1 **_Original Research Article_**

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3 Theoretical Insight into Preferential Interaction 4 Issues and Solution Structure, and Contentious 5 Apparent Hydrated Molar Volume of Cosolute.

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9 **ABSTRACT**

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Background: There seems to be a mathematical or a conceptual error in an equation whose substitution into other equations for the determination of an apparent hydrated molar volume (V_1) of a cosolute leads to incorrect answer.

Objectives: The objectives are 1) To undertake theoretical investigation into the issues of solution structure 2) reexamine various equations related to solution structure, 3) apply derived equation in the determination of V_1, and 4) determine *m*-values and cognate preferential interaction parameter (PIP).

Methods: The research is mainly theoretical and partly experimental. Bernfeld method of enzyme assay was adopted for the generation of data.

Result and discussion: The investigation showed that equation linking chemical potential of osmolyte to solution structure is dimensionally invalid; PIP was seen as a thermodynamically extensive quantity. Equations for the graphical determination of apparent hydrated molar volume (V_1) of osmolyte were determined. With ethanol alone, there were $-$ *m*-value and + PIP; with aspirin alone, there were + *m*-value and − PIP. There is a change in sign in *m*-value with sucrose and ethanol/aspirin mixture, and a change in sign in PIP when the latter is taken as function of [ethanol]/[aspirin] and [sucrose](C_3).

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Conclusion: A solution structure is as usual determined by either a relative excess or a deficit of the solution component either in the bulk or around the macromolecular surface domain; the PIP remains thermodynamically an extensive quantity. To be valid there is need to introduce a reference standard molar concentration or activity to some equations in literature. The slope $\partial\left(\frac{\gamma_3-1}{\gamma_3}\right)/\partial C_3$ from one of the equations seems to give a valid value for V_1 (this is «1; γ_3 is activity coefficient). A known destabiliser may behave as a stabiliser being excluded. Like ethanol, aspirin as cosolute is destabilising and opposed by sucrose.

Keywords: *Keywords: Porcine pancreatic alpha amylase; preferential interaction parameter; apparent hydrated molar volume; m – value; Kirkwood-Buff integrals; ethanol; aspirin; sucrose.*

1. INTRODUCTION

For many years according to Schurr et al [1] scholars have presented a theoretical discourse on the concept of cosolute (or cosolvent otherwise known as osmolytes, organic, and inorganic compounds) preferential interaction with macromolecules. There are several equations defined by the use of different symbols but all addressing the same issues. The issues are mainly solution structure, the change in such structure whenever an osmolyte or a macromolecule is introduced into any of such solution; the effect of the osmolytes on the macromolecular three dimensional (3-D) structure is often investigated using various biophysical instrument amenable to mainly biophysical studies [2]. There is also attempt to link the interaction parameters to Kirkwood-Bulk integrals and *m*-value [3, 4]. The catalytic activities of the enzymes are also studied in the presence and absence of the osmolytes with the hope of understanding or establishing what the effect of thermodynamic temperature increase in particular may be on the function of the enzyme [2, 5]. There were theoretical studies in the past [3, 6] all geared towards gaining theoretical insight into solution structure

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29 and thermodynamic properties. It seems that there are far more biophysical studies than
30 purely biochemical studies at the experimental front. Yet it is a greater theoretical insight that
31 can facilitate the interpretation of results. Hence this research is mainly theoretical with minor
32 experimentation for the generation of data for the evaluation of the derived equations.

33 Scholars have explained the mechanism of preferential interaction of osmolytes with
34 biomolecules often in the usual consistent way [3, 4, 7]. While preferential binding (otherwise
35 called solvation by binding) leads to unfolding that accompanies displacement of water of
36 hydration and perhaps water of preferential hydration, preferential hydration leads to the
37 folding of unfolded protein which, results from the preferential exclusion of osmolyte.
38 Recently, a different mechanism as opposed to preferential hydration has been advanced for
39 the (re)folding of biomolecules. The Lifshitz's dispersion forces play a strong role in solute-
40 induced stabilization/destabilization of globular proteins [8]. The positive and/or negative
41 electrodynamic pressure (perhaps due to such forces) generated by the solute-protein
42 interaction across the water medium seems to be the fundamental mechanism by which
43 solutes affect protein stability [8]. There is also the concept of translational entropy (TE) [9]
44 regarded as the driving force that opposes conformational entropy connected to unfolding
45 thereby forcing (re)folding. Hydrophobic effect is also known to promote folding [8, 10].

46 The issue remains effects of hydration and solvation or osmolation. But there are
47 models used to separate the effect of hydration from those of solvation of proteins. Those
48 models according to Rösgen et al [3] and cited references are the exchange model, osmotic
49 stress model, the local domain model, and constant solvation model. There is attempt to
50 bypass model-dependent assumptions while targeting Kirkwood-Buff (KB) – based protein
51 solvation model to describe protein stability [3]. However, there seems to be error,
52 typographical or conceptual in nature. Most of the models are at the far end of biophysics
53 with cognate biophysical methods. The hi-tech instruments for achieving the intended
54 measurements are circular dichroism spectroscopy, infrared spectroscopy, differential
55 scanning calorimetry, Fourier transform infrared spectroscopy etc [2]. An example of

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56 biochemical methods is the assay of any enzyme whose velocity of action can be monitored
57 using spectrophotometer of any kind that may be suitable. Adequate understanding of issues
58 regarding preferential interaction parameters, protein folding, and unfolding or misfolding are
59 important to biological scientist, biochemist, pharmacist etc. This is so because of the effects
60 that may be (in) compatible to health. To this end, there is need to achieve greater
61 theoretical insight regarding molecular interaction through far reaching or robust analysis of
62 the issues involved. There is need also to shift from so much emphasis on biophysical
63 approaches to biochemical methods.

64 As indicated earlier so much research on the biophysics of cosolvent, water, and
65 protein interaction has been carried out. The objectives of this research are: 1) To undertake
66 theoretical investigation into the issues of solution structure 2) reexamine various
67 mathematical equations related to solution structure, 3) apply derived equation in the
68 determination of apparent hydrated molar volume of cosolute, V_1, and 4) determine *m*-values
69 and cognate preferential interaction parameter (PIP).

70 2.0 THEORY AND CONSEQUENCES OF PREFERENTIAL INTERACTION OF 71 SOLUTION COMPONENT WITH A BIOMOLECULE

72 There are various forms of preferential interactions implied in the radial distribution
73 function. They are water-water, solvent-solvent (in this case osmolyte), protein-water,
74 protein-protein, and osmolyte-protein interactions. Interactions may be positive or negative.
75 What Timasheff [6] called epithet, "preferential" refers to the relative affinities of the
76 interacting loci on the protein for ligand and water. Using *C* as molarity symbol, the
77 preferential hydration parameter (Γ_{21}) [11] and preferential osmolation parameter (Γ_{23}) [6]
78 can be given respectively as:

79 $$\Gamma_{21} = \left(\frac{\partial C_1}{\partial C_2}\right)_{\mathrm{T,P,\mu_1}} = -\left(\frac{\partial \mu_2}{\partial \mu_1}\right)_{\mathrm{T,P,C_2}} \quad (1)$$

80 $$\Gamma_{23} = \left(\frac{\partial C_3}{\partial C_2}\right)_{\mathrm{T,P,\mu_3}} = -\left(\frac{\partial \mu_2}{\partial \mu_3}\right)_{\mathrm{T,P,C_2}} \quad (2)$$

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81 $$\Gamma_{23} = -\left(\frac{\partial \mu_3}{\partial C_2}\right)_{C_3} \Big/ \left(\frac{\partial \mu_3}{\partial C_3}\right)_{C_2} \quad (3)$$

82 Where μ_{i} stands for chemical potential of any solution components. The preceding equations
83 are in the furtherance of the reason why $\Gamma_{2\mathrm{i}}$ cannot be a measureable quantity and a slope
84 at the same time as previously reported [12]. According to Timasheff [6],

85 $$\Gamma_{21} = -\left({}^{C_1}/_{C_3}\right)\Gamma_{23} \quad (4a)$$

86 $$= \left({}^{C_1}/_{C_3}\right)\left(\frac{\partial \mu_3}{\partial C_2}\right)_{C_3} \Big/ \left(\frac{\partial \mu_3}{\partial C_3}\right)_{C_2} \quad (4b)$$

87 $$\Gamma_{23} = -\,\Gamma_{21}\left({}^{C_3}/_{C_1}\right) \quad (5a)$$

88 $$= \left(\frac{\partial \mu_2}{\partial \mu_1}\right)_{\mathrm{T,P,C_2}}\left({}^{C_3}/_{C_1}\right) \quad (5b)$$

89 A close look at Eqs (4a) and (5a) shows that Γ_{23} cannot remain constant at different values
90 of C_3 and the latter is the only independent variable. The parameters, Γ_{23} and Γ_{21}, are known
91 to be measurable by biophysical methods such as dialysis equilibrium [6, 11], sedimentation
92 equilibrium [11], and pressure osmometry [6]. The change of Γ_{21} or Γ_{23} as the case may be
93 seems to be more important to the biochemist, pharmacist, and related specialist other than
94 biophysicist. Such changes may compromise or inhibit the function of the biomolecule as a
95 result of conformational changes, the unfolding, partial folding and dysfunctional rigidification
96 that may arise depending on the kind of cosolvent and its concentration. The change of
97 Γ_{21} is directly related to the effect of water activity, a_1 or the osmolyte osmotic pressure Π on
98 the equilibrium constant $K_{\mathrm{eq(1)}}$ of the reaction which may be conformational change [11].

99 $$\left(\frac{\partial \mathrm{In} K_{\mathrm{eq(1)}}}{\partial \mathrm{In} a_1}\right)_{\mathrm{T,P,C_2}} = -\frac{RT}{\bar{v}_1}\left(\frac{\partial \mathrm{In} K_{\mathrm{eq(1)}}}{\partial \Pi}\right)_{\mathrm{T,P,C_2}} = \Delta\Gamma_{21} \quad (6)$$

100 Where $R, T, P,$ and C_2 are gas constant, thermodynamic temperature, standard pressure, and
101 molarity of the biomolecule; $\bar{v}_1$ is the partial molar volume of water. Integrating the derivative
102 Eq. (6), gives the following.

