent with their dimension, 2) derive electrostatic intermolecular potential energy equation, (ξ_e), 3) calculate the intrinsic rate constants for forward (k_1) and reverse (k_2) reactions, and 4) define the dependence or otherwise of kinetic constants on diffusion and deduce the catalytic efficiency.

Results and discussion: The ultimate quantitative results are $\sim 64.69 \pm 0.49 \exp (+3)/ \text{min}$ (k_2) (and k_d (σ) = $\sim 60.66 \exp (+3)/ \text{min}$), $\sim 1594.48 \pm 11.99 \exp (+3) \text{ L/mol.min}$ (k_1) (and k_a (σ) = $\sim 1482.47 \exp (+3) \text{ L/mol.min}$), $\sim 58.00 \pm 10.83 \exp (+3) / \text{min}$, the apparent rate constant for reverse reaction (k_b), and $\sim 75.83 \pm 10.83 \exp (+3) / \text{min}$, the rate constant for product formation (k_3). The catalytic efficiency is: $3.025 \exp (+9) \text{ L / mol}$.

Conclusion: The relevant equations were derived. Based on the derived equations the intrinsic rate constants can be calculated. Since k_3 is $> k_b$, the k_3 is diffusion controlled and it appears that the enzyme has reached kinetic perfection. Evaluation of rate constants being it either from the perspective of diffusion dependency or independency cannot be valid without Avogadro number.

8
9 **Keywords:** *Aspergillus oryzae* alpha-amylase, apparent rate constants, intrinsic rate constants,
10 intermolecular electrostatic potential energy, diffusion control, kinetic perfection.

11 1. INTRODUCTION

12 Researchers have shown interest in what has been termed diffusion controlled and non-diffusion
13 controlled enzyme catalysed reactions [1-3]. The confusion that may arise is that there could not be an
14 encounter complex formation without any form of motion. Coupled to this is the concept of intrinsic rate
15 constants [1, 2] whose equations need to be redetermined. Solute molecules which are immobile in their
16 various positions cannot form an encounter complex let alone solute-solute (e.g. enzyme-substrate)
17 complex. However, with reference to the works of Allison and McCammon [4] and Elcock *et al.* [5], Lu and
18 McCammon [6] posit that electrostatically steered diffusion-reaction processes exist widely in chemistry
19 and biochemistry. Similar to this is the view that “to bind at an enzyme’s active site, a ligand must diffuse
20 or be transported to the enzyme’s surface, and, if the binding site is buried, the ligand must diffuse
21 through the protein to reach it [3].

22 Although the driving force for ligand binding is often ascribed to the hydrophobic effect,
23 electrostatic interactions, also, influence the binding process of both charged and nonpolar ligands [3].
24 Although Wade *et al* [3] claimed that electrostatic steering is particularly relevant for diffusion controlled
25 reaction, it needs to be made clear that binding (or preferential interaction by binding) cannot occur
26 without ultimate attractive electrostatic interaction. In line with this view is the claim that “as time
27 increases, the rate coefficient decreases because the enzymes must diffuse to the substrate in order for
28 reaction to occur” [7] following binding which brings motion close to zero.

29 Besides, it has been observed that for an enzyme in solution, the rate-determining step in
30 catalysis will be either k_f , the rate of *ES* formation, or simply k_3 , the rate of product formation [8]. If k_3 is
31 rate limiting, the catalytic events that occur after substrate binding are slower than the rate of the
32 formation of the *ES* complex [8]. If, however, k_f is rate limiting, the enzyme turns over essentially
33 instantaneously once the *ES* complex has formed [8]. In either case, according to Copeman [8], the
34 fastest rate of catalysis for an enzyme in solution is limited by the rate of diffusion of molecules in the
35 solution. This statement seems to imply that regardless of the rate limiting step, there must be initial
36 translational motion (this may exclude randomness within certain intermolecular distance).

37 Many years ago, in the 20th century, the concept of intrinsic rate constants was advanced.
38 Recently, the paper by Eser and Fitzpatrick [9], “Measurement of intrinsic rate constants of tyrosine

39 hydroxylase reaction”, showed greater clarity for the effect of viscosogens on catalytic efficiency than was
40 the case for intrinsic rate constant. Since rates and rate constants in particular are important
41 characteristics of enzyme catalysed reactions for industrial applications, therapeutics (activation and
42 detoxification of drugs), digestion, a step to the end of nutrition *etc*, there is a need to rederive or restate
43 the equation for the determination of such intrinsic rate constants with consistency in the units of the
44 kinetic constants. This is against the backdrop of the observation in the literature to the effect that, in
45 Shurr’s [1] equation, if generalisability is possible, the parameter $\rho_{eq}(r)$ designated as g , is $\exp(+U(r)/k_B T)$
46 unlike Vijaykumar *et al* [10] who defined $\rho_{eq}(r)$, given as $\exp(-U(r)/k_B T)$, as the equilibrium probability that
47 they (the substrate and the enzyme for instance) are at the distance, r from each other. One may wish to
48 know if this observation is on the basis of conceptual differences. “It could not be ideological differences”.
49 What is ultimately important is the need for equations with which to compute the intrinsic rate constants
50 that can further characterise enzyme catalysed reactions despite what seems to be conventional
51 difference in methodology in the literature which indicates a possibility of a two-part approach for the
52 determination of intrinsic rate constants. Nevertheless, redefinition of K_D and its application and, the
53 adoption of Vijaykumar *et al* [10] approach are to be addressed in the appendix section. Thus, the
54 objectives of this research are: 1) To derive an alternative intrinsic rate constants equations consistent
55 with their dimension, 2) derive electrostatic intermolecular potential equation, 3) calculate based on
56 derived equations the intrinsic rate constants, and 4) define the dependence or otherwise of kinetic
57 constants on diffusion.

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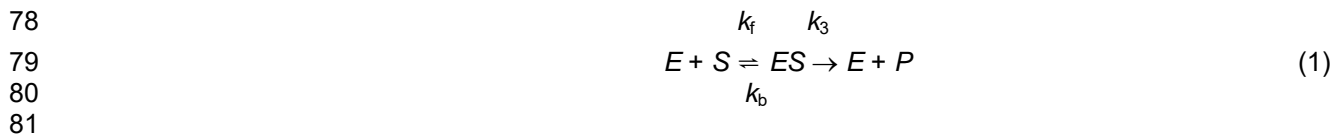
59 **2.0 Theory**

60 In this investigation one begins with the premise that if the solute-solute attraction is greater than
61 solute-solvent attraction, the aqueous solute particles may coalesce and precipitate out of solution; this
62 may be applicable to a dilute solution which becomes concentrated due to substantial partial evaporation.
63 It may arise due to continuous addition of the soluble solute. This is applicable to all solutes, the ionic and
64 nonionic solutes. Indeed any solvent, be it either polar organic – or nonpolar organic – solvent in which a
65 solute is dissolved is applicable. This is also against the backdrop of the claim that the intermolecular

66 potential ($U(r)$) is concentration-dependent] [1]. In this research however, the literature material of
67 immense interest and relevant is the work by Shurr [2].

