Original Research Article

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

ABSTRACT

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 an incorrect answer.

Objectives: The objectives are 1) To show theoretically that the preferential interaction parameter (PIP) is an extensive thermodynamic quantity, 2) rederive new equations and reexamine various equations related to solution structure, 3) apply derived equation for 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 V_1 of the osmolyte were determined. With ethanol alone, there were $-$ *m*-value and $+$ PIP; with aspirin alone, there were $+$ *m*-value and $-$ PIP. There was 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).

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 a 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 (V_1 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 that are the 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 an attempt to link the interaction parameters to Kirkwood-Bulk integrals and *m*-value (this is the slope of the plot of free energy of folding to unfolding transition versus cosolvent concentration) [3-7]. The catalytic activities of the enzymes are also studied in the presence

and absence of the osmolytes with the hope of understanding or establishing the effect of thermodynamic temperature increase in particular may be on the function of the enzyme [2, 8]. There were theoretical studies in the past [3, 9] all geared towards gaining theoretical insight into the solution structure and thermodynamic properties. It seems that there are far more biophysical studies than purely biochemical studies at the experimental front. Yet it is a greater theoretical insight that can facilitate the interpretation of results. Hence this research is mainly theoretical with minor experimentation for the generation of data for the evaluation of the derived equations.

Scholars have explained the mechanism of preferential interaction of osmolytes with biomolecules often in the usual consistent way [3, 7, 10]. While preferential binding (otherwise called solvation by binding) leads to unfolding that accompanies displacement of water of hydration and perhaps water of preferential hydration, preferential hydration leads to the folding of unfolded protein. The folding of the unfolded protein results from the preferential exclusion of the osmolyte from the surface (the peptide back born) of the protein. Recently, a different mechanism as opposed to preferential hydration has been advanced for the (re)folding of biomolecules [11]. The Lifshitz's dispersion forces play a strong role in solute-induced stabilisation/destabilisation of globular proteins [11]. The positive and/or negative electrodynamic pressure (perhaps due to such forces) generated by the solute-protein interaction across the water medium seems to be the fundamental mechanism by which solutes affect protein stability [11]. There is also the concept of translational entropy (TE) [12] regarded as the driving force that opposes conformational entropy connected to unfolding thereby forcing (re)folding. Hydrophobic effect is also known to promote folding [11, 13].

The issue remains effects of hydration and solvation or osmolation. But there are models used to separate the effect of hydration from those of solvation of proteins. Those models according to Rösgen *et al* [3-7] are the exchange model, osmotic stress model, local domain model, and constant solvation model. There is an attempt to bypass model-

dependent assumptions while targeting Kirkwood-Buff (KB) – based protein solvation model to describe protein stability [3]. However, there seems to be an error, typographical or conceptual in nature. Most of the models are at the far end of biophysics with cognate biophysical methods. The hi-tech instruments for achieving the intended measurements are those for circular dichroism spectroscopy, infrared spectroscopy, differential scanning calorimetry, Fourier transform infrared spectroscopy *etc* [2]**.** An example of biochemical method is the assay of any enzyme whose velocity of action can be monitored using spectrophotometer of any kind that may be suitable. Adequate understanding of the issues regarding preferential interaction parameters, protein folding, and unfolding or misfolding are important to biological scientist, biochemist, pharmacist *etc*. This is so because of the effects that may be (in) compatible to health. To this end, there is a need to achieve greater theoretical insight regarding molecular interaction through far reaching or robust analysis of the issues involved. There is a need also to shift from so much emphasis on biophysical approaches to biochemical methods.

The objectives of this research are: 1) To show theoretically that the preferential interaction parameter (PIP) is an extensive thermodynamic quantity, 2) rederive new equations and reexamine various mathematical equations related to solution structure, 3) apply derived equation in the determination of apparent hydrated molar volume of cosolute, V_1, and 4) determine *m*-values and the PIP.