103 $$\mathrm{In} K_{\mathrm{eq(1)}} = \Delta\Gamma_{21} \mathrm{In} a_1 \quad (7)$$

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104 $$\text{In}K_{\text{eq}(1)} = -\frac{\bar{v}_1\Pi}{RT}\Delta\Gamma_{21} \quad (8)$$

105 Timasheff [6] gives:

106 $$\text{In}a_1 = C_3\phi_3/55.56 = -\frac{\bar{v}_1\Pi}{RT} \quad (9)$$

107 Where, the parameter ϕ_3 is the osmotic coefficient of the osmolyte.

108 The following equation may hold for preferential osmolation.

109 $$\Delta\Gamma_{23} = \left(\frac{\partial \text{In}K_{\text{eq}(3)}}{\partial \text{In}a_3}\right)_{\text{T,P,C}_2} \quad (10a)$$

110 Equation appears to be a slope against the backdrop of the fact that $\Delta\Gamma_{23}$ is also a
111 measureable parameter. This issue has been raised and concluded in favour of the position
112 that the parameter cannot be an instrument based measurable parameter and a slope at the
113 same time [12]. Thus, Eq. (10a) gives,

114 $$\text{In}K_{\text{eq}(3)} = \Delta\Gamma_{23}\text{In}a_3 \quad (10b)$$

115 There are fundamental issues arising from Eq. (7), Eq. (8), and Eq. (10b). No devise
116 or equipment is known to measure $K_{\text{eq(i)}}$ directly. Rather absorbance of the biomolecule is
117 measured with variety of available biophysical equipment such as circular dichroism
118 spectroscopy, infrared spectroscopy, differential scanning calorimetry, Fourier transform
119 infrared spectroscopy etc [2]. These measurements can be taken at different concentration
120 of the osmolyte. The function of the biomolecule, enzyme for instance, may also be
121 monitored by taking the absorbance as a measure of the concentration of the product of
122 enzymatic action at different concentration of the osmolyte. Hence, the combined biophysical
123 model and biochemical model expressed via kinetic model. This issue will be readdressed
124 subsequently. It is not certain in literature, if the measuring device can measure Γ_{23} and Γ_{21}
125 simultaneously for every given concentration of the osmolyte. Devise such as pressure
126 osmometry is relevant to measurement of $\text{In}\left(\text{P}_1^{C_3}/\text{P}_1^0\right)$ or $\text{In}a_1$ [6] where, $\text{P}_1^{C_3}$ and P_1^0 are the
127 vapour pressures of water for the solution of any osmolyte (or it may be protein, whose
128 concentration may be C_2) and water free cosolute respectively.

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129 Given the information implied in Eq. (11) above, a plot of $\text{In}K_{\text{eq(i)}}$ versus Ina_1 or Ina_3

130 yields slopes, $\Delta\Gamma_{21}$ or $\Delta\Gamma_{23}$ respectively. However, if Γ_{23} is measured directly at 2 different

131 values of C_3, then, $\Gamma_{23-2} - \Gamma_{23-1} \neq \Delta\Gamma_{23}$ where Γ_{23-2} and Γ_{23-1} are the Γ_{23} values at higher

132 and lower concentration of osmolyte respectively, if by definition, $\Delta\Gamma_{23}$ is the slope as implied

133 in Eq. (10b). It seems $\Delta\Gamma_{21}$ and $\Delta\Gamma_{23}$ may represent parameters different from what they

134 were meant to be. Meanwhile a_3 and a_1 are calculated after taking measurement of relevant

135 parameters. The parameter $K_{\text{eq(3)}}$ is also calculated after taking measurement of needed

136 parameters either by biophysical or biochemical methods. In other words there are different

137 values of $K_{\text{eq(i)}}$, a_3 or a_1 which are osmolyte concentration dependent. The ratio, $\frac{\text{In}K_{\text{eq(i)}}}{\text{In}a_{\text{i}}}$ gives

138 value of $\Delta\Gamma_{2\text{ical}}$ (calculated value) that represents the preferential interaction parameter at a

139 defined C_3. This may be a mere speculation, the essence of theoretical contribution. The

140 parameter $\Delta\Gamma_{2\text{i}}$ as a slope may possess sign and magnitude that merely reflects the degree

141 of osmolation or hydration due to exclusion of osmolyte. However, according to Timasheff [

142 6], applying Eq. (4) gives, for the calculated $\Delta\Gamma_{21}$, $\Delta\Gamma_{21\text{cal}} = -\left({C_1}/{C_3}\right)\frac{\text{In}K_{\text{eq(3)}}}{\text{In}a_3}$ and for the

143 slope,

144 $$\Delta\Gamma_{21} = -\left({C_1}/{C_3}\right)\left(\frac{\partial \text{In}K_{\text{eq(3)}}}{\partial \text{In}a_3}\right)_{\text{P,T,C}_2}. \qquad (12)$$

145 The implication of Eq. (12) is that there should be different values of $\Delta\Gamma_{21}$ for different C_3

146 because $\left(\frac{\partial \text{In}K}{\partial \text{Ina}_1}\right)_{\text{P,T,C}_2}$ or $\Delta\Gamma_{23}$ is taken as slope and C_1 being molar concentration of water is

147 constant. Applying similar method to $\Delta\Gamma_{21}$ gives

148 $$\Delta\Gamma_{23} = -\left({C_3}/{C_1}\right)\left(\frac{\partial \text{In}K_{\text{eq(1)}}}{\partial \text{In}C_1}\right)_{\text{P,T,C}_2} \qquad (13)$$

149 Another implication is that,

150 $$-\left({C_1}/{C_3}\right)\frac{\text{In}K_{\text{eq(3)}}}{\text{In}C_3} \neq -\left({C_1}/{C_3}\right)\left(\frac{\partial \text{In}K_{\text{eq(3)}}}{\partial \text{In}a_3}\right)_{\text{P,T,C}_2}; -\left({C_3}/{C_1}\right)\frac{\text{In}K_{\text{eq(1)}}}{\text{In}a_1} \neq -\left({C_3}/{C_1}\right)\left(\frac{\partial \text{In}K_{\text{eq(1)}}}{\partial \text{In}C_1}\right)_{\text{P,T,C}_2}.$$

151 This analysis confirms the earlier suggestion that, the slopes may represent a parameter

152 with meaning different from what it is meant to be. This is against the backdrop of Wyman's

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153 equation known as the basic Wyman linkage equation which, according to Timasheff [6],
154 states that, “at any ligand concentration, the gradient of the equilibrium constant with respect
155 to ligand activity is equal to the change in the binding of the ligand to the biological system
156 during the course of the reaction (at constant temperature and pressure that will be
157 maintained throughout)”. Nothing seems to suggest that there is Wyman’s equivalent
158 equation for preferential hydration. The slope as the change in the binding of the ligand may
159 not give the same result of preferential exclusion according to Eq. (12). Besides, a
160 measurable quantity such as $\Delta\Gamma_{2\mathrm{i}}$ for the change or $\Gamma_{2\mathrm{i}}$ at different finite concentrations of
161 the osmolytes, extensive quantities, is also thermodynamically an extensive quantity unlike a
162 slope which is definitely an intensive quantity under clearly defined conditions.

163 As explained elsewhere [12], another reason, why calculation of $\Delta\Gamma_{2\mathrm{i}}$ may be more
164 useful for the determination of parameters is obtainable from the following equations [6, 11].
165 In their contributions, Shimizu [11] and Rösgen et al [3] attempted to relate preferential
166 interaction parameters with Kirkwood – Buff integrals (KBI). Beginning with Shimuzu [11] is
167 the equation:

168 $$\Gamma_{21} = N_{21} - \frac{C_1}{C_3} N_{23} \quad (14)$$

169 Where $C_{\mathrm{i}}(\text{or } n_{\mathrm{i}} \text{ as in literature})$ and $N_{2\mathrm{i}}$ represent respectively the density (molarity) of any
170 chemical species and the excess number of component *i* around the biomolecule, though
171 Eq. (14) is directly applicable to preferential hydration. The counterpart of Eq. (14) is the
172 osmolation case given as [6]:

173 $$\Gamma_{23} = N_{23} - \frac{C_3}{C_1} N_{21} \quad (15)$$

174 Equations (14) and (15) show that, the plot of measureable parameters versus either $1/C_3$ or
175 C_3 gives C_1N_{23} and N_{21}/C_1 respectively as slope. The equations for the change are given as
176 [Timasheff, 2002]

177 $$\left(\frac{\partial \mathrm{In} K_{\mathrm{eq}(1)}}{\partial \mathrm{In} a_1}\right)_{\mathrm{P,T,C_2}} = \Delta N_{21} - \frac{C_1}{C_3} \Delta N_{23} = \Delta\Gamma_{21} \quad (16)$$

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178 $$\left(\frac{\partial \text{In}K_{\text{eq(3)}}}{\partial \text{In}a_3}\right)_{\text{P,T,C}_2} = \Delta N_{23} - \frac{C_3}{C_1}\Delta N_{21} = \Delta\Gamma_{23} \quad (17)$$

179 Before this time and recent publication [13], $K_{\text{eq(3)}}$ and $K_{\text{eq(1)}}$ are taken symbolically to be K_{eq}
180 which would have implied that $\partial\text{In}a_3 = \Delta\Gamma_{21}\partial\text{In}a_1/\Delta\Gamma_{23}$. This is also quite different
181 from $\Delta\Gamma_{21} = -\left({C_1}/{C_3}\right)\Delta\Gamma_{23}$. Perhaps it may not be intended to be so, but nothing in literature
182 tells the story on the contrary. If the parameter, $\Delta\Gamma_{2\text{i}}$ from the plot of $\text{In}K_{\text{eq(i)}}$ versus $\text{In}a_{\text{i}}$
183 suggests that $\Delta N_{21} - \frac{C_1}{C_3}\Delta N_{23}$ or $\Delta N_{23} - \frac{C_3}{C_1}\Delta N_{21}$ is a slope then, as posited elsewhere [12] a
184 slope, such as $(\partial\text{In}K/\partial\text{In}a_1)_{\text{P,T,C}_2}$ or $(\partial\text{In}K/\partial\text{In}a_3)_{\text{P,T,C}_2}$ must not contain independent
185 variable such as C_3 given that molar concentration of water, C_1 is constant at a given
186 thermodynamic temperature.

187 **2.1 Examination of mathematical models connected to solution structure**

188 Solution structure involving the proteins can affect the function of the latter. Hence
189 the *m*-value need to be considered at all times. There are however, mathematical models or
190 equations that seem to create different forms of working equations when substituted into
191 initial equations, the derivative of the chemical potential of the osmolyte with respect to
192 osmolyte concentration. There is also relationship between the derivative of the chemical
193 potential of protein with respect to osmolyte concentration and the difference between
194 Kirkwood-Buff integral (KBI) for hydration and KBI for osmolation [3]. In this protein related
195 issue, the mathematical equations which appear in the derivatives lead to what seems to be
196 inconsistent equations. Because of the central role of *m*-value, it is reviewed here before,
197 examination of mathematical equations that affects its derivation.

198 The extent to which the interaction of different osmolytes may cause changes in the
199 structure and function of proteins in particular may differ. To Poklar *et al*. [14], the physical
200 significance of the factor, *m*-value, is not completely clear despite its wide spread use in
201 recent time, though it has been viewed as the difference in the amount of the denaturant
202 interacting with the native and denatured states of the polypeptide chain [14]. As stated

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elsewhere [15], if $C_{½}$ represent the concentration of the osmolyte needed to cause denaturation of half the given protein concentration then high *m*-value and low $C_{½}$ values indicate high effectiveness of a given denaturant [14]. Similar definition may be applicable to an osmolyte that can force folding.