68 2.1 Diffusion – dependent and diffusion – independent rates

69 According to the Shurr [2] “the reactions for which the rate constant for product formation and
70 release, k_3 is not diffusion-dependent include almost all reactions whose equilibria lie far to the side of the
71 products, since the rate of association of enzyme, E and products, P to form enzyme-substrate complex,
72 ES will generally be sufficiently slow in these cases that it is not diffusion dependent, and consequently
73 the conjugate dissociation step k_3 will not be diffusion dependent either”. “Only extremely rapid over-all
74 reactions have the possibility of a diffusion-dependent k_3 when the equilibrium greatly favors the products,
75 and such cases cannot be experimentally characterised by the usual steady-state methods in any case”.
76 This view seems to go against the earlier view because far right position of equilibria implies that the
77 product formation is more favoured and, as such, k_3 in the scheme below may be $\gg k_b$.



82 Where, k_f and k_b are the rate constant for the forward reaction, rate constant for the dissociation of ES to
83 free enzyme, E and free substrate, S .

84 Based on the assumption that k_3 is not diffusion controlled, the Michaelis – Menten constant, $K_M =$
85 $(k_b + k_3)/k_f$ is the only part of the expression which depends upon the diffusion coefficient. “Clearly, for
86 saturation (i.e. $[S_0] \gg K_M$) conditions, K_M may be neglected and diffusion plays no role in the reaction,
87 however fast” [1]. Also, if $k_3 \ll k_b$, then $K_M \cong k_f/k_b = k_2/k_1$ (where k_2 and k_1 are the intrinsic rate constants
88 for the backward reaction, $ES \rightarrow E + S$ and for the formation of ES respectively) so that the K_M and also
89 velocity of catalytic action, Φ are independent of diffusion processes [1]. Going by these statements,
90 despite what seems to be a contradictory statement at the beginning of the paragraph, it seems that the
91 K_M and Φ are independent of diffusion only when $k_3 \ll k_b$. Also, if k_3 is $\gg k_b$, k_3 should be diffusion
92 dependent. Finally, if the k_f and k_b are not appreciably diffusion controlled (i.e. $f k_1 \ll g k_D$), then K_M and,
93 hence, Φ will not be diffusion dependent [1]. The concern in this statement is that while g and f to be

94 given shortly, are dimensionless, k_1 and k_D (to be given shortly) are neither dimensionless nor of the same
 95 dimension. The factor, g is given as

$$96 \quad g = \exp(U(r)/k_B T) \quad (2)$$

97 Where, $U(r)$, k_B and T are the intermolecular potential energy, Boltzmann constant and thermodynamic
 98 temperature respectively.

99 Meanwhile,

$$100 \quad f = R \int_R^\infty \exp(U(r)/k_B T) \frac{dr}{r^2} \quad (3)$$

101 Where $R (= R_E + R_S)$ is the reaction radius where R_E and R_S are the hydrodynamic radii of the enzyme
 102 and substrate respectively.

$$103 \quad k_D = 4 \pi (R_E + R_S) (D_E + D_S) \quad (4)$$

104 The unit of k_D is m^3/s because the diffusion coefficients for the enzyme, D_E and substrate, D_S is m^2/s and
 105 the unit of R_E or R_S is the metre; the unit of k_1 is $1/M \cdot \text{min}$. Therefore, $f k_1$ and $g k_D$ cannot be compared
 106 qualitatively.

107 For a diffusion-dependent reaction in the absence of forces

$$108 \quad K_M = \frac{k_2}{k_1} + \frac{k_3}{k_f} = K_{eq}^{-1} + \frac{k_3}{k_D} \left(\frac{k_D + k_1}{k_1} \right) \quad (5)$$

109 However, if it is known that $k_3 \gg k_b$, then if $k_3/k_D \approx K_M$ (which may not be case because the unit of k_3 is
 110 $1/\text{min}$ and that of k_D is m^3/s), it may be concluded that the reaction is diffusion dependent. While the
 111 forces in question are not specified in the original work [1], nevertheless the equation $K_M = \frac{k_2}{k_1} + \frac{k_3}{k_f}$ may be

112 likely, unlike the equation, $K_M = K_{eq}^{-1} + \frac{k_3}{k_D} \left(\frac{k_D + k_1}{k_1} \right)$ because k_1 and k_D do not possess the same unit.

113 However, it may be speculated that such force may be intermolecular potential force referred to in paper
 114 one [1]. Besides, k_2 and k_3 are 1^{st} order rate constants ($\text{mol}/\text{mol}/\text{unit time}$) while k_1 and k_f are 2^{nd} order
 115 rate constants ($(\text{dm}^3/\text{mol})/\text{unit time}$).

116 Summarising, Shurr [1] posits that the usual enzyme reaction is independent of diffusion and,
 117 hence, the medium viscosity under the following circumstances: (a) $[S_0] \gg K_M$ and (b) $k_3 \ll k_b$; the enzyme
 118 reaction is diffusion-dependent under the following circumstances: (a) $[S_0] \lesssim K_M$ and (b) $k_3 \gg k_b$. Based on

119 this summary, one can evaluate the experimental data generated under the given conditions so as to
120 determine any dependence or otherwise on diffusion.

121 In the first paper by Shurr [2] the equations of k_b , the effective steady-state reverse rate constant
122 and k_f , the effective steady-state forward rate constant were stated without any derivational process. The
123 equations are given as

$$124 \quad k_b = \frac{k_D k_2 \exp(U(r)/k_B T)}{f k_1 + g k_D} \quad (6)$$

$$125 \quad k_f = \frac{k_D k_1}{f k_1 + g k_D} \quad (7)$$

126 Once again, it is necessary to restate that the concept of intrinsic rate constants may be
127 quantitatively useful in biochemical, medical, and biological sciences in general. Intrinsicity may be an
128 inherent tendency for a physicochemical process to occur. This may be applicable to biochemical
129 reactions leading to one or more effects such as enhanced rate constants in general if factors that can
130 enable the process exist. Thus, while Eq. (6) and Eq. (7) contain the intrinsic rate constants, the
131 presence of only a single k_D in the denominator in both equations renders them dimensionally invalid. As
132 in the literature [2], the equations may be restated with k_D appearing twice in the denominator by
133 replacing the k_1 in Eq. (6) with K_D leading to the following results.

$$134 \quad k_b = \frac{k_2 \exp(U(r)/k_B T)}{f + g} \quad (8)$$

135 Looking critically at Eq. (8) one sees that the nominator contains g such that $f + g$ as denominator should
136 be $> g$; this implies that the k_b is a fraction of the ' k_2 '. Applying the same procedure to Eq. (7) gives