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

There are various forms of preferential interactions implied in the radial distribution function. They are water-water, solvent-solvent (in this case osmolyte), protein-water, protein-protein, and osmolyte-protein interactions. Interactions may be positive or negative. What Timasheff [9] called epithet, ‘‘preferential’’ refers to the relative affinities of the interacting loci on the protein for ligand and water. Using *C* as molarity symbol, the

preferential hydration parameter (Γ_{21}) [14] and preferential osmolation parameter (Γ_{23}) [9] can be given respectively as:

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

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

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

Where μ_i stands for chemical potential of any solution components. The preceding equations are in the furtherance of the reason why Γ_{2i} cannot be a measureable quantity and a slope at the same time as previously reported [15]. According to Timasheff [9],

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

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

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

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

A close look at Eqs (4a) and (5a) shows that Γ_{23} cannot remain constant at different values of C_3 and the latter is the only independent variable. The parameters, Γ_{23} and Γ_{21}, are known to be measurable by biophysical methods such as dialysis equilibrium [9, 14], sedimentation equilibrium [14], and pressure osmometry [9]. The change in Γ_{21} or Γ_{23} as the case may seem to be, seems more important to the biochemist, pharmacist, and related

specialist other than biophysicist. Such changes may compromise or inhibit the function of the biomolecule as a result of conformational changes, the unfolding, partial folding and dysfunctional rigidification that may arise depending on the kind of cosolvent and its concentration. The change in Γ_{21} is directly related to the effect of water activity, a_1 or the osmolyte osmotic pressure Π on the equilibrium constant $K_{\mathrm{eq}(1)}$ of the reaction which may be conformational change [14].

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

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

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

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

Timasheff [9] gives:

$$\mathrm{In}a_1 = C_3\phi_3/55.56 = -\frac{\bar{\mathrm{v}}_1\Pi}{RT} \tag{9}$$

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

The following equation may hold for preferential osmolation.

$$\Delta\Gamma_{23} = \left(\frac{\partial \mathrm{In}K_{\mathrm{eq}(3)}}{\partial \mathrm{In}a_3}\right)_{\mathrm{T,P,C_2}} \tag{10a}$$

Equation appears to be a slope against the backdrop of the fact that $\Delta\Gamma_{23}$ is also a measureable parameter. This issue has been raised and concluded in favour of the view that the parameter cannot be an instrument based measurable parameter and a slope at the same time [15]. Thus, Eq. (10a) gives,

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

There are fundamental issues arising from Eq. (7), Eq. (8), and Eq. (10b). No devise or equipment is known to measure $K_{\mathrm{eq(i)}}$ directly. Rather absorbance of the biomolecule is measured with variety of available biophysical equipment such as those for circular dichroism spectroscopy, infrared spectroscopy, differential scanning calorimetry, Fourier transform infrared spectroscopy etc [2]. These measurements can be taken at different concentration of the osmolyte. The function of the biomolecule, enzyme for instance, may also be monitored by taking the absorbance as a measure of the concentration of the product of enzymatic action at different concentration of the osmolyte. Hence, the combined biophysical model and biochemical model expressed via kinetic model. This issue will be readdressed subsequently. It is not certain in literature, if the measuring device can measure Γ_{23} and Γ_{21} simultaneously for every given concentration of the osmolyte. Devise such as pressure osmometry is relevant to measurement of $\mathrm{In}\left(\mathrm{P}_1^{C_3}/\mathrm{P}_1^{0}\right)$ or $\mathrm{In}a_1$ [9] where, $\mathrm{P}_1^{C_3}$ and P_1^{0} are the vapour pressures of water for the solution of any osmolyte (or it may be protein, whose concentration may be C_2) and water free from cosolute respectively.

Given the information implied in Eq. (11) above, a plot of $\mathrm{In}K_{\mathrm{eq(i)}}$ versus $\mathrm{In}\boldsymbol{a}_1$ or $\mathrm{In}\boldsymbol{a}_3$ yields slopes, $\Delta\Gamma_{21}$ or $\Delta\Gamma_{23}$ respectively. However, if Γ_{23} is measured directly at 2 different 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 and lower concentration of osmolyte respectively, if by definition, $\Delta\Gamma_{23}$ is the slope as implied in Eq. (10b). It seems $\Delta\Gamma_{21}$ and $\Delta\Gamma_{23}$ may represent parameters different from what they were meant to be. Meanwhile, a_3 and a_1 are calculated after taking measurement of relevant parameters. The parameter $K_{\mathrm{eq}(3)}$ is also calculated after taking measurement of needed parameters either by biophysical or biochemical methods. In other words there are different values of $K_{\mathrm{eq(i)}}$, a_3 or a_1 which are osmolyte concentration