Once again the *m*-value is a measure of the effect of an osmolyte on protein stability. It is the slope ($m - \text{value} = \text{dG}/\text{d}C_3$) of a plot of the native to denatured free energy change as a function of osmolyte concentration (C_3). This is the opinion of Marcelo et al [16] and as cited by Harries and Rösgen. [17]. The *m*-value is a reflection of the effect that a change in the concentration of the osmolyte (co-solute) has on the stability of the protein and it is a good measure of the effectiveness of the osmolyte's ability to force the protein either to fold or unfold. Meanwhile, the preferential interaction can also be used as an alternative descriptor for the $m - value$ [17]. This is to say that there could be a link between preferential interaction parameter and $m - \text{value}$. This can be achieved via the KBI as indicated by Rösgen *et al* [3], although with reservation due to what seems to be a mathematical mistake or perhaps, misconception in an effort to define the structural basis for the $m - \text{value}$ as found in literature [3].

In this research the slope, $\left(-\left(\frac{\partial \text{In}K_{\text{eq}(3)}}{\partial \text{C}_3}\right)_{\text{T,P}} = \frac{m}{RT}\right)$ [4], whose magnitude and sign indicate the capacity of the osmolyte to (re) fold or unfold a protein is adopted. In this regard, the protecting osmolyte has positive $m - \text{value}$ while a destabilising osmolyte has a negative $m - \text{value}$ [4].

Mathematically the structural basis for the $m - \text{value}$ is according to Rösgen *et al* [3] given as

$$-\left(\frac{\partial \text{InK}}{\partial \text{C}_3}\right)_{\text{T,P}} = \frac{m}{RT} = \frac{\Delta_{\text{N}}^{\text{D}}(G_{21}-G_{23})}{1-C_3(G_{13}-G_{33,})} \quad (18)$$

Where, $G_{13} - G_{33}$, and G_{13} and G_{33} are the apparent hydrated molar volume of the osmolyte, KBI for osmolyte hydration and osmolyte self osmolation (correlation) respectively; G_{21} and G_{23} are respectively the KB integral for hydration and osmolation of the protein. The issue in

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229 contention is about the equation [3] which perhaps is mistakenly given as

230 $$\frac{1}{1-C_3(G_{13}-G_{33})} = \frac{1}{RT}\left(\frac{\partial \mu_3}{\partial C_3}\right)_{\mathrm{T,P}} \quad (19a)$$

231 Equation (19a) has issue with dimension if the unit (L/mol) of $G_{13} - G_{33}$ is taken into account.

232 Besides, if $\left(\frac{\partial \mu_3}{\partial C_3}\right)_{\mathrm{T,P}}$ is taken as slope, any calculation to obtain $(G_{13} - G_{33})$, leads to highly

233 contentious result. Nonetheless, it is to be substituted into all relevant equations to enable

234 the verification of any claim regarding the invalidity of whatever equations that arise in this

235 research as well as in literature. However, there is a need to point out the fact that C_3 is the

236 same at the left - and right - hand sides of Eq. (19a); but the introduction of standard-state

237 molarity given as $C_i = C_i^0 = 1\ \mathrm{mol/L}$ at the right-hand side corrects the dimensional

238 inconsistency. The corollary is that there should be the expression given as $a_i = a_i^0 =$

239 $1\ \mathrm{mol/L}$. Thus Eq. (19a) can be rewritten as $\frac{1}{1-C_3(G_{13}-G_{33})} = \frac{1}{RTC_3^0}\left(\frac{\partial \mu_3}{\partial C_3}\right)_{\mathrm{T,P}}$, thereby eliminating

240 dimensional inconsistency. According to Rösgen *et al* [3], the derivative is given as

241 $$\frac{1}{RT}\left(\frac{\partial \mu_3}{\partial C_3}\right)_{\mathrm{T,P}} = \frac{1}{C_3} + \frac{G_{13}-G_{33}}{1-C_3(G_{13}-G_{33})} \quad (19b)$$

242 It is important to realise that the denominator at the left hand side also appears in the

243 derivative relating the chemical potential of the protein to the osmolyte concentration and to

244 the KBI for the hydration and osmolation of protein. This is given for the protein as follows

245 [3].

246 $$\frac{1}{RT}\left(\frac{\partial \mu_2}{\partial C_3}\right)_{\mathrm{T,P}} = \frac{G_{21}-G_{23}}{1-C_3(G_{13}-G_{33})} \quad (19c)$$

247 Henceforth, $G_{13} - G_{33}$ is designated as V_1, the apparent hydrated molar volume of the

248 osmolyte. If Eq. (19a) is substituted into Eq. (19b) one obtains

249 $$\frac{1}{RT}\left(\frac{\partial \mu_3}{\partial C_3}\right)_{\mathrm{T,P}} = \frac{1}{C_3} + \frac{V_1}{RT}\left(\frac{\partial \mu_3}{\partial C_3}\right)_{\mathrm{T,P}} \quad (20)$$

250 Rearrangement followed by integration gives

251 $$\Delta\mu_3 = \frac{RT}{1-V_1}\mathrm{In}C_3 \quad (21)$$

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252 None of these equations, Eq. (20) and Eq. (21) can be valid because the dimension or unit
253 of final result is incorrect just like the result from the original equation, Eq. (19a). If
254 thermodynamic principle is valid, then, for an ideal solution, $\Delta\mu_3 = RT\text{In}C_3$. This makes the
255 denominator in Eq. (21a or 21b) irrelevant. But under such ideal condition, $V_1 = 0$ thereby,
256 confirming the issue of relevance or validity. However, the ideal situation does not give
257 absolute equality between C_3 and a_3; this implies that, though $\Delta\mu_3 \cong RT\text{In}(C_3)$, nevertheless,
258 the difference may be important in the determination of V_1 in Eq. (21). It is important noting is
259 taken for granted. But that is not all because if ideality is precluded, the issue of dimensional
260 inaccuracy cannot be precluded.

261 If Eq. (19a) is substituted into Eq. (19c) one obtains

262 $$\frac{1}{RT}\left(\frac{\partial\mu_2}{\partial C_3}\right)_{T,P} = \frac{G_{21}-G_{23}}{RT}\left(\frac{\partial\mu_3}{\partial C_3}\right)_{\mathrm{T,P}} \qquad (22a)$$

263 Rearrangement gives

264 $$\left(\frac{\partial\mu_2}{\partial\mu_3}\right)_{T,P} = G_{21} - G_{23} = -\frac{\Gamma_{23}}{C_3} \qquad (22b)$$

265 The denominator, C_3 in Eq. (22b) makes the latter different from Eq. (2) [2]. Substitution of
266 Eq. (19a) into Eq. (18) gives

267 $$m = \Delta_{\mathrm{N}}^{\mathrm{D}}(G_{21} - G_{23})\left(\frac{\partial\mu_3}{\partial C_3}\right)_{\mathrm{T,P}} \qquad (23)$$

268 Equation (23) like any other equation arising from the use of Eq. (19a), is dimensionally
269 inaccurate.

270 On the other hand, Eq. (19a) may be rewritten as

271 $$\frac{1}{1 - C_3(G_{13}-G_{33})} = \frac{1}{RT}\left(\frac{\partial\mu_3}{\partial \text{In}C_3}\right)_{T,P} \qquad (24)$$

272 In the paper by Rösgen *et al* [3] $\left(\frac{\partial\mu_3}{\partial\text{In}C_3}\right)_{T,P}$ was used in the determination of the structural
273 basis of the *m*-value (*m* for short), which is, seemly suggestive of an initial technical error.
274 There is no issue of dimensional inaccuracy in Eq. (24) if $\text{In}C_3$ is rewritten as $\text{In}(C_3/C_3^0)$. But
275 the independent parameter cannot appear as a constant and as a variable considering the

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276 partial differential $\partial \text{In}(C_3/C_3^0)$ even if $\left(\frac{\partial \mu_3}{\partial \text{In}(C_3/C_3^0).}\right)_{T,P}$ is taken as slope. However, the

277 continuous appearance of C_3 in the equations, demands examination shortly. Before this,

278 there is need to realise that $\left(\partial \mu_3/\partial \text{In}C_3\right)_{T,P,C_3=0} = RT$ if $C_3 \rightarrow 0$ (i.e. a case of infinite dilution).

279 This seems to be the valid view of Rösgen *et al* [3]. If this is the case most of the preceding

280 equations where C_3, instead of $\text{In}C_3$, appears cannot be valid. The implication is that $G_{13} -$

281 $G_{33} = 0$ ($G_{13} = G_{33}$). However, in subsequent derivations, C_3 is regarded as one which is » 0.

282 But before this, issue regarding ideality is reexamined as follows.

283 Substitution of Eq. (24) into Eq. (19b) gives

284 $$\frac{1}{RT}\left(\frac{\partial \mu_3}{\partial C_3}\right)_{T,P} = \frac{1}{C_3} + \frac{V_1}{RT}\left(\frac{\partial \mu_3}{\partial \text{In}C_3}\right)_{\text{T,P}} \quad (25)$$

285 Rearrangement and integration gives (note that, $\partial \mu_3/\partial \text{In}C_3 = RT$) for an ideal case

286 $$\frac{\Delta \mu_3}{RT} = \text{In}C_3 + \Delta C_3 V_1 \quad (26)$$

287 But $\frac{\Delta \mu_3}{RT} = \text{In}C_3$ (or more appropriately, $\text{In}(C_3/C_3^0)$ for an ideal case, such that, $\Delta C_3 V_1 = 0$: This

288 is as often stated in literature [3]. What the value of ΔC_3 should be needs to be ascertained.

289 One cannot shy away from the fact that the adoption of standard-state molarity implies a

290 transition from 1 mol/L to values C_3« $1\ \text{mol/L}$ or > 1 mol/L as the case may be. But as stated

291 earlier, the infinitesimal difference between C_3 and a_3 may be useful for the determination

292 of V_1. In such situations, the value of V_1 obtained by calculation may be negative if activity

293 coefficient is < 1 mol/L. Ideal case is to be applied to dilute solution of the protein as follows.

294 Substitution of Eq. (24) into Eq. (19c) gives

295 $$\frac{1}{RT}\left(\frac{\partial \mu_2}{\partial C_3}\right)_{T,P} = \frac{G_{21}-G_{23}}{RT}\left(\frac{\partial \mu_3}{\partial \text{In}C_3}\right)_{\text{T,P}} \quad (27a)$$

296 $$\iint \partial\ \text{In}C_3.\left(\partial \mu_2\right)_{\text{T,P}} = (G_{21} - G_{23}).\iint \partial \mu_3.\partial \text{C}_3 \quad (27b)$$

297 Rearrangement of Eq. (27a) and integration as shown in Eq. (27b) gives

298 $$\text{In}C_3.\left(\Delta \mu_2\right)_{\text{T,P}} = (G_{21} - G_{23})\Delta \mu_3.\Delta C_3 \quad (27c)$$

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299 Once again if standard state molarity is taken into account, then $\text{In}C_3 - \text{In}C_3^0 = \text{In}C_3$: the
300 question is, what is the expression for the change in [C_i] if it cannot be defined by $\Delta C_3 = C_3 -$
301 C_3^0? Therefore, for the ideal case,

302 $$\left(\Delta\mu_2\right)_{\text{T,P}} = RT\Delta C_3(G_{21} - G_{23}) \qquad (28a)$$

303 If in Eq. (28a), $\left(\Delta\mu_2\right)_{\text{T,P}}/\Delta C_3$ $\left(\text{or } \partial\mu_2/\partial C_3\right)$ is taken as slope from the plot of $\left(\Delta\mu_2\right)_{\text{T,P}}$
304 versus C_3, the difference between the KBI for hydration of protein and KBI for its osmolation,
305 $G_{21} - G_{23}$ should be equal to slope/RT or $\left(\left(\Delta\mu_2\right)_{\text{T,P}}/\Delta C_3/RT\right)$. Considering that $C_3(G_{21} -$
306 $G_{23}) = -\Gamma_{23}$ then, the following equation may be applicable.