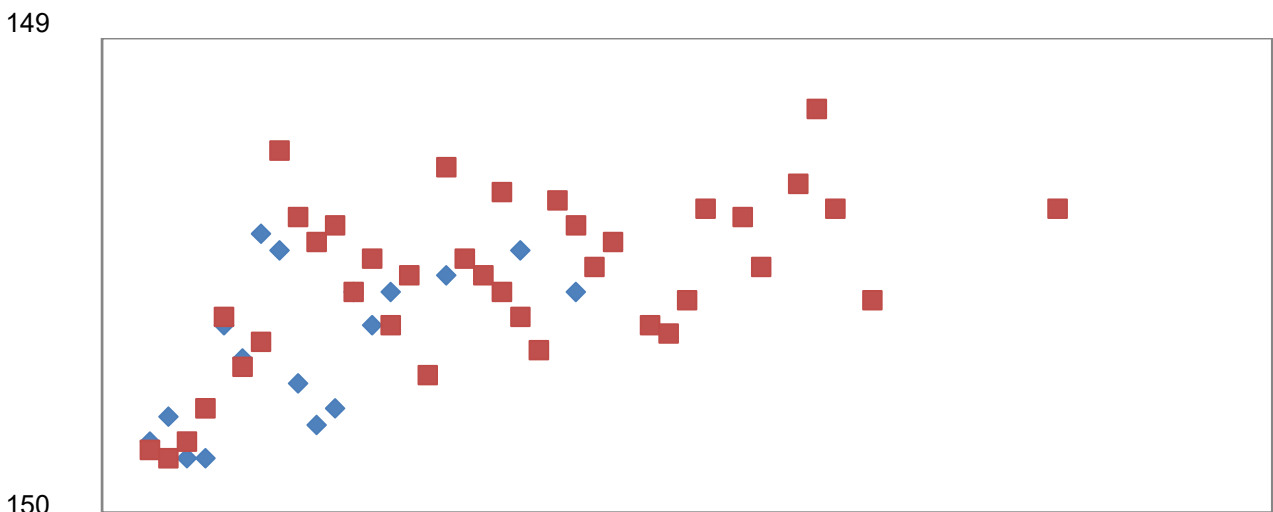
$$137 \quad k_f = \frac{k_1}{f + g} \quad (9)$$

138 Before further comment, it needs to be stated that g may be constant for a given system. Thus, making
139 the denominator in Eq. (6), Eq. (7), Eq. (8), and Eq. (9) subject of the formula and upon rearrangement
140 one obtains

$$141 \quad g = \frac{k_1 k_b}{k_2 k_f} \quad (10)$$

142 Meanwhile for a reaction to occur the reactants must be within reach of each other as applicable
143 to binding interaction between the substrate and enzyme. The substrate molecules are randomly
144 distributed (Fig. 1). The introduction of an aliquot of enzyme solution starts a reaction after translational

145 motion, the effect of swirling notwithstanding. Figure 2 illustrates the intermolecular distance in which
 146 randomness is negligible as the molecules approach each other. This is where diffusion becomes
 147 universally relevant the distinction between diffusion – dependent and diffusion – independent catalytic
 148 action of enzymes notwithstanding.

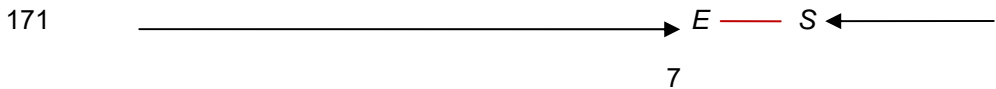


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 152 **Fig.1: Reaction mixture containing the molecules of enzyme and substrate.** Blue (\diamond) and red (\square)
 153 stand for the enzyme and the substrate molecules respectively. The positions of the symbols depict
 154 randomness. The substrate molecules are previously in random distribution before the addition of the
 155 aliquot of the enzyme solution, though swirling rapidly distributes the molecules of enzyme.



160 **Fig. 2: Initial intermolecular distance (R_x):** Initial intermolecular distance where there is infinitesimal
 161 tendency for randomness; the velocity of the solute is bulk-like.

163 If one is not mistaking, Eq. (3) seems to suggest that two particles are to be separated from an
 164 intermolecular distance equal to R to an infinite intermolecular distance; however in this research the
 165 coming together of the enzyme and substrate is also of interest because there is a need to be aware of
 166 the electrostatic force of attraction between the enzyme and the substrate. The equation for this is given
 167 in method subsection. Since the reaction radii can be seen to be constant, there must be a distant apart
 168 from which mutual weak electrostatic perturbation of the combining molecules occurs (Fig. 3); attractive
 169 perturbative interaction cannot occur at an infinite intermolecular distance if consideration is given to
 170 conservative forces.



172 **Fig. 3: Change in intermolecular distance with time**
 173 *The linear interval — illustrates the minimum intermolecular distance (R_0) needed for the*
 174 *commencement of electrostatic attraction. The longer arrow portrays the fact that the smaller molecule,*
 175 *the enzyme made a longer displacement than the substrate.*
 176 The outcome of Eq. (3) (whose physical meaning was not stated in the original work [2]) based on

177 attractive interaction can be stated as

$$178 \quad f = Rg \left(\frac{1}{r_1} - \frac{1}{r_2} \right) \quad (11)$$

179 Where, $r_2 > r_1$ and $r_1 = R$; $r_2 > R$. A careful examination of Eq. (11) should reveal that f is < 1 but > 0 .
 180 Although R has been defined, but in this research, it is replaced with the intermolecular distance (R_{ter})
 181 where terminal velocity is attained. On the basis of this and the fact that g is always > 1 if the
 182 intermolecular potential energy is negative, then k_b and k_f are fractions of k_2 and k_1 respectively. Next is
 183 the determination of the alternative equation of g based on what has been described as diffusion –
 184 dependent and diffusion – independent kinetic constants.

185 2.2 Determination of alternative equation of the intermolecular potential energy

186 The intermolecular potential energy for the diffusion-independent case depends on the
 187 relationship given as [1].

$$188 \quad \frac{K_M}{M_{\text{alt}}} = \frac{k_2}{k_1} \quad (12)$$

189 Making k_2 subject of the formula and substitute same into Eq. (10) gives

$$190 \quad g = \frac{k_b M_{\text{alt}}}{k_f K_M} \quad (13)$$

191 It is clear here that g must always be > 0 and, it could be > 1 but $\ll \infty$ if $U(r)$ is negative.

192 Therefore,

$$193 \quad U(r) = k_B T \ln (M_{\text{alt}} k_b / K_M k_f) \quad (14)$$

194 The intermolecular potential energy for the diffusion-dependent case requires the following equation [1].
 195 However, this may appear to be a contradiction considering the fact that Eq. (15) below refers to a case in
 196 the absence of forces that are explicitly defined. Clearly, subsequent derivation may confirm this absence
 197 of forces. Thus,

$$198 \quad \frac{K_M}{M_{\text{alt}}} = \frac{k_2}{k_1} + \frac{k_3}{k_f} \quad (15)$$