dependent. The ratio, $\frac{\mathrm{In}K_{\mathrm{eq(i)}}}{\mathrm{In}a_{\mathrm{i}}}$ gives value of $\Delta\Gamma_{2\mathrm{ical}}$ (calculated value) that represents the preferential interaction parameter at a defined C_3. This may be a mere speculation, the essence of theoretical contribution. The parameter $\Delta\Gamma_{2\mathrm{i}}$ as a slope may possess sign and magnitude that merely reflects the degree of osmolation or hydration due to exclusion of osmolyte. However, according to Timasheff [9], applying Eq. (4) gives, for the calculated $\Delta\Gamma_{21}$, $\Delta\Gamma_{21\mathrm{cal}} = -\left({}^{C_1}/_{C_3}\right)\frac{\mathrm{In}K_{\mathrm{eq(3)}}}{\mathrm{In}a_3}$ and for the slope,

$$\Delta\Gamma_{21} = -\left({}^{C_1}/_{C_3}\right)\left(\frac{\partial \mathrm{In}K_{\mathrm{eq(3)}}}{\partial \mathrm{In}a_3}\right)_{\mathrm{P,T,C_2}} . \quad (12)$$

The implication of Eq. (12) is that there should be different values of $\Delta\Gamma_{21}$ for different C_3 because $\left(\frac{\partial \mathrm{In}K}{\partial \mathrm{In}a_1}\right)_{\mathrm{P,T,C_2}}$ or $\Delta\Gamma_{23}$ is taken as slope and C_1 being molar concentration of water is constant. Applying similar method to $\Delta\Gamma_{21}$ gives

$$\Delta\Gamma_{23} = -\left({}^{C_3}/_{C_1}\right)\left(\frac{\partial \mathrm{In}K_{\mathrm{eq(1)}}}{\partial \mathrm{In}C_1}\right)_{\mathrm{P,T,C_2}} \quad (13)$$

Another implication is that, $-\left({}^{\mathrm{C}_1}/_{C_3}\right)\frac{\mathrm{In}K_{\mathrm{eq(3)}}}{\mathrm{In}C_3} \neq -\left({}^{C_1}/_{C_3}\right)\left(\frac{\partial \mathrm{In}K_{\mathrm{eq(3)}}}{\partial \mathrm{In}a_3}\right)_{\mathrm{P,T,C_2}}$ and $-\left({}^{C_3}/_{C_1}\right)\frac{\mathrm{In}K_{\mathrm{eq(1)}}}{\mathrm{In}a_1} \neq -\left({}^{C_3}/_{C_1}\right)\left(\frac{\partial \mathrm{In}K_{\mathrm{eq(1)}}}{\partial \mathrm{In}C_1}\right)_{\mathrm{P,T,C_2}}$. This analysis confirms the earlier suggestion that, the slopes may represent a parameter with meaning different from what it is meant to be. This is against the backdrop of Wyman's equation known as the basic Wyman linkage equation which, according to Timasheff [9], states that, "at any ligand concentration, the gradient of the equilibrium constant with respect to ligand activity is equal to the change in the binding of the ligand to the biological system during the course of the reaction (at constant temperature and pressure that will be maintained throughout)". Nothing seems to suggest that there is Wyman's equivalent equation for preferential hydration. The slope as the change in the binding of the ligand may not give the same result of preferential exclusion

according to Eq. (12). Besides, a measurable quantity such as $\Delta\Gamma_{2i}$ for the change or Γ_{2i} at different finite concentrations of the osmolytes, extensive quantities, is also thermodynamically an extensive quantity unlike a slope which is definitely an intensive quantity under clearly specified conditions, temperature and pressure.

As explained elsewhere [15], another reason, why calculation of $\Delta\Gamma_{2i}$ may be more useful for the determination of parameters is obtainable from the following equations [9, 14]. In their contributions, Shimizu [14] and Rösgen *et al* [3] attempted to relate preferential interaction parameters with Kirkwood – Buff integrals (KBI). Beginning with Shimuzu [14] is the equation:

$$\Gamma_{21} = N_{21} - \frac{C_1}{C_3} N_{23} \tag{14}$$

Where $C_i (\text{or } n_i \text{ as in literature})$ and N_{2i} represent respectively the density (molarity) of any chemical species and the excess number of component *i* around the biomolecule, though Eq. (14) is directly applicable to preferential hydration. The counterpart of Eq. (14) is the osmolation case given as [9]:

$$\Gamma_{23} = N_{23} - \frac{C_3}{C_1} N_{21} \tag{15}$$

Equations (14) and (15) show that, the plot of measureable parameters versus either $1/C_3$ or C_3 gives C_1N_{23} and N_{21}/C_1 respectively as slope. The equations for the change are given as [9]

$$\left(\frac{\partial \text{In} K_{\text{eq}(1)}}{\partial \text{In} a_1}\right)_{\text{P,T,C}_2} = \Delta N_{21} - \frac{C_1}{C_3}\Delta N_{23} = \Delta\Gamma_{21} \tag{16}$$