307 $$C_3\left(\Delta\mu_2\right)_{\text{T,P}}/\Delta C_3/RT = -\ \Gamma_{23} \qquad (28b)$$

308 The chemical potential of the protein (enzyme) can be determined if the concentration of
309 unfolded enzyme is known; the fraction of the total concentration of the cosolute-treated
310 enzyme that is unfolded multiplied by total concentration of the enzyme can used to
311 determine $\left(\Delta\mu_2\right)_{\text{T,P}}$.

312 Looking at Eq. (28b) one sees that the chemical potential of the protein can either be
313 positive or negative if respectively, the preferential interaction parameter by exclusion or
314 binding is the case. There is need to recall that for stabilising cosolute, the preferential
315 interaction parameter is negative while for the destabilising cosolute it is positive [6]. This
316 view notwithstanding, Eq. (28b) represents a precedence whose validity or scientific merit
317 remains a matter for feature investigation. Considering that the concentration (ranging from
318 nanoscale−milli−scale mol/L) of the enzyme is very low in most laboratory/clinical
319 investigation, one can correctly admit that ideality should be the case: One may need to
320 recall that Eq. (28b) is an outcome of contentious equations, namely Eq. (19a) and Eq. (24).

321 In terms of structural basis for the $m-\text{value}$

322 $$m = \Delta_{\text{N}}^{\text{D}}(G_{21} - G_{23})RT \qquad (29a)$$

323 $$m = -RT\Delta_{\text{N}}^{\text{D}}\Gamma_{23}/\ C_3 \qquad (29b)$$

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324 It seems that with respect to the $m-\text{value}$, the place of ideality may not be ruled out
325 probably on account of the fact that $\text{In}\left(1/K_{\text{eq}}\right)$ is plotted versus C_3. With the end of the
326 consideration for ideal situation, subsequent derivations take into account nonideal cases.
327 This was implied in previous research [12] but it was not explicitly stated.

328 The nonideal case is hereby considered beginning with the dependence of the
329 osmolyte's chemical potential on the osmolyte concentration. Rearrangement of Eq. (25) for
330 integration gives

331 $$\frac{1}{RT}\iint \partial\,\text{In}C_3.\left(\partial\mu_3\right)_{\text{T,P}} = \iint \frac{\partial C_3}{C_3}\partial\text{In}C_3 + \iint \frac{V_1\partial\mu_3(\partial C_3)_{\text{T,P}}}{RT} \quad (30a)$$

332 But in the light of other parameters that need to be determined, InC_3 should be replaced by
333 Ina_3 for the nonideal case (N.B. $\Delta\mu_3 = RT\text{In}a_3$). Rearrangement and integration of Eq. (25)
334 as shown in Eq. (30a) gives

335 $$\frac{1}{RT}\Delta\mu_3\text{In}a_3 = (\text{In}a_3)^2 + \frac{V_1\Delta\mu_3\Delta C_3}{RT} \quad (30b)$$

336 If V_1 is known, then the chemical potential of the osmolyte is given as

337 $$\Delta\mu_3 = \frac{(\text{In}a_3)^2}{\left(\frac{\text{In}a_3}{RT} - \frac{V_1\Delta C_3}{RT}\right)} \quad (30c)$$

338 $$V_1 = \frac{\text{In}a_3.\text{In}a_3 - (\text{In}a_3)^2}{\Delta C_3.\text{In}a_3} \quad (31)$$

339 A closer view of Eq. (31) should reveal that after substituting relevant parameters into it, the
340 calculable value of V_1 is equal to zero. This situation may not be suitable for the
341 determination of the Kirkwood-Buff integral for hydration and osmolation. The dependence of
342 chemical potential of dilute protein on the osmolyte concentration (for nonideal case) initially
343 given in Eq. (27c) is restated as (N.B. In Eq. (27c), $\Delta\mu_3 = RT\text{In}a_3$)

344 $$\left(\Delta\mu_2\right)_{\text{T,P}} = \frac{(G_{21}-G_{23})\Delta C_3 RT\text{In}a_3}{\text{In}a_3} \quad (32a)$$

345 $$= (G_{21}-G_{23})\Delta C_3 RT \quad (32b)$$

346 In the light of the Eq. (2) [6], there is need to revisit Eq. (27a). Rearranging the latter gives

347 $$\left(\frac{\partial\mu_2}{\partial\mu_3}\right)_{\text{T,P}} = \frac{(G_{21}-G_{23})RT}{RT}\left(\frac{\partial C_3}{\partial\text{In}C_3}\right)_{\text{T,P}} \quad (33a)$$

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348 $$= (G_{21} - G_{23})\left(\frac{\partial C_3}{\partial \text{In} C_3}\right)_{\text{T,P}} \quad (33b)$$

349 The implication is that

350 $$\left(\frac{\partial C_3}{\partial C_2}\right)_{\text{T,P},\mu_3} = -(G_{21} - G_{23})\left(\frac{\partial C_3}{\partial \text{In} C_3}\right)_{\text{T,P}} \quad (33c)$$

351 Rearrangement of Eq. (33c) gives

352 $$(\partial C_2)_{\text{T,P},\,\mu_3} = -\,\partial \text{In} C_3/(G_{21} - G_{23}) \quad (33d)$$

353 Looking at Eq. (33d), one sees that $(\partial C_2)_{\text{T,P},\,\mu_3}/\,\partial \text{In} C_3$ looks like a slope, appropriately from
354 the plot of C_2 versus $\text{In}(C_3/C_3^0)$. Therefore, it may not be out of place to rewrite Eq. (33d) as
355 follows:

356 $$C_2 = -\text{In}(C_3/C_3^0)/(G_{21} - G_{23}) \quad (33e)$$

357 Due to the effect and the presence of a cosolute, there may be the occurrence of a
358 preponderance of either the unfolded or (re)folded enzyme such that a plot of the
359 concentration of (un)folded versus (C_3/C_3^0) gives a slope equal to $1/(G_{21} - G_{23})$; this remains
360 conjecturally possible.

361 The nonideal case for the determination of the structural basis of the *m*-value is
362 given by rewriting Eq. (23) as follows.

363 $$m = \Delta_{\text{N}}^{\text{D}}(G_{21} - G_{23})\left(\frac{\partial \mu_3}{\partial \text{In} C_3}\right)_{\text{T,P}} = \Delta_{\text{N}}^{\text{D}}(G_{21} - G_{23})RT\left(\frac{\partial \text{In} a_3}{\partial \text{In} a_3}\right)_{\text{T,P}} \quad (34a)$$

364 $$= \frac{-RT\Delta_{\text{N}}^{\text{D}}\Gamma_{23}}{C_3}\left(\frac{\partial \text{In} a_3}{\partial \text{In} a_3}\right)_{\text{T,P}} \quad (34b)$$

365 $$\frac{C_3 m}{RT} = -\Delta_{\text{N}}^{\text{D}}\Gamma_{23} \quad (34c)$$

366 Looking closely at Eq. (34a) and Eq. (34b), it would appear that there are 3 slopes
367 viz: $\Delta_{\text{N}}^{\text{D}}(G_{21} - G_{23})$, $m - \text{value}$' and $\Delta_{\text{N}}^{\text{D}}\Gamma_{23}$. If the values of $G_{21}(= \Delta N_{21}/C_1)$ and $G_{23}(= \Delta N_{23}/C_3)$
368 are obtained from the plot of $\Delta\Gamma_{2\text{i}}$ versus C_3 or $1/\,C_3$, as the case may be, according to Eq.
369 (17) and Eq. (16) respectively then, $\Delta_{\text{N}}^{\text{D}}(G_{21} - G_{23})$ may speculatively be taken as a constant
370 or slope. Therefore, $\Delta_{\text{N}}^{\text{D}}\Gamma_{23}$ can be calculated for different values of C_3, thereby justifying the
371 claim that the former cannot be a constant quantity or slope and equipment based

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372 measurable parameter. It is definitely obvious that $a_i \neq C_3$ and as such a plot of $\text{In}a_3$ versus

373 $\text{In}C_3$ cannot be equal to one; the coefficient of determination may be one. An equation

374 relating $\text{In}a_3$ to $\text{In}C_3$ may be expressed as: $\text{In}a_3 = \text{Ş}\text{In}C_3 - \text{Í}$ where Ş and Í are the slope and

375 intercept respectively. However, this is not to justify the place of $\frac{\partial \text{In}a_3}{\partial \text{In}C_3}$ or $\left(\frac{\partial \mu_3}{\partial \text{In}C_3}\right)_{\text{T,P}}$. Previous

376 publication [12] and, as pointed out earlier in the text, has strongly shown that all except

377 $m - \text{value}$ are not slope and consequently they are extensive quantity; the other two, $\Delta_{\text{N}}^{\text{D}}\Gamma_{23}$

378 and Γ_{23} cannot be a devise based measurable parameter and constant quantities at the

379 same time. In previous research [12] the change of solvation preference upon unfolding in

380 terms of *m*-value equation was determined by eliminating the apparent hydrated molar

381 volume of the osmolyte. But if V_1 is relevant and correctly known, it may be used to calculate

382 the same parameter at different values of C_3. Thus,

383 $$\mu_3 = \mu_3^0 + RT\text{In}\left(\frac{\text{C}_3}{1 - V_1 C_3}\right) \quad (35a)$$

384 Equation (35a) is obtained by integrating the derivative (Eq. (19b)) given by KB theory [3]

385 with respect to C_3 while holding V_1 constant. Rearrangement of Eq. (35a) gives

386 $$\frac{\Delta\mu_3}{RT} = \text{In}\left(\frac{\text{C}_3}{1 - V_1 C_3}\right) \quad (35b)$$

387 **2.2.0 Apparent hydrated molar volume, a variable or a constant?**

388 Here apparent hydrated molar volume of cosolutes is to be determined based on

389 different principles. There are arguments about the validity of derived equations based on

390 fundamental equations and recent equations in this research.

391 **2.2.1 Determination based on the presumed relationship with activity coefficient.**

392 In line with Timasheff equation [6] but on the basis of molar concentration,

393 $$\frac{\Delta\mu_3}{RT} = \text{In}C_3\gamma_3 = \text{In}\left(\frac{\text{C}_3}{1 - V_1 C_3}\right) \quad (36a)$$

394 Where, the parameter γ_3 is the osmolyte activity coefficient. Although the standard reference

395 concentration can be introduced into Eq. (36a), its presence both at the right - and left - hand

396 sides makes it unnecessary.