199 The reason for the appearance of the molar mass of maltose, the product of amylolysis has been
200 explained elsewhere [11]. Making k_2 subject of the formula and substituting into Eq. (10) gives:

$$201 \quad g = \frac{k_b}{(K_M/M_{alt} - k_3/k_f)k_f} \quad (16)$$

202 Therefore,

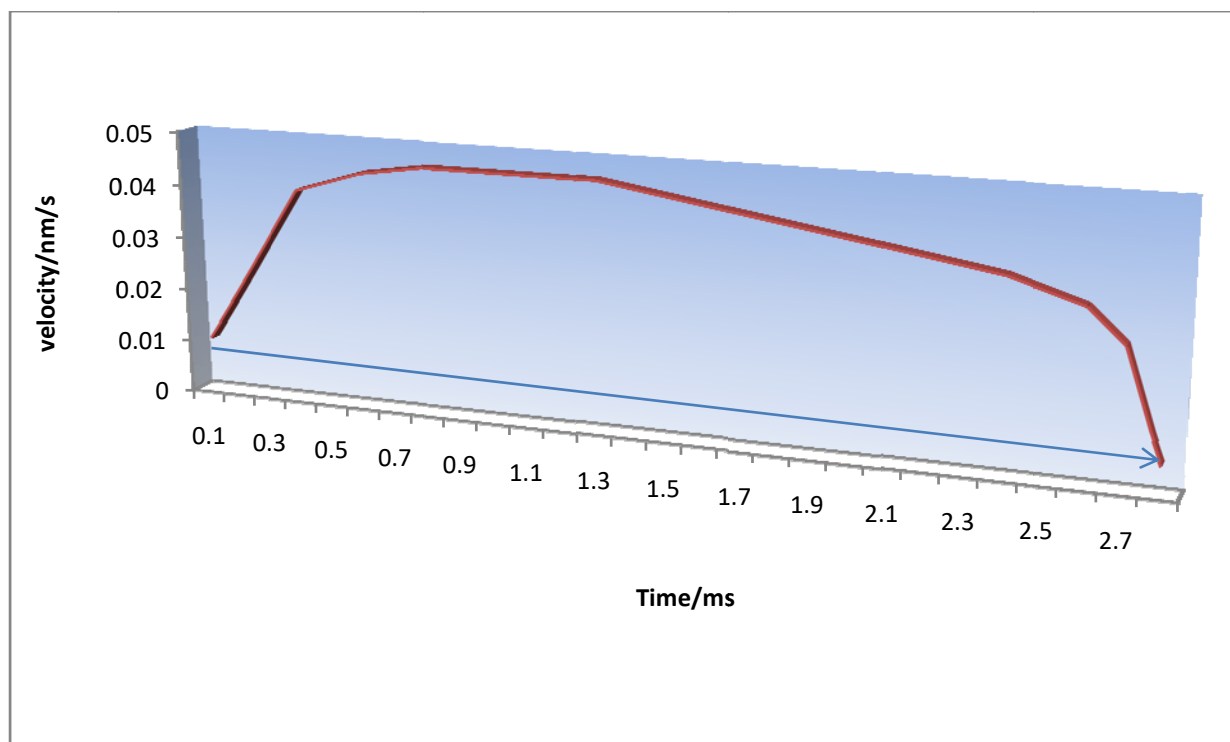
$$203 \quad U(r) = k_B T \ln \frac{k_b}{(K_M/M_{alt} - k_3/k_f)k_f} \quad (17)$$

204 However, $k_b = (K_M/M_{alt} - k_3/k_f)k_f$ confirming the fact that $U(r) = 0$. Additional reason is advanced in
205 method section. It is however, difficult to concede to the notion of zero intermolecular force if not mistaken
206 for something else. But the implication is that, the intermolecular distance approaches infinity if not
207 infinite, going by the concept of conservative field forces. Biochemical transformation is unlikely at infinite
208 dilution. The values of the k_b and k_f can be determined by fitting the equations in literature [11] to the data
209 generated experimentally. The equations are given in the method subsection.

210 **2.3 Considering electrostatic kinetic energy as a key factor in the catalytic function of enzyme.**

211 While it is obvious that potential and kinetic energies are convertible, the interest in the latter is
212 due to its direct link with translational motion which ensures delivery to target. Attraction begins when
213 minimum intermolecular distance is reached. There should be an initial increase in velocity, a decrease
214 due to viscosity, a steady velocity and sudden decrease to \approx zero velocity (Fig. 4).

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Fig. 4. Hypothetical time course of the velocity changes as solute molecules approach each other attractively: At the commencement of attractive interaction there is an increase in velocity, followed by a decrease to velocity > than bulk, and finally to \approx zero velocity as complex formation occurs. The decrease is due to solvent resistance otherwise called viscosity. The initial bulk-like and final (which \rightarrow 0) velocities are more important and relevant than the increase in velocity. Once again the blue background symbolises the fact that the reaction occurred in aqueous medium.

In a previous investigation [12], the need for minimum intermolecular distance for the commencement of attractive electrostatic interaction was established. This is relevant to very dilute reaction mixture of the enzyme and substrate in laboratory test tubes unlike *in vivo* cases where the concentration of pancreatic [13] plus intestinal alpha-amylase [14-15] is known to be very high. It is known that "substrate concentrations within cells are in the neighbourhood of their K_M values ($\exp(-6) - \exp(-2)$ M); with reference to Cha [15], Goldstein [16] and Srere [17], Schnell and Maini [14] posit that this scenario enhances the full potential of the enzymes or the intrinsic capacity of the enzyme to executes its function as may be expressible via the intrinsic reverse and forward rate constants [2]. The 1st step in this regard is to derive electrostatic interaction energy otherwise called interaction potential equation. The derivation is based on the assumption that the total work down in transit between a position in bulk before

237 collision and after collision is equal to the sum of the work down within the electrostatic field and outside
 238 the field. Thus,

$$239 \quad F_{\text{Tot}}(\mathfrak{R} - R) = \frac{k_{\text{B}}T}{L}(\mathfrak{R} - R_{\text{ter}}) + F_{\text{Elect}}(R_{\text{ter}} - R) \quad (18a)$$

240 Where, \mathfrak{R} , F_{Tot} , L , F_{Elect} and R_{ter} are the concentration-dependent bulk intermolecular distance where $U(r)$
 241 $\rightarrow 0$, total force, the cube root of the molar volume of water, the electrostatic force of attraction, and the
 242 intermolecular distance where terminal velocity is attained. F_{Tot} is given as in manuscript in preparation
 243 as:

$$244 \quad F_{\text{Tot}} = \frac{\left(\frac{\gamma(\mathfrak{R}-R)}{m} + \sqrt{\left(\frac{\gamma(\mathfrak{R}-R)}{m} \right)^2 + \left(\frac{Lu_0^2}{k_{\text{B}}T} \right)^2} \right) (k_{\text{B}}T)^2}{(u_0L)^2} \quad (18b)$$

245 $\gamma = 0.97471916$ [12] and u_0 is determined as described in the literature [18] and manuscript in
 246 preparation; $u_0 = \sqrt[2]{\frac{3\sqrt{4m(k_{\text{B}}T D_{\text{E}}/L)^2}}{m}}$ where D_{E} and m are respectively, the diffusion coefficient and mass of
 247 the enzyme molecule. The electrostatic force is given as:

$$248 \quad F_{\text{Elect}} = \frac{F_{\text{Tot}}(\mathfrak{R}-R) - \frac{k_{\text{B}}T}{L}(\mathfrak{R}-R_0)}{R_0-R} \quad (18c)$$

249 The electrostatic energy (ξ_{Elect}) is given as:

$$250 \quad \xi_{\text{Elect}} = F_{\text{Elect}}R_0 \quad (18d)$$

251 **3 MATERIALS AND METHODS**

252 **3.1 Materials**

253 **3.1.1 Chemicals**

254 *Aspergillus oryzae* alpha-amylase (EC 3.2.1.1) and soluble potato starch were purchased from
 255 Sigma – Aldrich, USA. Tris 3, 5 – dinitrosalicylic acid, maltose, and sodium potassium tartrate
 256 tetrahydrate were purchased from Kem light laboratories Mumbai, India. Hydrochloric acid, sodium
 257 hydroxide, and sodium chloride were purchased from BDH Chemical Ltd, Poole England. Distilled water
 258 was purchased from local market. The molar mass of the enzyme is ~ 52 k Da [19].