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

Before this time and recent publication [16], $K_{\text{eq}(3)}$ and $K_{\text{eq}(1)}$ are taken symbolically to be K_{eq} 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

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 tells the story on the contrary. If the parameter, $\Delta\Gamma_{2i}$ from the plot of $\mathrm{In}K_{\mathrm{eq(i)}}$ versus $\mathrm{In}a_{\mathrm{i}}$ 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 [15] a slope, such as $(\partial \mathrm{In}K/\partial \mathrm{In}a_1)_{\mathrm{P,T,C_2}}$ or $(\partial \mathrm{In}K/\partial \mathrm{In}a_3)_{\mathrm{P,T,C_2}}$ must not contain independent variable such as C_3 given that molar concentration of water, C_1 is constant at a given thermodynamic temperature.

2.1 Examination of mathematical models connected to solution structure

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

The extent to which the interaction of different osmolytes may cause changes in the structure and function of proteins in particular may differ. To Poklar *et al*. [17], the physical significance of the factor, *m*-value, is not completely clear despite its wide spread use in recent time, though it has been viewed as the difference in the amount of the denaturant interacting with the native and denatured states of the polypeptide chain [17]. As stated elsewhere [18], 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 [17]. 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* [19] and as cited by Harries and Rösgen. [20]. 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 $m-value$ [20]. 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, $\left(-\left(\frac{\partial \text{In}K_{\text{eq}(3)}}{\partial \text{C}_3}\right)_{\text{T,P}}=\frac{m}{RT}\right)$ [7], 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 $m-\text{value}$ [7].

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

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

Equation (19a) has issue with dimension if the unit (L/mol) of $G_{13}-G_{33}$ is taken into account. 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 contentious result. Nonetheless, it is to be substituted into all relevant equations to enable the verification of any claim regarding the invalidity of whatever equations that arise in this research as well as in literature. However, there is a need to point out the fact that C_3 is the same at the left - and right - hand sides of Eq. (19a); but the introduction of standard-state molarity given as $C_i = C_i^0 = 1\ \mathrm{mol/L}$ at the right-hand side corrects the dimensional inconsistency. The corollary is that there should be the expression given as $a_i = a_i^0 = 1\ \mathrm{mol/L}$. Thus Eq. (19a) can be rewritten as $\frac{1}{1-C_3(G_{13}-G_{33})} = \frac{1}{RT/C_3^0}\left(\frac{\partial \mu_3}{\partial C_3}\right)_{\mathrm{T,P}}$, thereby eliminating dimensional inconsistency. According to Rösgen *et al* [3], the derivative is given as

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

It is important to realise that the denominator at the left hand side also appears in the derivative relating the chemical potential of the protein to the osmolyte concentration and to the KBI for the hydration and osmolation of protein. This is given for the protein as follows [3].

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

Henceforth, $G_{13}-G_{33}$ is designated as V_1, the apparent hydrated molar volume of the osmolyte. If Eq. (19a) is substituted into Eq. (19b) one obtains

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

Rearrangement followed by integration gives

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

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

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

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

Rearrangement gives

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

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

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

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

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

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

In the paper by Rösgen *et al* [3] $\left(\frac{\partial \mu_3}{\partial \mathrm{In} C_3}\right)_{T,P}$ was used in the determination of the structural basis of the *m*-value (*m* for short), which is, seemly suggestive of an initial technical error. There is no issue of dimensional inaccuracy in Eq. (24) if $\mathrm{In} C_3$ is rewritten as $\mathrm{In}(C_3/C_3^0)$. But the independent parameter cannot appear as a constant and as a variable considering the partial differential $\partial \mathrm{In}(C_3/C_3^0)$ even if $\left(\frac{\partial \mu_3}{\partial \mathrm{In}(C_3/C_3^0).}\right)_{T,P}$ is taken as slope. However, the continuous appearance of C_3 in the equations, demands examination shortly. Before this, there is need to realise that $\left(\partial \mu_3/\partial \mathrm{In} C_3\right)_{T,P,C_3=0} = RT$ if $C_3 \rightarrow 0$ (i.e. a case of infinite dilution). This seems to be the valid view of Rösgen *et al* [3]. If this is the case most of the preceding equations where C_3, instead of $\mathrm{In} C_3$, appears cannot be valid. The implication is that $G_{13} - G_{33} = 0$ $(G_{13} = G_{33})$. However, in subsequent derivations, C_3 is regarded as one which is » 0. But before this, the issue regarding ideality is reexamined as follows.