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397 $$C_3\gamma_3 = \frac{C_3}{1-V_1C_3} \quad (36b)$$

398 $$\gamma_3 = \frac{1}{(1-V_1C_3)} \quad (36c)$$

399 One advantage of Eq. (36a or 36b) is that, *ab initio*, there is no dimensional issue, pointing to
400 a probable validity. In order to determine V_1 graphically, Eq. (36c) can be transformed into,
401 first,

402 $$\frac{1}{\gamma_3 V_1} = \frac{1}{V_1} - C_3 \quad (37a)$$

403 Rearrangement of Eq. (37a) gives

404 $$\frac{\gamma_3 - 1}{\gamma_3} = V_1C_3 \quad (37b)$$

405 A plot of $\frac{\gamma_3-1}{\gamma_3}$ versus C_3 gives a positive slope with increasing γ_3 and, if γ_3 <1 the calculated
406 values should be negative in sign. This raises question as to the validity of V_1 if it must
407 always be a positive quantity. The issue of validity is strongly applicable to Eq. (31). The
408 values of V_1 can also be determined directly from Eq. (35b) and Eq. (37b); the values
409 obtainable may be slightly higher than those obtainable from Eq. (31). This is not to support
410 the negative value of V_1, a parameter that differs for different values of C_3.

411 **2.2.2 Apparent hydrated molar volume based on alternate equations to Eq. (19a)**

412 At this juncture, an alternative first view is to be given to Eq. (19a). In the first place,
413 there was no indication as to whether or not Eq. (19a) was the original contribution of the
414 authors [3]. Besides, no reference was made to literature. The side to be taken is that the
415 equation is the original contribution of the authors. The issue of dimensional inconsistency
416 has been established and it is very apparent. The equation is restated as

417 $$\frac{1}{1-C_3(G_{13}-G_{33})} = \left(\frac{\partial\Delta\mu_3}{R\partial T}\right)_P \quad (38)$$

418 Equation (38) expresses accurate dimension because C_3V_1 is dimensionless. The value
419 of V_1, in this case is taken at a fixed concentration of the osmolyte at varying temperature
420 which expectedly affects the chemical potential of the osmolyte at constant pressure. The

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421 concentration of the osmolyte can also be affected because, the density of the aqueous
422 solvent changes with temperature. But at a fixed thermodynamic temperature and pressure,

423 $$\frac{1}{1-C_3(G_{13}-G_{33})}=\left(\frac{\Delta\mu_3}{RT}\right)_{\text{P,T}} \quad (39)$$

424 There is a deduction from Eq. (39) which is the issue of generalisation to both ideal and
425 nonideal solution of the osmolyte. The variable $\left(\frac{\Delta\mu_3}{RT}\right)_{\text{P,T}}$ may be equal to InC_3 or Ina_3, if ideal
426 or nonideal case is applicable. However, in line with Levine [18], it seems more appropriate
427 to use In X_3 (for the idea case solution) and In $\gamma_3 X_3$ (for the nonideal case). Nonetheless,
428 rearrangement of Eq. (39) gives equation which shows clearly again that V_1 can only be seen
429 as constant quantity if obtained as a slope. The equation is

430 $$\frac{1-\text{In}a_3}{\text{In}a_3}=-C_3V_1 \quad (40a)$$

431 $$1-\frac{1}{\text{In}a_3}=C_3V_1 \quad (40b)$$

432 However, if calculation is carried out, the value that is obtainable from Eq. (40a) and from the
433 slope, if a plot is carried out, is much larger and positive compared to values that may be
434 obtained from Eq. (31), Eq. (35b), and Eq. (37b).

435 An alternative 2nd view is hereby given to Eq. (19a). For the core chemical physicist
436 to proof is the introduction of apparent hydrated molar volume into Eq. (19a) to give

437 $$\frac{V_1}{1-C_3V_1}=\frac{1}{RT}\left(\frac{\partial\mu_3}{\partial C_3}\right)_{\text{T,P}} \quad (41)$$

438 Since $\frac{1}{RT}\left(\frac{\partial\mu_3}{C_3}\right)_{\text{T,P}}=\partial\text{In}a_3/\partial C_3\neq\frac{1}{1-C_3V_1}$, then what may be postulated is

439 $$(\partial\text{In}a_3/\partial C_3)_{\text{T,P}}=\frac{V_1}{1-C_3V_1} \quad (42)$$

440 Next, one integrates as follows:

441 $$\int\partial\text{In}a_3=\int\frac{V_1}{1-C_3V_1}\partial C_3 \quad (43a)$$

442 $$\text{In}a_3=-\text{In}(1-C_3V_1) \quad (43b)$$

443 $$1-\frac{1}{a_3}=C_3V_1 \quad (44)$$

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444 Nonetheless Eq. (44) remains conjectural until firmly proven by the core chemical physicist.
445 The slope of the plot of left-hand side versus right-hand side will always yield a positive
446 slope. The implication is that the apparent hydrated molar volume yielded from such plot can
447 be exceptionally large for very dilute solution of the cosolute given that for ideal solution $a_3 \cong$
448 C_3 unlike nonideal solution. The same is applicable, but to a greater extent, if mole fraction
449 is taken in place of a_3. Having used C_3 directly and InC_3 where applicable and having seen a
450 clear dimensional inconsistency, there is need to consider the use of mole fraction of
451 solution component as in literature [18]. Doing so is very likely to give very large slope as the
452 apparent hydrated molar volume for the dilute solutions well above the values obtained using
453 a_3 and γ_3.

454 The place of standard reference molar concentration or activity has general
455 implication. It is necessary to note that $\Delta\mu_3 = RT\text{In}C_\text{i} \neq RT\text{In}x_\text{i}$ where, x_i is the mole fraction
456 of the solution component given as $n_\text{i}/(n_1 + n_\text{i})$ where respectively, n_i and n_1 are the
457 number of moles of any solution components and water (usually ≅ 55.5556). For reason
458 stated elsewhere [12], the equation, $\Delta\Gamma_{2\text{i}} = \frac{\text{In}K_\text{eq}}{\text{In}a_\text{i}}$ may need to be rewritten as $\Delta\Gamma_{2\text{i}} = \frac{\text{In}K_\text{eq}}{\text{In}a_\text{i}/a_\text{i}^0}$
459 which is never equal to $\Delta\Gamma_{2\text{i}} = \frac{\text{In}K_\text{eq}}{\text{In}(\gamma_\text{i}x_\text{i})}$.

460 **2.3 The reexamination of the model equations for the determination of the**
461 **equilibrium constant, for the transition from folded to unfolded protein.**

462 Meanwhile, there is the need to make further modification of Baskakov and Bolen
463 equation [19]. The equation seems to suggest that the equilibrium constant for folding-
464 unfolding transition may be increasing with increasing concentration of the protecting
465 osmolyte in particular in the presence of a known destabilizing cosolute. This is against the
466 backdrop of the fact that the specific activity of the enzyme may be increasing with
467 increasing concentration of the protecting osmolyte. The paradox is that $[U]/[F] > 1$ for such
468 a case. Here, *U* and *F* are respectively the unfolded and folded protein. The conformational
469 adjustment by partial unfolding does not amount to instability. The issue of conformational

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470 flexibility for function dictated by the environment is well studied [20, 21]. The 2nd paradox is
471 that the *m*-value should also be negative even if the specific activity of the enzyme is > the
472 native activity.

473 Another aspect is that the specific activities though > native activity are nevertheless
474 decreasing with increasing concentration of the protecting osmolyte. In this case the values
475 of the equilibrium constant would be decreasing against what is expected from the general
476 relation $K_{eq} = [U]/[N]$. The implication is that the *m*-value would be positive. While such
477 positive sign may agree with the definition of *m*-value for a protecting osmolyte it will not
478 correlate with the result from the plot of In (1/K_{eq}) versus $[C_{\text{os}}]$ if increasing specific activities
479 may be observed with increasing concentration of the protecting osmolyte and if the original
480 equation given below is used for the calculation of K_{eq} after substituting velocity data into it.

481 $$K_{\text{eq}} = \frac{V_{\text{Max}} - V_{\text{N}}}{V_{\text{N}} - V_{\text{Min}}} = [U]/[N] \quad (45)$$

482 Increasing magnitude of V_{Max}, a function of $[N]$, appears to suggest that the magnitude of $[U]$
483 is increasing which may be incorrect. Therefore, if V_{Max} is increasing with increasing $[C_3]$, an
484 alternative equation is needed so that calculated values of K_{eq} with increasing $[C_3]$ (for
485 protecting osmolyte in particular) should be decreasing because in such a case, $[U]$ may be
486 decreasing; this should be expected from calculations using such an equation that
487 corresponds to $K_{\text{eq}} = [U]/[N]$ if the indicator of folded or refolding enzyme, the velocity of
488 catalytic action, is increasing.

489 Another scenario is the specific activity which may be > unfolded enzyme specific
490 activity but < the native enzyme activity even with increasing concentration of the protecting
491 osmolyte in the presence of strong destabiliser. The specific activities may also be
492 increasing but < native activity. In this case, the original Baskakov and Bolen equation [19]
493 cannot apply. While the equations that are to be derived shortly may not be sacrosanct, the
494 preceding issues cannot easily be ignored.

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The equilibrium constant (K_{eq}) for the process folded (*F*)→unfolded (*U*) is adapted from Pace equation [28] and modified Baskakov and Bolen equation [29] as in previous publications [12, 13, 15]. First is the equation for the assay in which the catalytic velocity of the enzyme is increasing with increasing concentration of the osmolyte. Such velocities may be < velocity of the native enzyme in a reaction mixture containing destabilising cosolute and increasing concentration of the protecting osmolyte. There may be increasing velocities with increasing concentration of the destabilising osmolyte only, but such velocities may also be < velocity of the native enzyme. This is to say that the observed velocities are < the velocity of the native enzyme which is either in a binary mixture or a ternary mixture of osmolyte. Pace defines mathematically [*U*] (this is however a fraction of the protein that is unfolded, the symbol [*U*] notwithstanding; this applicable to [*N*] for the folded) as

$$\frac{A_{\mathrm{N}}-A_{\mathrm{OBS}}}{A_{\mathrm{N}}-A_{\mathrm{MIN}}}=[U] \tag{46}$$

Where A_{N}, A_{OBS}, and A_{MIN} are absorbance of the native enzyme, the observed absorbance used to follow unfolding in the transition region, and the absorbance of the unfolded protein respectively. In place of the absorbance of the protein the absorbance of the product (within the visible region of the spectrophotometer) is taken and converted to the molar concentration of the product. Equation (46) takes the form for the case just described as follows.

$$\frac{V_{\mathrm{N}}-V_{\mathrm{OBS}}}{V_{\mathrm{N}}-V_{\mathrm{MIN}}}=[U] \tag{47}$$

Therefore,

$$[N]=1-[U] \tag{48a}$$

Substitution of Eq. (47) into Eq. (48a) gives after rearrangement

$$[N]=\frac{V_{\mathrm{OBS}}-V_{\mathrm{MIN}}}{V_{\mathrm{N}}-V_{\mathrm{OBS}}} \tag{48b}$$

Therefore, Eq. (46) takes the modified form after replacing [*U*] and [*N*] with Eq. (47) and Eq. (48b) respectively to give

$$K_{\mathrm{eq}}=\frac{V_{\mathrm{N}}-V_{\mathrm{OBS}}}{V_{\mathrm{OBS}}-V_{\mathrm{MIN}}} \tag{49}$$

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521 Here, in Eq. (49), $V_{\mathrm{N}} > V_{\mathrm{OBS}} > V_{\mathrm{MIN}}$.