259 **3.1.2 Equipment**

260 Electronic weighing machine was purchased from Wensar Weighing Scale Limited and 721/722
 261 visible spectrophotometer was purchased from Spectrum Instruments, China; pH meter was purchased
 262 from Hanna Instruments, Italy.

263 3.2 Methods

264 3.2.1 Preparation of solution of reactants and assay.

265 The enzyme was assayed according to Bernfeld method [20] using gelatinised potato starch
 266 whose concentration range is 4-10 g/L; the weight average molecular weight of the insoluble potato
 267 starch is 7.73 (+7) g/mol [21]. Reducing sugar produced upon hydrolysis of the substrate using maltose
 268 as standard was determined at 540 nm with extinction coefficient equal to ~ 181 L/mol.cm. Concentration
 269 equal to 1 g/100 mL of potato starch was gelatinised at 100°C for 3 min and subjected to serial dilution
 270 after making up for the loss of moisture due to evaporation to give concentrations ranging between 4
 271 and 10 g/L. Concentration equal to 0.1 g/100 mL of *Aspergillus oryzae* alpha-amylase was prepared by
 272 dissolving 0.1 g of the enzyme (as the stock) in 100 mL of Tris HCl buffer at pH = 6.9. Assay of the
 273 enzyme was carried out with an enzyme concentration = 1 mg/L. The duration of assay was 3 minutes at
 274 25 °C.

275 3.2.2 The determination of kinetic constants

276 Alternative direct linear plot [22] was explored for the determination of K_M , the Michaelis – Menten
 277 constant and v_{max} , the maximum velocity of amylolysis. The equations used for the determination of
 278 kinetic constants are [11, 23]:

$$279 \quad k = M_{alt} \left(\frac{v_{max} \pm \sqrt{v_{max}^2 - 4 S_{lope} [S_0]}}{2[S_0]} \right) \quad (19)$$

280 Where, v_{max} , S_{lope} , $[S_0]$, k , and M_{alt} are the maximum velocity of amylolysis, slope from the plot of velocity
 281 of amylolysis, v versus $[S_0]/(v_{max} - v)$, concentration of the substrate, first order rate constant for the
 282 utilisation of the substrate, S and molar mass of maltose as product respectively.

$$283 \quad \ln \frac{1}{1 - \frac{[E_0] M_{alt}}{[S_0]} \ln \frac{[E_0]}{[E_0] - [ES]}} = k t \quad (20)$$

284 Where, $[E_0]$, t , and $[ES]$, are the molar concentration of the enzyme at $t = 0$, duration of ES formation and
 285 molar concentration of enzyme-substrate complex.

286
$$\ln \frac{[E_0]}{[E_0]-[ES]} = \frac{(k_{-1}+k_2)[S_0]}{K_M k} (1 - \exp(-k t)) \quad (21)$$

287 However, without prejudice to Eq. (20) and the graphical approach in literature [11], it has been realised

288 that $\ln \frac{1}{1 - \frac{[E_0]M_{alt}}{[S_0]} \ln \frac{[E_0]}{[E_0]-[ES]}} = (1 - \exp(-k t))$ if the product of the calculated values of t (Eq. (20)) and

289 calculated values of k based on Eq. (19) is substituted into Eq. (21). This can be interpreted to mean that

290 $\ln \frac{[E_0]}{[E_0]-[ES]}$ can be plotted against $[S_0] \left(\ln \frac{1}{1 - \frac{[E_0]M_{alt}}{[S_0]} \ln \frac{[E_0]}{[E_0]-[ES]}} \right) / k$ to yield a slope = $\frac{(k_{-1}+k_2)}{K_M}$ from where,

291 $k_{-1} + k_2$ is given as slope $\times K_M$. Ultimately, the k_f is then given as: slope $\times M_{alt}$.

292 3.2.3 The determination of intermolecular distance for electrostatic attraction.

293 The determination of the minimum intermolecular distance for the commencement of electrostatic
294 attraction (being also the beginning of negative potential energy of interaction in line with conservative
295 field force principle) is as previously described. The equation is given below.

296
$$R_0 = R / \left(1 - \left(S_{lope(1)} / (S_{lope(2)})^2 \right) \right) \quad (22a)$$

297 Where R (which is = $R_E + R_S$ where R_E and R_S are taken as the radii of spheres whose diffusion
298 coefficients are equal to that of the species, the enzyme and substrate respectively being considered by
299 exploring Einstein-Stoke equation); $S_{lope(1)}$ is the 1st slope from the plot of the square of effective collision
300 frequency (ν) versus $1/\mathfrak{X}$ ($\mathfrak{X} - R$) and $S_{lope(2)}$ is the 2nd slope from the plot of ν versus $1/\mathfrak{X}$ where \mathfrak{X} is the
301 concentration-dependent intermolecular distance. The frequency of collision, $\nu \approx 2\pi RDC_E$ where C_E ($[ES]$
302 N_A) is expressed in number of molecules per cubic metres, where N_A is the Avogadro number), and D is
303 taken to be equal to the sum of the D_S and D_E . The D_S for potato starch was calculated using the
304 relationship: $D_S = D_v \sqrt[3]{(M_v/M_S)}$ where D_v , M_S and M_v are the diffusion coefficient (which is $1.31 \exp(-11)$
305 m^2/s) of tomato bushy virus [24] at 298.15 K, weight average molecular mass of potato starch, and molar
306 mass of virus given as $1.06 \exp(+7)/mol$ [24].