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

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

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

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

But $\frac{\Delta \mu_3}{RT} = \mathrm{In} C_3$ (or more appropriately, $\mathrm{In}(C_3/C_3^0)$ for an ideal case, such that, $\Delta C_3 V_1 = 0$: This is as often stated in literature [3]. What the value of ΔC_3 should be needs to be ascertained. One cannot shy away from the fact that the adoption of standard-state molarity implies a transition from 1 mol/L to values of C_3« 1 mol/L or > 1 mol/L as the case may be.

But as stated earlier, the infinitesimal difference between C_3 and a_3 may be useful for the determination of V_1. In such situations, the value of V_1 obtained by calculation may be negative if activity coefficient is < 1 mol/L. Ideal case is to be applied to dilute solution of the protein as follows. Substitution of Eq. (24) into Eq. (19c) gives

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

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

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

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

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

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

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

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

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

Looking at Eq. (28b) one sees that the chemical potential of the protein can either be positive or negative if respectively, the preferential interaction parameter by exclusion or binding is the case. Eq. (28b) represents a precedence whose validity or scientific merit remains a matter for feature investigation. Considering that the concentration (ranging from nanoscale–milli–scale mol/L) of the enzyme is very low in most laboratory/clinical investigation, one can correctly admit that ideality should be the case: One may need to recall that Eq. (28b) is an outcome of contentious equations, namely Eq. (19a) and Eq. (24).

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

$$m = \Delta_{\mathrm{N}}^{\mathrm{D}}(G_{21} - G_{23})RT \tag{29a}$$

$$m = -RT\Delta_{\mathrm{N}}^{\mathrm{D}}\Gamma_{23}/\,C_3 \tag{29b}$$

It seems that with respect to the $m-\text{value}$, the place of ideality may not be ruled out probably on account of the fact that $\mathrm{In}(1/K_{\mathrm{eq}})$ is plotted versus C_3. With the end of the consideration for ideal situation, subsequent derivations take into account nonideal cases. This was implied in previous research [15] but it was not explicitly stated.

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

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

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

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

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

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

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

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

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

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

In the light of the Eq. (2) [9], there is need to revisit Eq. (27a). Rearranging the latter gives

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

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

The implication is that

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

Rearrangement of Eq. (33c) gives

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

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

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

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

The nonideal case for the determination of the structural basis of the *m*-value is given by rewriting Eq. (23); instead of $\mathrm{In}C_3$, $\mathrm{In}a_3$ is used as follows.

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

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

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

Looking closely at Eq. (34a) and Eq. (34b), it would appear that there are 3 slopes viz: $\Delta_{\mathrm{N}}^{\mathrm{D}}(G_{21} - G_{23}), m - \mathrm{value}$ and $\Delta_{\mathrm{N}}^{\mathrm{D}}\Gamma_{23}$. If the values of $G_{21}(= \Delta N_{21}/C_1)$ and $G_{23}(= \Delta N_{23}/C_3)$ are obtained from the plot of $\Delta\Gamma_{2\mathrm{i}}$ versus C_3 or $1/C_3$, as the case may be, according to Eq. (17) and Eq. (16) respectively then, $\Delta_{\mathrm{N}}^{\mathrm{D}}(G_{21} - G_{23})$ may speculatively be taken as a constant or slope. Therefore, $\Delta_{\mathrm{N}}^{\mathrm{D}}\Gamma_{23}$ can be calculated for different values of C_3, thereby justifying the claim that the former cannot be a constant quantity or slope and equipment based measurable parameter. It is definitely obvious that $a_{\mathrm{i}} \neq C_3$ and as such a plot of $\mathrm{In}a_3$ versus $\mathrm{In}C_3$ cannot be equal to one even if the coefficient of determination may be one. An equation relating $\mathrm{In}a_3$ to $\mathrm{In}C_3$ may be expressed as: $\mathrm{In}a_3 = \text{Ş}\mathrm{In}C_3 - \text{Í}$ where Ş and Í are the slope and intercept respectively**.** However, this is not to justify the place of $\frac{\partial \mathrm{In}a_3}{\partial \mathrm{In}C_3}$ or $\left(\frac{\partial \mu_3}{\partial \mathrm{In}C_3}\right)_{\mathrm{T,P}}$. Previous publication [15] and, as pointed out earlier in the text, has