522 Next is the equation for the assay in which the catalytic velocity of the enzyme is
523 increasing with increasing concentration of the protecting osmolyte. Such velocities may be
524 > velocity of the native enzyme in a reaction mixture containing destabilising cosolute and
525 increasing concentration of the protecting osmolyte. The equation is also relevant to the
526 case in which the velocities are increasing for the treated enzyme with increasing
527 concentration of the osmolyte. Such velocities should also be > the velocity of the untreated
528 native enzyme. To begin with it is imperative to realise that the original equation by Pace [22]
529 concerns the unfolding enzyme. It can be adapted for the refolding case leading to
530 hydrodynamic radius equal to or less than the radius of the native enzyme (if there is extra-
531 rigidification that is not very common).

532 $$[N] = \frac{A_{\mathrm{MAX}} - A_{\mathrm{N}}}{A_{\mathrm{MAX}} - A_{\mathrm{MIN}}} \quad (50)$$

533 Where, the parameter A_{MAX} is the absorbance of the refolded or over-folded protein. Then
534 replacing the parameters with the velocity of catalytic action of the protein gives

535 $$[N] = \frac{V_{\mathrm{MAX}} - V_{\mathrm{N}}}{V_{\mathrm{MAX}} - V_{\mathrm{MIN}}} \quad (51)$$

536 Thus substitution of Eq. (51) into $[U] = 1 - [N]$ gives

537 $$[U] = \frac{V_{\mathrm{N}} - V_{\mathrm{MIN}}}{V_{\mathrm{MAX}} - V_{\mathrm{MIN}}} \quad (52)$$

538 Therefore, the equilibrium equation should be

539 $$K_{\mathrm{eq}} = \frac{V_{\mathrm{N}} - V_{\mathrm{MIN}}}{V_{\mathrm{MAX}} - V_{\mathrm{N}}} \quad (53)$$

540 In Eq. (53), $V_{\mathrm{MAX}} > V_{\mathrm{N}} > V_{\mathrm{MIN}}$. The nominator is constant for the system but the denominator
541 is increasing with increasing values of V_{MAX}; this means that [*U*] may be decreasing such that
542 K_{eq} or [*U*]/[*N*] is decreasing as expected for a refolding protein.

543 **3.0 MATERIALS AND METHODS**

544 **3.1 Materials**

545 The chemicals used were: The chemicals used were: Sucrose (St Lious France); raw
546 (native) potato starch (Sigma Chemicals Co, USA); ethanol, hydrochloric acid and sodium

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547 chloride (BDH Chemical Ltd, Poole England); 3,5-dinitrosalicylic acid (DNA) (Lab Tech
548 Chemicals, India); Tris (Kiran Light Laboratories, USA); porcine pancreatic alpha amylase (EC
549 3.2.1.1) (Sigma, Adrich, USA); all other chemicals were of analytical grade and solutions were
550 made in distilled water. Aspirin was purchased from CP Pharmaceuticals Ltd, Ash road North,
551 Wrexham, LL 13 9UF, U.K.

552 **3.2 Equipment**

553 *pH* meter (tester) from Hanna Instruments, Mauritius; electronic weighing machine
554 from Wensar Weighing Scale Ltd, Chennai; Centrifuge, 300D model from China; 721/722
555 visible spectrophotometer from Spectrum Instruments Co Ltd, China.

556 **3.3 Methods**

557 Bernfeld method [23] of enzyme assay was adopted for the assay of the enzyme,
558 porcine pancreatic alpha amylase (PAA). Preparation of substrate and enzyme was as
559 described elsewhere [13]. Equilibrium constant for folded to unfolded transition is either
560 according to Eq. (49) or Eq. (53) as the case may be. The calculation of preferential
561 interaction parameter for folded to unfolded transition is according to Eq. (34c or 29b). Plots
562 for the determination apparent hydrated molar volume, are according to Eq. (37b), Eq. (40b)
563 and E. (44). Determination of thermodynamic activity of solvent and solute and corresponding
564 activity coefficient was as described elsewhere [13].

565 **3.4 Statistical analysis**

566 The velocities of hydrolysis were determined in triplicates. The mean values were
567 used to determine the equilibrium constant for folded to unfolded protein transition. Microsoft
568 Excel (2007) was used to plot the dependent variable versus independent variable.

569 **4. RESULTS AND DISCUSSION**

570 The important purpose of the theoretical section, a major part of this research is to
571 proffer a proper basis of any interpretation of results obtained from the changes of the
572 biomolecular function. Such change may result from change of structure due to solution
573 composition. It is very imperative that mathematical models or equations used to qualitatively

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574 and in most cases quantitatively interpret results are valid. Thus as was observed in the
575 theoretical section, the appearance of $RT\ln C_3$ gives the impression of ideality with respect to
576 the osmolyte concentration. This leads to a situation where the apparent hydrated molar
577 volume, V_1 of the osmolyte is equal to zero. The continuous use of $RT\ln C_3$ demands that C_3,
578 though low, must be much greater than 0. Although the calculated values of V_1 are shown in
579 a table of values (Table 1), which shows different values. This is applicable to Eq. (31), Eq.
580 (37b), and Eq. (40b). Mathematically and from the standpoint of dimensionality in particular,
581 equations that are not valid are Eq. (20)-Eq. (23). Equations that appear valid from the same
582 stand point due to the substitution of Eq. (24) which appears dimensionally valid are Eq. (25)
583 to Eq. (30c). But this is mainly a dimensionality issue whose validity validates in part the
584 mathematical models or equations. Thus beyond dimensional validity, substitution of Eq.
585 (24) into a particular equation does not always produce a valid equation as observed in this
586 research. This is applicable to Eq. (33a-33d), where there is need to introduce the standard
587 reference concentration equal to 1 mol/L.

588 The slopes (see Figures 1, 2, & 3) for all are positive but unlike the slope from plot
589 based on Eq. (37b) the slopes from plots based on Eq. (40b) and Eq. (44) are very high in
590 magnitude (Table 1). The plots where the data are generated are shown as Figures 1, 2, and
591 3 respectively. This is strictly for the purpose of illustration; the order of magnitude is Eq.
592 (37b) < Eq. (40b) < Eq. (44).

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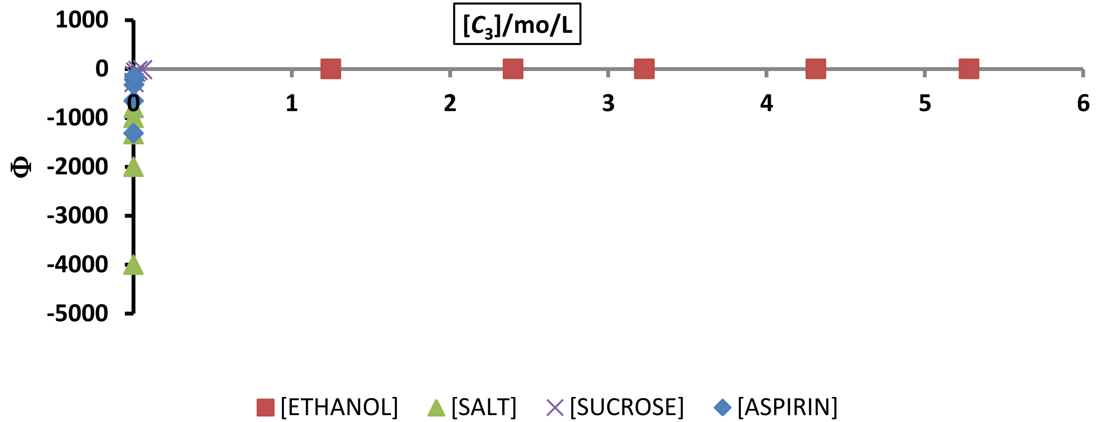
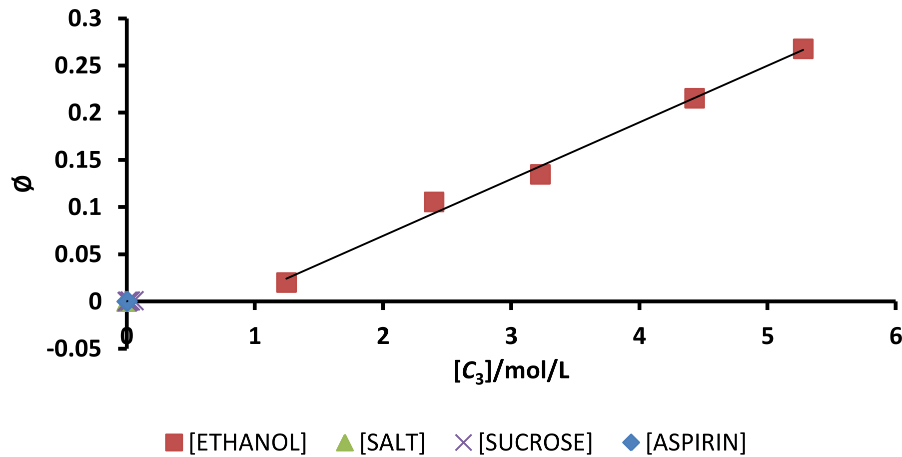
593



594

595 **Fig. 1. A plot for the determination apparent hydrated molar volume as function of C_3.**
596 *The symbol Φ stands for 1- (1/ a_3) and [C_3] is the molar concentration of cosolute. Note*
597 *curve along the axis is an expression of nonlinearity and it is also due to greater*
598 *concentration of ethanol than other cosolutes whose concentration « 1/10 mol/L.*



599

600 **Fig. 2. A plot for the determination apparent hydrated molar volume as function of**
601 **molar concentration of cosolute, C_3.** *The parameter Ø is 1– 1/γ. The shape of the graph is*
602 *as a result of the magnitude of Ø («1/100) for salt, sucrose and aspirin being « the magnitude*
603 *(» 1/100) for ethanol.*
604

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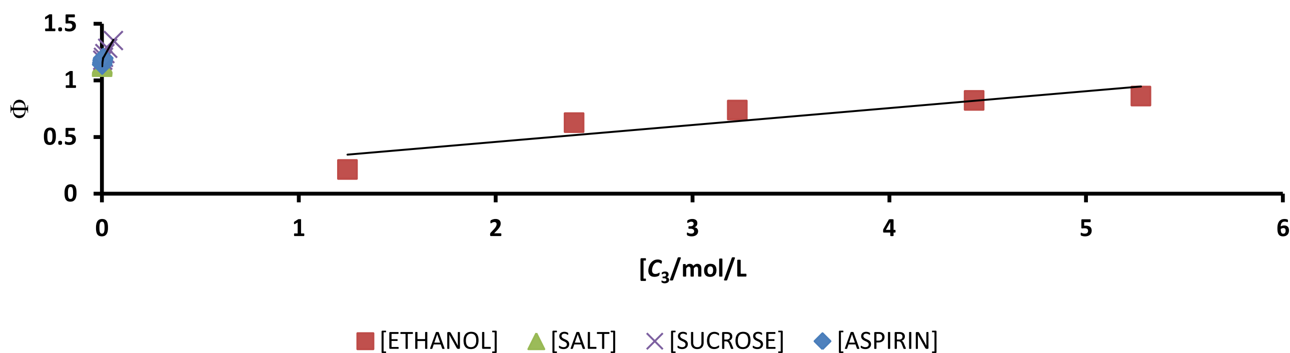

605
606
607 **Fig. 3. A plot for the determination apparent hydrated molar volume as function of C_3;**
608 *the shape of the curves is as explained under Fig. (1); the parameter Ø is 1−1/lna$_3$.*
609

610 Of particular note is the observed similar values obtained for ethanol based on Eq.
611 (40b) and Eq. (44) (Table 1). This goes to show that concentration regimes seem to create
612 different slopes and, most importantly the derived mathematical equations may not be
613 appropriate unlike Eq. (37b). The values of V_1 based on Eq. (37b) can better serve
614 calculational purpose that give positive result of other parameters when substituted into
615 relevant equations in literature [3]. For instance, but for the feature, the values based on Eq.
616 (37b) can be used to determine the change of solvation preference (this is given as $\Delta_N^D(G_{21} -$
617 $G_{23}))$ upon destabiliser denaturation if the *m*-value is known. It can also be used to
618 determine the chemical potential of osmolyte ($\Delta\mu_3$) given as $RT\text{In}\,(C_3/C_3^0)/(1 - C_3V_1)\,G_{23}$, the
619 modified form of Rösgen *et al* [‘] equation. It needs to be stated that this approach is slightly
620 different from conventional methods in literature [Levine], though it seems to enable the
621 determination of V_1 if $\Delta\mu_3$ is independently determined.