307 In order to determine the dimensionless factor f at intermolecular distance where terminal velocity
308 is reached, such intermolecular distance (R_{ter}) needs to be determined. Hence, as in the manuscript in
309 preparation, the R_{ter} can be calculated with Eq. (22b) below.

$$(R_{\text{ter}} - R)^2 \left((24\pi^2 \eta R_E \gamma_2 R D_{E \rightarrow \infty} C_E (u_0 L)^2)^2 - \frac{48\pi^2 \eta R_E \gamma_2^2 R D_{E \rightarrow \infty} C_E (u_0 L)^2 (k_B T)^2}{m_2} \right) = (L u_0^2 k_B T)^2 \quad (22b)$$

Where η , R_E , $C_E = [ES]N_A$ (where $[ES]$ is in mol/m³ and N_A is the Avogadro number), $\gamma = 0.97471916$ a factor which enables the calculation of distance covered by the smaller particle of two particles moving towards each other as described elsewhere [12] and $D_{E \rightarrow \infty}$ is given as in the same manuscript under preparation as:

$$D_{E \rightarrow \infty} = \frac{\left(\frac{48\pi^2 \eta R_E \gamma_2^2 R C_E (u_0 L)^2 (k_B \theta)^2}{m_2} + \sqrt{\left(\frac{48\pi^2 \eta R_E \gamma_2^2 R C_E (u_0 L)^2 (k_B \theta)^2}{m_2} \right)^2 + 4(24\pi^2 \eta R_E \gamma_2 R C_E (u_0 L)^2)^2 \frac{(L u_0^2 k_B \theta)^2}{(R - R)^2}} \right)}{2(24\pi^2 \eta R_E \gamma_2 R C_E (u_0 L)^2)^2} \quad (22c)$$

3.2.4 The generalisable equations for the determination of intrinsic rate constants.

Having determined the equation for ξ_e , the dimensionless factor g , given as Eq. (2) can be determined such that the 2nd dimensionless factor f can also be determined given the value of R_0 . The method for the determination of the latter is given as Eq. (22a). One can obtain the reverse intrinsic rate constant by rearranging Eq. (8) to give

$$k_2 = k_b(f + g)/g \quad (23)$$

Likewise the forward intrinsic rate constant is obtained by rearranging Eq. (9) to give

$$k_1 = k_f(f + g) \quad (24)$$

Having previously defined f as $g(1 - R/R_0)$, Eqs (23) and (24) is restated respectively as:

$$k_2 = k_b(2 - R/R_0) \text{ (Thus } k_2 \neq f(g)) \quad (25)$$

$$k_1 = k_f g(2 - R/R_0) \text{ (Thus } k_1 = f(g)) \quad (26)$$

Equations (25) and (26) can be applied in a straight forward manner in the determination of relevant intrinsic rate constant.

3.3 Statistical Analysis

The standard deviation was determined according to the method described by Hozo *et al* [25] and by means Microsoft Excel. The mean values of 3 determinations were used to determine all the effective kinetic constants.

4. RESULTS AND DISCUSSION.

334 This research clearly is not concerned with rate constants mainly but there is a need to state
335 equations of intrinsic rate constants that are dimensionally consistent as to be very much applicable to
336 biochemical and even biophysical processes that need quantification. There is also important need to
337 characterise enzyme catalysed reactions as either diffusion – dependent or diffusion – independent
338 reaction. In this regard, there is always a need to bear in mind that, be it diffusion – dependent or diffusion
339 – independent reaction, there is always initial intermolecular motion due to attractive interaction and
340 thermal energy. Hence there is the diffusion-limited rate constant, k_D which determines the rate at which
341 the two particles (*e.g.* enzyme and substrate) diffuse towards each other [26].

342 The parameter k_D is adopted for the determination of what may be termed apparent (or effective)
343 rate constants, the 2nd order rate constant, k_f for *ES* formation and the 1st order rate constants, k_b for the
344 dissociation of *ES*. In this regard, Vijaykumar *et al* [10] derived k_f and k_b in line with what they called
345 Agmon and Szabo [26] procedure to give equations (which are different from Eq. (6) and Eq. (7) [1])
346 where $\rho_{eq}(r)$ (i.e. $\exp(-U(r)/k_B T)$) was defined as the equilibrium probability that they are at the distance, r
347 from each other. In Shurr's [1] equation, if generalisability is possible, the parameter $\rho_{eq}(r)$ designated as
348 g , is $\exp(+U(r)/k_B T)$. One may wish to know if this observation is on the basis of conceptual differences.
349 "It could not be either conventional or ideological differences". Despite these commendable efforts, the
350 issue of dimension remains unresolved. This issue was intuitively resolved as shown in Eq. (8) and Eq.
351 (9). Based in part on the method in literature [11] it is possible to calculate the intermolecular potential
352 which enabled the calculation of the equilibrium probability [26], a necessary requirement for the
353 calculation of intrinsic rate constants (Table 1). Though not shown in any table, it needs to be revealed
354 that substitution of relevant data into Eq. (14) gave ~ -2.07 kJ/mol as interaction potential for a diffusion-
355 independent reaction or rate constant if that was the case; for the diffusion-dependent reaction or rate
356 constant, $U(r) = 0$. This implies that no *ES* may have been formed.

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362 **Table 1. Apparent and intrinsic rate constants**

Results obtained based in part on modified Shurr's approach			
k_3 /min	k_b /min	k_f /L/mol.min	g
75.83 ± 10.83 exp (+3)	58.00 ± 10.83 exp (+3)	1417.48 ± 0.20 exp (+3)	
$([S]+[E])$ /mol/L/exp (-8)	k_2 / min/exp (+3)	k_1 /L/mol.min	
7.098	63.83	1617.32	0.910
8.391	64.42	1596.00	0.900
9.685	65.13	1584.25	0.891
10.979	64.77	1593.19	0.896
12.272	65.05	1586.26	0.892
14.860	64.91	1589.86	0.894
Average ± SD	64.69 ± 0.49	1594.48± 11.99	0.897 ± 0.007
Results obtained based in part on modified Vijaykumar <i>et al</i> approach			
$k_{off}(\sigma)$	~ 60.66 exp (+3) /min		
$k_a(\sigma)$	~ 1482.47 exp (+3) /min		

363 *The rate constants, k_f , k_b , k_3 , k_2 ($k_{off}(\sigma)$), and k_1 ($k_a(\sigma)$) are the 2nd order rate constant for enzyme-substrate*
 364 *formation (ES), reverse rate constant for the dissociation of ES, rate constant for product formation, and*
 365 *the intrinsic rate constants, the dissociation rate constant for the formation of E (enzyme) and S*
 366 *(substrate) and the association rate constant for the formation of ES respectively. Total enzyme*
 367 *concentration is ~1.923 exp (- 8) mol/mL; [S] + [E] and g values are approximation to 3 decimal places*
 368 *while the rest are approximations to 2 decimal places. The Michaelis-Menten constant and maximum*
 369 *velocity of amylolysis are 32.29 ± 6.04 g/L and 1458.34 ± 208.35 μM/min; the catalytic efficiency is: 3.025*
 370 *exp (+9) L / mol. In line with Shurr's [1] approach, $U(r) = k_B T \ln g$.*
 371

372 Based on another approach in this research, Eq. (23) to be specific, the attractive energy per
 373 molecule was ~ 1.02 exp (- 21) J but need not be used further. Taking in part, the approach of Shurr [2],
 374 the values of $\rho_{eq}(r)$ (or g which is = exp (+ $U(r)/k_B T$)) were determined as shown in Table 1. Having known
 375 the value of a dimensionless parameter, f (Eq. (11)) - calculated after replacing r_2 with R_0 - and g , the
 376 intrinsic rate constants were calculated according to Eq. (25) (and Eq.A.15b) and Eq. (26) (and Eq. A.
 377 18b). The modified approach of Vijaykumar *et al* [10] yielded values that are similar (though the
 378 magnitudes differ) to any of the results from modified approach of Shurr [14]. Unlike modified Shurr's
 379 approach, modified approach of Vijaykumar *et al* did not require information about g (or $\rho_{eq}(r)$) for the
 380 computation of the intrinsic rate constants. The calculated values were > the apparent rate constants as
 381 shown in Table (I). These results seemed to suggest that enzymes can achieve higher rates if challenge
 382 of viscosity and greater stability of the enzyme can be attained. As shown in Table (1), the apparent rate
 383 constant for product formation is > the reverse rate constant for the dissociation of *ES* to free enzyme and
 384 substrate. This means that the substrate undergoes conversion to product as quickly as the *ES* is formed
 385 [8]. The rate limiting step is thus, the formation of *ES*. Cognate to this is the issue of catalytic perfection

386 [8] which requires the catalytic efficiency to be very high as in this research as shown as footnote under
387 Table 1.