strongly shown that all except $m-\text{value}$ are not slope and consequently they are extensive quantity; the other two, $\Delta_{\mathrm{N}}^{\mathrm{D}}\Gamma_{23}$ and Γ_{23} cannot be a devise based measurable parameter and constant quantities at the same time. In previous research [15] the change in solvation preference upon unfolding in terms of the *m*-value equation was determined by eliminating the apparent hydrated molar volume of the osmolyte. But if V_1 is relevant and correctly known, it may be used to calculate the same parameter at different values of C_3. Thus,

$$\mu_3 = \mu_3^0 + RT\mathrm{In}\left(\frac{\mathrm{C}_3}{1-V_1C_3}\right) \quad (35a)$$

Equation (35a) is obtained by integrating the derivative (Eq. (19b)) given by KB theory [3] with respect to C_3 while holding V_1 constant. Rearrangement of Eq. (35a) gives

$$\frac{\Delta\mu_3}{RT} = \mathrm{In}\left(\frac{\mathrm{C}_3}{1-V_1C_3}\right) \quad (35b)$$

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

Here apparent hydrated molar volume of cosolutes is to be determined based on different principles. There are arguments about the validity of derived equations based on fundamental equations and recent equations in this research.

2.2.1 Determination based on the presumed relationship with activity coefficient.

In line with Timasheff equation [9] but on the basis of molar concentration,

$$\frac{\Delta\mu_3}{RT} = \mathrm{In}C_3\gamma_3 = \mathrm{In}\left(\frac{\mathrm{C}_3}{1-V_1C_3}\right) \quad (36a)$$

Where, the parameter γ_3 is the osmolyte activity coefficient. Although the standard reference concentration can be introduced into Eq. (36a), its presence both at the right - and left - hand sides makes it unnecessary.

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

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

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

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

Rearrangement of Eq. (37a) gives

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

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

3.0 MATERIALS AND METHODS

3.1 Materials

The chemicals used were: The chemicals used were: Sucrose (St Lious France); raw (native) potato starch (Sigma Chemicals Co, USA); ethanol, hydrochloric acid and sodium chloride (BDH Chemical Ltd, Poole England); 3,5-dinitrosalicylic acid (DNA) (Lab Tech Chemicals, India); Tris (Kiran Light Laboratories, USA); porcine pancreatic alpha amylase (EC 3.2.1.1) (Sigma, Adrich, USA); all other chemicals were of analytical grade and solutions were made in distilled water. Aspirin was purchased from CP Pharmaceuticals Ltd, Ash road North, Wrexham, LL 13 9UF, U.K.

3.2 Equipment

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

3.3 Methods

Bernfeld method [21] of enzyme assay was adopted for the assay of the enzyme, porcine pancreatic alpha amylase (PAA). Preparation of substrate and enzyme was as described elsewhere [16]. Equilibrium constant for folded to unfolded transition is either according to Eq. (49) or Eq. (53) as the case may be. The calculation of preferential interaction parameter for folded to unfolded transition is according to Eq. (34c or 29b). Plots for the determination apparent hydrated molar volume, are according to Eq. (37b), Eq. (40b) and E. (44) (Eq. (40b) and E. (44) are in the supplementary section). Determination of thermodynamic activity of solvent and solute and corresponding activity coefficient was as described elsewhere [16]. Microsoft Excel (2007) was used to plot the dependent variable versus independent variable.

3.4 Statistical analysis

The velocities of hydrolysis were determined in triplicates. The mean values were used to determine the equilibrium constant for folded to unfolded protein transition.

4. RESULTS AND DISCUSSION

The important purpose of the theoretical section, a major part of this research is to proffer a proper basis of any interpretation of results obtained from the changes of the biomolecular function. Such change may result from change of structure due to solution composition. It is very imperative that mathematical models or equations used to qualitatively and in most cases quantitatively interpret results are valid. Thus as was observed in the theoretical section, the appearance of $RT\ln C_3$ gives the impression of ideality. This leads to a situation where the apparent hydrated molar volume, V_1 of the osmolyte is equal to zero. The continuous use of $RT\ln C_3$ demands that C_3, though low, must be much greater than 0.