622 **Table 1. Determination of apparent hydrated molar volumes of cosolutes**

Equations	[Ethanol]	[Salt]	[Sucrose]	[Aspirin]
	V_1			
Eq. (37b)	0.060	0.147	0.014	0.067
r^2	0.995	0.954	0.966	0.847
Eq. (40b)	0.148	28.500	3.076	10.39
r^2	0.831	0.972	0.970	0.968
Eq. (44)	0.150	3 exp (+6)	3646	18918
r^2	0.832	0.813	0.566	0.749

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623 *The parameter V_1 is the apparent hydrated molar volume of cosolutes. The coefficient of*
624 *determination (r^2) is indicated so as to emphasise the departure from linearity where*
625 *applicable rather than only the occurrence of outliers arising from imperfection in the assay.*
626

627 The capacity of cosolute to force refolding or unfolding, the *m*-value was determined
628 either with a single or multiple cosolute. With ethanol alone unlike with a mixture of the
629 former and sucrose, the *m*-value was positive in sign (Table 2a). With respect to ethanol
630 alone, the positive *m*-value is similar to the result achieved in the past [13]. There has been
631 report that an organic solvent which should have been destabilising may become a stabiliser
632 [24]. To this end, "low water – content ethanol is preferentially excluded from the protein
633 surface" [24]. If this is the case, there may have been positive *m*-value for such solvent,
634 ethanol as in this research. However, the interest in this research is to use alternative
635 equation to determine the preferential parameters via Eq. (29c) and Eq. (34c).

636 **Table 2a. The *m*-values arising from cosolutes' and aqueous solvent's interactions**
637 **with the enzyme, in a reaction mixture, containing sucrose and ethanol-RTln$K_{eq(3)}$ as a**
638 **function of [Ethanol].**

[Sucrose]/mmol/L	0.00	3.57	7.19	14.38	28.76	57.75
m – value (kJL/mol^2)	+1.60	–1.78	– 3.03	– 1.67	– 0.69	– 0.44
r^2	0.855	0.969	0.932	0.943	0.992	1.000^z

639 *The data is obtained from the plot of ln1/$K_{eq(3)}$ versus [Ethanol] with different concentration of*
640 *sucrose; the superscript z indicates datum from a straight line of two-data points; (r^2) is the*
641 *coefficient of determination.*
642

643 The fact that there were negative *m*-values with a mixture of ethanol and sucrose,
644 points to the possibility that sucrose may either have reduced the solubility of water insoluble
645 native potato starch or has reduced the conformational flexibility of the enzyme needed for
646 function. According to Kurkal *et al* and references cited by the authors [25] proteins,
647 dynamics otherwise called 'loosening up' facilitates biological function of enzymes. In the
648 same vein, according to Affleck *et al* and references therein [26] the increased
649 conformational flexibility due in part, to the reduced interaction of charged and /or polar
650 amino acid residues within the enzyme molecules is caused by water's ability to effect
651 dielectric screening: This prevents unfavourable interactions between charged and /or polar

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652 residues within the protein molecule. This explains the residual biological function of the
653 enzyme. It appears therefore, that apart from water – striping effect of ethanol which
654 compromises the role of water as plasticiser, that ought to promote conformational flexibility,
655 the sucrose content may have rigidified the enzyme's three-dimensional structure. But there
656 is apparent paradox considering the fact that sucrose is known as a folding stabilizer and
657 classified as an additive which shifts the folding equilibrium from the partially unfolded state
658 toward the native state [27]. It seems generally any plot versus folding destabiliser and
659 folding stabiliser should respectively give negative and positive *m*-value.

660 One may wish to add that, it is the enzyme primary structure that can determine the
661 effectiveness of a cosolute to unfold or rigidify its structure. Without residual biological
662 function of the enzyme, the determination of *m*-value based on kinetics/velocity of biological
663 function will be impossible. There is also the need to add that where there is negative *m*-
664 value there is preferential dehydration [3, 6]. There is need also to suggest that the presence
665 of sucrose "unusually enhanced the effectiveness of ethanol to act as destabiliser" (this is
666 however, mere speculation) by rather, decreasing the solubility of the substrate. But the plot
667 versus sucrose, due perhaps to the concentration regime, exhibited in all except with lowest
668 concentration of ethanol, the usual positive *m*-values [Table 2b].

669 **Table 2b. The *m*-values arising from cosolutes' and aqueous solvent's interactions**
670 **with the enzyme, in a reaction mixture, containing sucrose and ethanol-RTIn$K_{eq(3)}$ as a**
671 **function of [Sucrose].**

[Ethanol]/mol/L	1.247	~3.223	5.279
m – value (kJL/mol^2)	− 27.93	28.55	276.69
r^2	0.870	0.533	0.955

672 *The data is obtained from the plot of In1/ $K_{eq(3)}$ versus [sucrose] with different concentration*
673 *of ethanol; (r^2) is the coefficient of determination.*
674
675 Unlike ethanol, aspirin showed what it may be, a folding destabiliser, having no
676 effect on substrate solubility which is unexpected considering the fact that while ethanol is a
677 solvent, aspirin is not. The *m*-values generated from the plot versus [Aspirin] with and
678 without sucrose yielded negative *m*-values (Table 3a). It thus, appears that aspirin is a

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679 folding destabiliser to the enzyme porcine pancreatic alpha-amylase. Therefore, as
680 explained by Singh et al including their references [28], the critical factor is the partitioning
681 between water and osmolyte (in this case aspirin) at solvent-exposed surfaces of a protein
682 whereby denaturing cosolute accumulate or bind at the surface and promote unfolding as
683 applicable to the effect of aspirin on the enzyme.

684 **Table 3a. The *m*-values arising from cosolutes' and aqueous solvent's interactions**
685 **with the enzyme, in a reaction mixture, containing sucrose and aspirin-RTln$K_{eq(3)}$ as a**
686 **function of [Aspirin].**

[Sucrose]/mmol/L	0.00	7.19	14.38	28.76	57.75
m – value (kJL/mol^2)	– 188.55	– 3754.56	– 4177.46	– 2453.36	– 2174.34
r^2	0.865	1.000^z	1.000	0.993	0.989

687 *The data is obtained from the plot of In1/ $K_{eq(3)}$ versus [Aspirin] with different concentration of*
688 *sucrose; the superscript z indicates datum from a straight line of two-data points; (r^2) is the*
689 *coefficient of determination.*
690
691 With a mixture of aspirin and sucrose the *m*-values from the plot versus [Sucrose]
692 were all positive (Table 3b) in line with the view that stabilizing osmolytes have an
693 overwhelming tendency to be excluded from the protein surface, forcing the polypeptide to
694 adopt a compactly folded structure with a minimum of exposed surface area. On this issue of
695 *m*-values, it is pertinent to note that it may not be unusual that sucrose was unable to totally
696 refold rather than over-rigidify because it has been observed that similar observation was
697 made in respect of chymotrypsin, chymotrypsin, and ribonuclease [29].

698 **Table 3b. The *m*-values arising from cosolutes' and aqueous solvent's interactions**
699 **with the enzyme, in a reaction mixture, containing sucrose and aspirin-RTln$K_{eq(3)}$ as a**
700 **function of [Sucrose].**

[Aspirin]/mol/L	0.76	3.05	6.10
m – value (kJL/mol^2)	41.10	96.39	57.45
r^2	0.738	0.797	1.000^z

701 *The data is obtained from the plot of In1/ $K_{eq(3)}$ versus [sucrose] with different concentration*
702 *of aspirin; the superscript z indicates datum from a straight line of two-data points; (r^2) is the*
703 *coefficient of determination.*
704
705 Next is the issue of preferential solvation, hydration and osmolation, which has been
706 described as a thermodynamic quantity that describes the protein occupancy by the

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707 cosolvent/water molecules [24]. The results in this research are based on either Eq. (29b) or
708 Eq. (34c) which shows direct link between the *m*-value and change in preferential interaction
709 parameter (PIP). With ethanol alone, the PIP values were unexpectedly negative (Table 4a).

710 **Table 4a. Preferential interaction parameters in a reaction mixture containing ethanol**
711 **as the only cosolute.**

[Ethanol]/mol/L	1.247	~2.398	~3.228	4.311	5.279
$\Delta_N^D\Gamma_{23}$	– 0.776	–1.492	–2.008	– 2.681	– 3.283

712 *The symbol $\Delta_N^D\Gamma_{23}$ is the preferential interaction parameter for folding-unfolding transition.*
713
714 This has been observed for chymotrypsin elsewhere [24]; but with the presence of
715 sucrose as part of ternary mixture of cosolutes, PIP values as a function of [Ethanol],
716 showed positive sign (Table 4b) because, *ab initio* the *m*-values were negative in sign. This
717 is as expected if the known effect of ethanol is taking into account. Such effect includes the
718 change in the protein-water interactions and consequently, the modulation of the protein
719 stability. The stripping of weakly bound water [6, 30] due to the binding of ethanol is
720 inevitable, thereby leading to altered function of the enzyme. However, the PIP values as a
721 function of [Sucrose], gave in all, except with lowest [Ethanol], negative values of PIP (Table
722 4c). This may be as a result of the greater solubilising effect of ethanol at its higher
723 concentrations, on the insoluble raw starch.