388 5. CONCLUSION

389 The equations for the calculation of intrinsic rate constants and were re-stated with dimension
390 consistent with the kinetic parameters determined. The equation for intermolecular electrostatic potential
391 energy is exactly derivable. The intrinsic rate constants could be higher than the apparent rates
392 constants. The apparent rate constant (k_3) for product formation and release is > the apparent reverse
393 rate constant for the release of free enzyme, E and free substrate, S . Thus, the k_3 may be diffusion
394 controlled. With reservation it seems the enzyme has attained kinetic perfection under the assay
395 condition. Besides, the research has shown that certain parameters cannot be validly quantified, without
396 Avogadro number.

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449 APPENDIX

450 Further redefinition of intrinsic rate constants

451 In this section Vijaykumar *et al* approach is introduced so as to enable the creation of results that
 452 may compare with the results obtained using Shurr's approach. Meanwhile taken k_D given as $k_D = 4 \pi (R_E$
 453 $+ R_s)(D_E + D_s)$ leaves one with a dimensional issue that has been addressed in the main text. But its
 454 application has been tied however, to the caveat that, when intermolecular distance is chosen to be
 455 beyond the range r_c (intermolecular distance allowing for mutual electrostatic interaction) of the interaction
 456 potential, then an exact expression given above for K_D , the Smoluchowski diffusion-limited reaction rate
 457 constant is applied [10]. However, one need to know of what value this could be if in line with
 458 conservative field principle, the potential energy of interaction is zero as to imply that no electrostatic
 459 attraction of the enzyme for the substrate occurs. The application is reflected in Eq. (A.3) and Eq. (A.4) for
 460 intrinsic association rate constant and intrinsic dissociation rate constant respectively. Meanwhile in line
 461 with Vijaykumar *et al* [10] the corresponding equations expected if Shurr's approach is taken into account
 462 are:

$$463 k_b = \frac{k_2 \exp(-U(r)/k_B T)}{f + \exp(-U(r)/k_B T)} \quad (\text{A.1})$$

$$464 k_f = \frac{k_1}{f + \exp(-U(r)/k_B T)} \quad (\text{A.2})$$

465 Where, in line with Vijaykumar *et al* approach [10], g (or $\rho_{eq}(r)$) is given as $\exp(-U(r)/k_B T)$.

$$466 k_{on} = \frac{k_a(\sigma)k_D(\sigma)}{k_a(\sigma) + k_D(\sigma)} \quad (\text{A.3})$$

467 Again the unit of association rate constant (k_{on}) and its corresponding intrinsic rate constant ($k_a(\sigma)$) is
 468 dm^3/mol per unit time, while $k_D(\sigma)$ is strictly dm^3 per unit time. The same dimensional issue is applicable
 469 to Eq. (A.4) below because the unit of first order rate constants, apparent or effective rate constant and
 470 the corresponding intrinsic rate constant is a dimensionless quantity per unit time. The mole concept must
 471 not be precluded!

$$472 \quad k_{off} = \frac{k_d(\sigma)k_D(\sigma)}{k_a(\sigma)+k_D(\sigma)} \quad (\text{A.4})$$

473 Where k_{off} and $k_d(\sigma)$, the effective dissociation rate constant and the corresponding intrinsic dissociation
 474 rate constant are in dimensionless quantity per unit time. Making $k_a(\sigma) = k_D(\sigma)$ leads to k_{on} being = $k_a(\sigma)/2$
 475 and k_{off} being = $k_d(\sigma)/2$. These are definitely different from Eq. (24) and Eq. (23) respectively. This is
 476 despite the fact that the intrinsic values remain > than the effective rate constants. The bone of contention
 477 is therefore, the concern for validity. However, in the literature [27] is the equation given as:

$$478 \quad k_D = 4 \pi N_A(R_E + R_s)(D_E + D_s) \quad (\text{A.5})$$

479 Where, N_A is the Avogadro constant.

480 Making $k_a(\sigma)$ and $k_d(\sigma)$ in Eq. (3) and Eq. (4) respectively subject of the formula gives
 481 respectively

$$482 \quad k_a(\sigma) = \frac{k_{on}k_D(\sigma)}{k_D(\sigma)-k_{on}} \quad (\text{A.6})$$

$$483 \quad k_d(\sigma) = \frac{k_{off}(k_a(\sigma)+k_D(\sigma))}{k_D(\sigma)} \quad (\text{A.7})$$

484 In order that Eq. (A.6) to be valid, $k_D(\sigma)$ must be > k_{on} . To be noted is the fact that application of $4\pi(R_E +$
 485 $R_s)(D_E + D_s)$ as the k_D makes it of no consequence because it is « 1. This could be seen clearly if
 486 Vijaykumar *et al* approach (Eq. (A.6) and Eq. (A.7)) is critically examined. Such a scenario in addition to
 487 the issue of dimension calls to question the approaches of Vijaykumar *et al* [10] and Shurr [1]. However,
 488 the substitution of $4\pi N_A (R_E + R_s)(D_E + D_s)$ in place of k_D should give a dimensionally and scientifically
 489 more consistent result. It is the introduction of Avogadro number that gives a correctional effect. This is
 490 thus, effected beginning from Eq. (A.6) as follows:

$$491 \quad k_a(\sigma) = \frac{4\pi k_{on}(R_E + R_s)(D_E + D_s)N_A}{4\pi(R_E + R_s)(D_E + D_s)N_A - k_{on}} \quad (\text{A.8})$$

492 The function (σ) is dropped in order to avoid technical confusion. Since $k_a(\sigma)$ is never practicably a
 493 negative parameter, $4\pi(R_E + R_S)(D_E + D_S)N_A$ must always be $> k_{on}$ which may not be the case without
 494 N_A . Moreover, $\frac{4\pi(R_E + R_S)(D_E + D_S)N_A}{4\pi(R_E + R_S)(D_E + D_S)N_A - k_{on}} > 1$ so that $k_a(\sigma)$ is always greater than k_{on} .