The different calculated values of V_1 are shown in Table 1. This is applicable to Eq. (31), Eq. (37b), and Eq. (40b). Mathematically and from the standpoint of dimensionality in particular, equations that are not valid are Eq. (20)-Eq. (23). Equations that appear valid from the same stand point due to the substitution of Eq. (24) which appears dimensionally valid are Eq. (25) to Eq. (30c). But this is mainly a dimensionality issue whose validity validates in part the mathematical models or equations. Thus beyond dimensional validity, substitution of Eq. (24) into a particular equation does not always produce a valid equation as observed in this research. This is applicable to Eq. (33a-33d), where there is need to introduce the standard reference concentration equal to 1 mol/L.

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

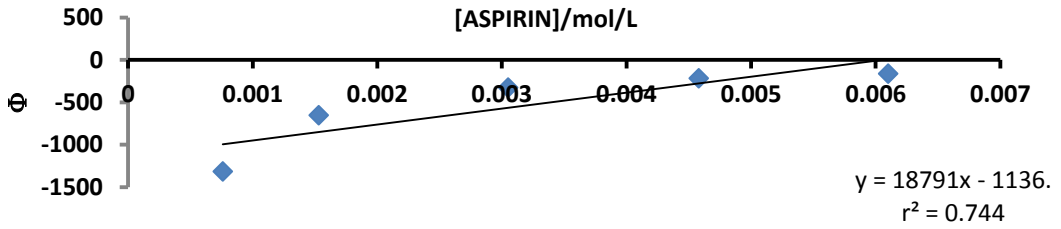

Fig. 1a. A plot for the determination apparent hydrated molar volume as function of *[Aspirin]*. *The symbol Θ stands for 1-(1/a$_3$). Note that the coefficient of determination r^2 (0.744<0.900) expresses nonlinearity.*

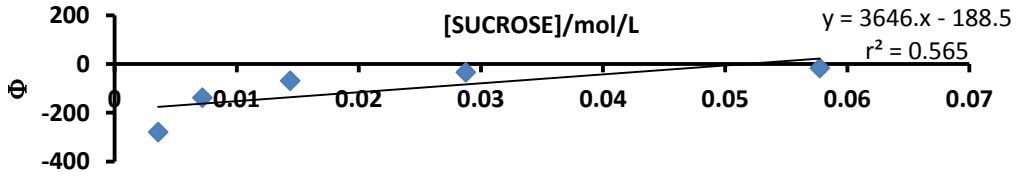

Fig. 1b. A plot for the determination apparent hydrated molar volume as function of [Sucrose]. *The symbol Φ stands for 1-(1/a_3). The coefficient of determination r^2 (0.565 < 0.900) expresses nonlinearity.*

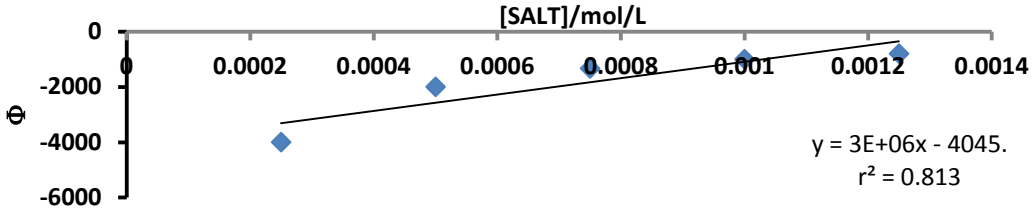

Fig. 1c. A plot for the determination apparent hydrated molar volume as function of *[Salt]*. *The symbol Φ stands for 1-(1/a_3).The salt is calcium chloride. Note that the coefficient of determination r^2 (0.813 < 0.900) expresses nonlinearity.*

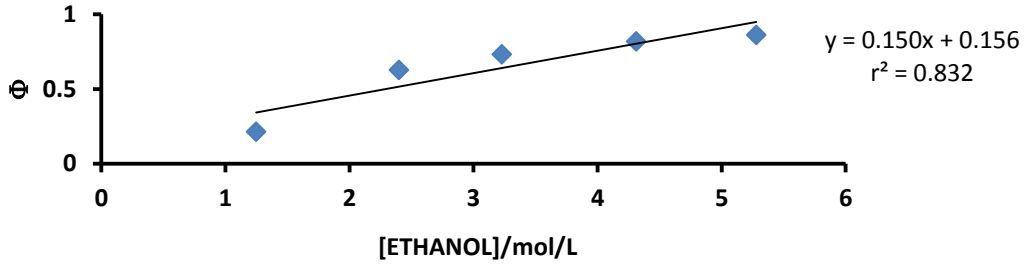

Fig. 1d. A plot for the determination apparent hydrated molar volume as function of [Ethanol]. *The symbol Φ stands for 1-(1/a_3). The coefficient of determination r^2 (0.832 < 0.900) expresses nonlinearity.*