724 **Table 4b. Preferential interaction parameters in a reaction mixture containing ethanol**
725 **and sucrose- $\Delta_N^D\Gamma_{23}$ is taken as a function of ethanol concentration.**

[Ethanol]/mol/L	[Sucrose]/mmol/L				
	3.57	7.19	14.38	28.76	57.75
	$\Delta_N^D\Gamma_{23}$				
1.247	~0.859	1.464	0.808	0.332	0.213
~3.228	~2.224	3.789	2.092	0.859	0.552
~5.279	3.637	6.197	3.421	1.404	0.903

726 *The symbol $\Delta_N^D\Gamma_{23}$ is the preferential interaction parameter for folding-unfolding transition; it*
727 *is obtained as a function of ethanol concentration (See either Eq. (34b) or Eq. (28b)) with*
728 *different concentration of sucrose.*
729
730
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733 **Table 4c. Preferential interaction parameters in a reaction mixture containing ethanol**
734 **and sucrose- $\Delta_N^D \Gamma_{23}$ is taken as a function of sucrose concentration.**

[Sucrose]/mmol/L	[Ethanol]/mol/L		
	1.247	3.228	5.279
	$\Delta_N^D \Gamma_{23}$		
3.57	0.039	– 0.040	– 3.83
7.19	0.078	– 0.080	– 0.771
14.38	0.156	– 0.159	– 1.543
28.76	0.311	– 0.318	– 3.086
57.75	0.625	– 0.639	– 6.197

735 *The symbol $\Delta_N^D \Gamma_{23}$ is the preferential interaction parameter for folding-unfolding transition; it*
736 *is obtained as a function of sucrose concentration (See either Eq. (34b) or Eq. (28b)) with*
737 *different concentration of ethanol.*
738

739 The PIP values as a function of [Aspirin] only conformed to conventional expectation
740 of being positive thereby suggesting a binding interaction with enzyme. The magnitudes
741 showed increasing trend (Table 5a). Also, the PIP values as a function of [Aspirin] with
742 different [Sucrose] were positive pointing to the fact that aspirin has a strong affinity for the
743 enzyme despite the presence of sucrose (Table 5b).

744 **Table 5a. Preferential interaction parameters in a reaction mixture containing aspirin**
745 **as the only cosolute.**

[Aspirin]/mmol/L	1.247	~2.398	~3.228	4.311	5.279
$\Delta_N^D \Gamma_{23}$	0.556	1.119	2.230	3.349	4.460

746 The symbol $\Delta_N^D \Gamma_{23}$ *is the preferential interaction parameter for folding-unfolding transition.*
747

748 **Table 5b. Preferential interaction parameters in a reaction mixture containing aspirin**
749 **and sucrose- $\Delta_N^D \Gamma_{23}$ is taken as a function of aspirin concentration.**

[Aspirin]/mmol/L	[Sucrose]/mmol/L			
	7.19	14.38	28.76	57.75
	$\Delta_N^D \Gamma_{23}$			
0.76	1.107	1.231	0.723	0.641
3.05	4.441	4.941	2.902	2.572
6.10	8.882	9.882	5.804	5.143

750 *The symbol $\Delta_N^D \Gamma_{23}$ is the preferential interaction parameter for folding-unfolding transition; it*
751 *is obtained as a function of aspirin concentration with different concentration of sucrose.*
752

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753 The PIP values as a function of [Sucrose] with different [Aspirin] were negative
754 (Table 5c); this again conforms to the conventional behaviour of sucrose as a stabilising
755 osmolyte. This seems to suggest that the concentration regime of sucrose is sufficient to
756 cancel the initial effect of aspirin if the enzyme was incubated in an aqueous solution of
757 aspirin. Meanwhile, there are theories of preferential interaction which are Kirkwood-Buff,
758 cavity formation, solvophobic/solvophilic, surface tension theories *etc* with which to elucidate
759 the results. By being excluded sucrose unlike ethanol and aspirin, from the peptide back
760 bone as to imply solvophobic effect, the protein is said to fold, leaving, as a consequence,
761 excess of the cosolute in the bulk solution. Here, according to Rösgen *et al* and reference
762 cited [3] the Kirkwood-Buff theory comes into relevance. Thus an enrichment or relative
763 excess of water around protein corresponds to a positive G_{21} (positive correlation resulting
764 from exclusion), whereas a depletion of water around protein corresponds to a negative G_{21}
765 (negative correlation which is due to preferential binding) [4].

766 There is a recent theory implicating Lifshitz's dispersion forces which are inextricably
767 involved in solute-induced stabilization/destabilization of globular proteins [8]. The positive
768 and/or negative electrodynamic pressure generated by the solute–protein interaction
769 (perhaps as implied in Lifshitz's dispersion forces) across the water medium seems to be the
770 fundamental mechanism by which solutes affect protein stability [8] as against preferential
771 hydration or exclusion of cosolute.

772 As stated elsewhere [15] another aspect of the effect of sucrose is the energy cost
773 of cavity formation in order to accommodate the expanded conformation of the unfolded
774 enzymes. The free energy needed to accommodate the expanded form in the presence of
775 sucrose is high. Therefore, in line with Lech atelier principle, there was a shift towards the
776 direction of less expanded or more compacted species within native state ensemble [29, 31].
777 This may be as a result of exclusion of sucrose from enzyme due to increase in surface
778 tension of water occasioned by sucrose in a manner dependent on the proteins' surface
779 area. Increase in surface tension may increase the free energy cost for cavity formation for

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780 the accommodation the unfolded if it exists. If the case of glycerol is a general one [32] then
781 sucrose, by all the means enunciated may have achieved partial refolding of the enzyme by
782 strengthening hydrophobic interaction and by overcoming the unfavourable electrostatic
783 interaction between charged residues [32]. Since destabilisers and stabilisers have opposing
784 effects, one may conjecture that unlike sucrose, ethanol, in particular, and aspirin which
785 binds may be decreasing the surface tension, reducing the energy cost for cavity formation
786 for the accommodation of the expanded unfolded enzyme.

787 **Table 5c. Preferential interaction parameters in a reaction mixture containing aspirin**
788 **and sucrose- $\Delta_N^D \Gamma_{23}$ is taken as a function of sucrose concentration.**

[Sucrose]/mmol/L	[Aspirin]/mmol/L		
3.57	0.76	3.05	6.10
	$\Delta_N^D \Gamma_{23}$		
7.19	– 0.115	– 0.269	– 0.160
14.38	– 0.229	– 0.537	– 0.320
28.76	– 0.458	– 1.075	– 0.641
57.75	– 0.921	– 2.159	– 1.287

789 *The symbol $\Delta_N^D \Gamma_{23}$ is the preferential interaction parameter for folding-unfolding transition; it*
790 *is obtained as a function of sucrose concentration with different concentration of aspirin.*
791
792 In summary, it is pertinent to state that lack of details occasioned by what may have
793 been considered as basic principles requiring less attention results in perceived technical or
794 conceptual error in well-intended research papers in literature. Although a dimensionally
795 consistent equation may be the case, it does not necessarily imply that the equation/model is
796 suitable for the qualitative and quantitative analysis of issues being addressed. On the other
797 hand the issue/concept being addressed may be clear, the theoretical background, both
798 qualitative and mathematical may become invalid if in particular, the mathematical models,
799 give results that are dimensionally inconsistent with the parameters to be determined. This is
800 the hallmark of various observations in literature that motivated this research. The
801 contentious issue was precipitated by the observation in Eq. (19a), as found in literature,
802 which shows that the left hand side is dimensionless while the right hand side is not (unit is

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803 litre/mol.). The appearance of $\left(\frac{\partial\mu_3}{\partial \text{In} C_3}\right)_{\text{T,P}}$ and $\frac{\partial\mu_3}{\partial C_3}$ in some equations in literature is one such

804 evidence of inconsistence making the value of hydrated molar volume of cosolute

805 contentious. Both parameters can be dimensionless if the mole fractions were to be the case

806 otherwise, some of the equations where they appear, become invalid. For instance Eq. (21)

807 and Eq. (23) are dimensionally inaccurate.

808 Combining Timasheff equation (Eq. (2)) with derived equation (Eq. (27a)) results in a

809 different slope and consequently the value of $G_{21} - G_{23}$which appeared as a reciprocal equal

810 to the slope is also different from what is expected from Eq. (28a). Also, the introduction of

811 apparent molar volume, V_1 into Eq. (41) for instance creates, *ab initio*, a dimensionally

812 consistent equation, including the derived equation for the determination of V_1. The

813 introduction of RT Ina_3 into Eq. (39) and V_1 into Eq. (41) gives after integration equations

814 which are dimensionally valid but not necessarily suitable equations for the determination of

815 V_1. Taking 1-1/γ_3 as a function of C_3 gives a better correlation, where V_1 is a slope. The

816 equation of unfolding has also been revisited, and deriving in the process, alternative

817 equations that are suitable for different situations in which velocity of amylolysis as observed

818 is either greater or less than the velocity for native untreated enzyme, with a caveat that the

819 observed velocity of hydrolysis for the treated enzyme is greater than for the unfolded

820 enzyme. The concept of preferential interaction and *m*-value were investigated by treating

821 the enzyme with three cosolutes, ethanol, aspirin, and sucrose.

822 This summary is imperatively terminated with following comment. The fact that

823 ethanol has been implicated in the aetiology of distinct intermediate protein states

824 responsible for numerous neurodegenerative diseases such as Alzheimer's disease,

825 Parkinson's disease, and Huntington's disease [24] should motivate the need for appropriate

826 models that can be used to quantify the physico-chemical and biophysical effect of ethanol

827 so as to establish a standard. This does not rule out improvisation as was the case in the

828 thesis that generated the data; but the truth needs to be told as to the degree of precision of

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829 instrumentation. Stating otherwise to gain acceptance or evade censorship render
830 quantitative result invalid and below standard in the light of the wishes of Strenda and what
831 is expected of high precision instrumentation.

832 **CONCLUSION**

833 A major theoretical investigation was carried out on the issue of solution structure
834 with a conclusion that it is as usual determined by either a relative excess or a deficit of the
835 solution component either in the bulk or around the macromolecular surface domain; the
836 preferential interaction coefficient or parameter remains thermodynamically an extensive
837 parameter. Some of the derived equations may remain dimensionally invalid if standard
838 reference concentration/activity is not substituted into such equations. All derived equations
839 based on speculation or assumption except the equation derived from first principle may be
840 useful for the determination of (G_{13} − G_{33}), the apparent hydrated molar volume of the
841 osmolyte/cosolute. As with ethanol unlike aspirin, the *m*-values exhibit positivity contrary to
842 the usual; the cognate preferential interaction coefficient has sign other than the usual with
843 ethanol unlike with aspirin alone and with sucrose. In the light of earlier comment, it is
844 hereby recommended that for feature research, scholars or researchers should against the
845 backdrop of the theoretical exposition in this research carry out experiment with a-state-of-
846 the-act high precision instrumentation so as generate very high quality data.

847

848 **COMPETING INTERESTS DISCLAIMER:**

849

850 Authors have declared that no competing interests exist. The products used for this
851 research are commonly and predominantly use products in our area of research and
852 country. There is absolutely no conflict of interest between the authors and
853 producers of the products because we do not intend to use these products as an
854 avenue for any litigation but for the advancement of knowledge. Also, the research
855 was not funded by the producing company rather it was funded by personal efforts
856 of the authors.

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858

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COMPETING INTERESTS

There is no competing interests.

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