495 Subjecting Eq. (A.7) to similar treatment gives:

$$496 \quad k_d(\sigma) = \frac{k_{off}(k_a(\sigma) + 4\pi(R_E + R_S)(D_E + D_S)N_A)}{4\pi(R_E + R_S)(D_E + D_S)N_A} \quad (\text{A.9})$$

497 Once again, the intrinsic dissociation constant, a 1st order rate constant, is always $> k_{off}$
 498 because $\frac{(k_a(\sigma) + 4\pi(R_E + R_S)(D_E + D_S)N_A)}{4\pi(R_E + R_S)(D_E + D_S)N_A} > 1$. Though this claim is mathematically valid for both Eqs. (A.8) and
 499 (A.9), the issue of potential energy of interaction being zero as the maximum value, in line with
 500 conservative field principle, remains relevant because, $U(r)$ is zero at infinite dilution as to imply that
 501 enzyme-substrate interaction may be nonexistence.

502 In order that binding can take place there must be a form of attractive interaction between the
 503 bullet and target molecule such as enzyme and substrate, drug and deadly pathogen *etc*; this
 504 presupposes that there should be kinetic energy and consequently negative potential of interaction, a key
 505 characteristics of conservative field principle. As applied to equations arising from Shurrs approach, the
 506 equation of the equilibrium probability that two molecules are at the distance, r from each other is derived
 507 as follows:

$$508 \quad k_a(\sigma) + k_D(\sigma) = \frac{k_a(\sigma)\rho_{eq}(\sigma)k_D(\sigma)}{k_{on}} = \frac{k_d(\sigma)k_D(\sigma)}{k_{off}} \quad (\text{A.10})$$

509 Simplification and rearrangement of Eq. (A.10) gives as follows an equation exactly the same as that
 510 derived from Shurr's given equations:

$$511 \quad \rho_{eq}(\sigma) = \frac{k_d(\sigma)k_{on}}{k_a(\sigma)k_{off}} \quad (\text{A.11})$$

512 The equations, $k_a = k_a(\sigma)$, $k_d = k_d(\sigma)$ and $k_D = k_D(\sigma)$ means that these rate constants, in contrast to the
 513 effective rate constants k_{on} and k_{off} , depend on the choice of σ [Vijaykumar *et al*]. This simply means that
 514 any of the intrinsic rate constants is a function of ' σ '.

515 Meanwhile, the scientist, the biochemist in particular in the subfield, enzymology, professionals
 516 such as medics, pharmacists, dieticians and nutritionist is interested on the fate of food or drug as the
 517 case may be; this may preclude the value of σ that is beyond the range r_c of the interaction potential,

518 where $U(r)$ may be equal to zero. In such situation, binding of the enzyme to food substrate or drug as
 519 may be applicable and the drug to the pathogen may be impossible due to over dilution. Thus a negative
 520 $U(r)$ (or equivalently the kinetic energy) is desirable. This implies that the equilibrium probability $\rho_{eq}(\sigma)$
 521 (Eq. (A.11)) needs to be reintroduced. Thus,

$$522 \quad k_{on} = \frac{k_a(\sigma)\rho_{eq}(\sigma)k_D(\sigma)}{k_a(\sigma)\rho_{eq}(\sigma)+k_D(\sigma)} \quad (A.12)$$

$$523 \quad k_{off} = \frac{k_d(\sigma)k_D(\sigma)}{k_a(\sigma)\rho_{eq}(\sigma)+k_D(\sigma)} \quad (A.13)$$

524 From Eq. (A.12),

$$525 \quad k_a(\sigma) = \frac{k_{on}k_D(\sigma)}{(k_D(\sigma)-k_{on})\rho_{eq}(\sigma)} \quad (A.14)$$

526 Upon substitution of Eq. (A.11) into Eq. (A.14) and simplification one obtains,

$$527 \quad k_d(\sigma) = \frac{k_D(\sigma)k_{off}}{k_D(\sigma)-k_{on}} \quad (A.15a)$$

528 In order not to slip into former confusion, $k_D(\sigma)$ as $4\pi(R_E + R_s)(D_E + D_s)N_A$ is substituted into Eq. (A.15a) to
 529 give:

$$530 \quad k_d(\sigma) = \frac{4\pi(R_E + R_s)(D_E + D_s)N_A k_{off}}{4\pi(R_E + R_s)(D_E + D_s)N_A - k_{on}} \quad (A.15b)$$

531 From Eq. (A.13), $k_d(\sigma)$ is also given as:

$$532 \quad k_d(\sigma) = \frac{k_{off}(\sigma)(k_a(\sigma)\rho_{eq}(\sigma)+k_D(\sigma))}{k_D(\sigma)} \quad (A.16)$$

533 Substitution of Eq. (A.11) into Eq. (A.16) gives respectively 1st after rearrangement and 2nd after making
 534 $(k_a^2)(\sigma)$ subject of resulting equation the following:

$$535 \quad \frac{k_{off}^2 k_a^2}{k_d k_{on}} = \frac{k_D^2 k_{off}}{k_D - k_{on}} - k_{off} k_D \quad (A17a)$$

$$536 \quad k_a(\sigma) = \sqrt{\left(\frac{k_D(\sigma)}{k_D(\sigma)-k_{on}} - 1\right) \frac{k_d(\sigma)k_{on}k_D(\sigma)}{k_{off}}} \quad (A.17b)$$

537 Simplification of Eq. (A.17 b) gives finally

$$538 \quad k_a(\sigma) = \frac{k_{on}k_D(\sigma)}{k_D(\sigma) - k_{on}} \quad (A.18a)$$

$$539 \quad k_a(\sigma) = \frac{4\pi(R_E + R_s)(D_E + D_s)N_A k_{on}}{4\pi(R_E + R_s)(D_E + D_s)N_A - k_{on}} \quad (A.18b)$$

540 Equations (A.15a)/(15b) and (A.18a)/(A.18b) have the same denominator and most importantly as usual,
541 all the independent variables (or parameters) can either be theoretically (in particular with respect to k_D
542 (σ)) or experimentally with respect to k_{on} and k_{off} determined. While it is obvious that binding interaction is
543 a function of attractive kinetic energy which must diminish in favour of increasing potential energy during
544 dissociation, the determination of intrinsic rate constants does not require information about the potential
545 energy of interaction for their determination as long as the background approach of Vijaykumar is the
546 case. One should not shy away from the fact that the equilibrium probability is equal to one if potential
547 energy of interaction is substantially negative (or substantial and sustained mutual electrostatic attraction
548 yielding kinetic energy) as to engender enzyme-substrate formation for instance, leading to catalysis of
549 whatever kind. Substitution of Eqs (A.15b) and (A.18b) into Eq. (A.11) verifies this view.

550 With respect to Vijaykumar *et al* [10], Eqs (8) and (9) may imply that where $U(r)$ is equal to zero,
551 on account of σ being $> r_c$, a case of infinite dilution, there can never be any form of association or
552 encounter complex formation preceding enzyme-substrate complex formation, and, if there has never
553 been association there could never be any dissociation. On the other hand with respect to Shurr [2], Eqs
554 (25) and (26) show respectively that where $R_0 \rightarrow \infty$, $k_b \approx k_2 / 2$ and $k_1 = 0$ because $g = 0$ ($U(r) = 0$ at infinite
555 dilution). However, there is no question of $k_b \approx k_2 / 2$ because if there was no association, there can never
556 be dissociation of *ES*.

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