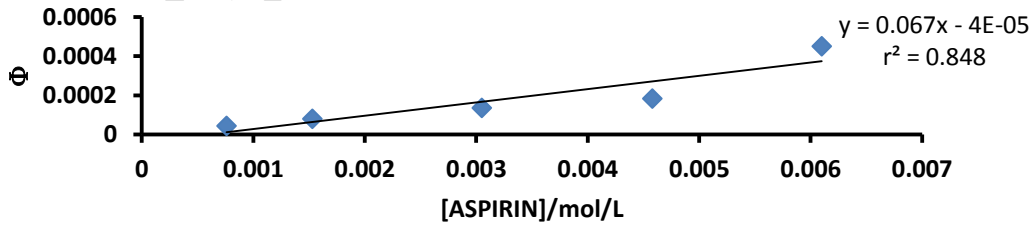

Fig. 2a. A plot for the determination apparent hydrated molar volume as function of molar concentration of [Aspirin]; *the parameter Φ is 1–1/γ. The coefficient of determination r^2 (0.848 < 0.900) expresses nonlinearity.*

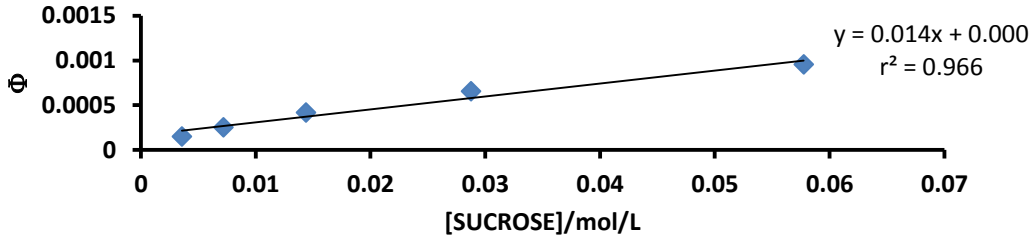

Fig. 2b. A plot for the determination apparent hydrated molar volume as function of molar concentration of [Sucrose]; *the parameter Φ is 1–1/γ.*

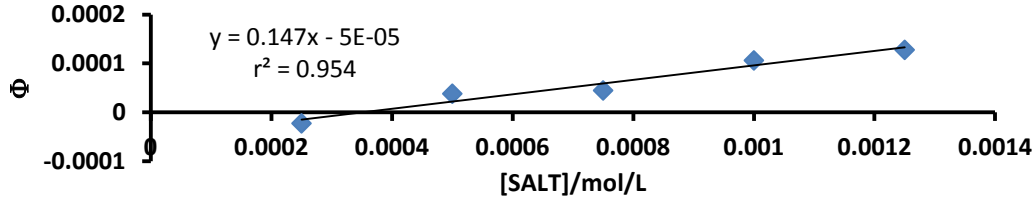

Fig. 2c. A plot for the determination apparent hydrated molar volume as function of molar concentration of [Salt]; *the parameter Φ is 1–1/γ.*

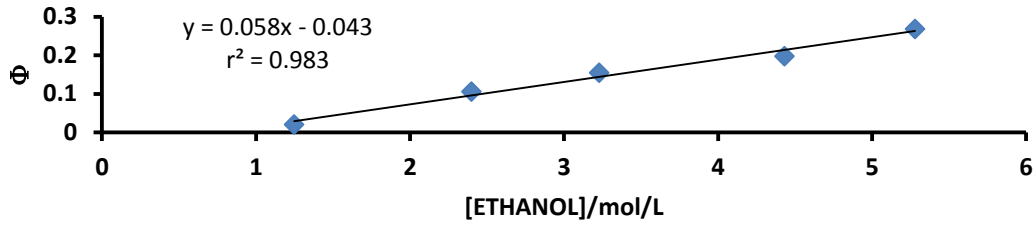

Fig. 2d. A plot for the determination apparent hydrated molar volume as function of molar concentration of [Ethanol]; *the parameter Φ is 1–1/γ.*

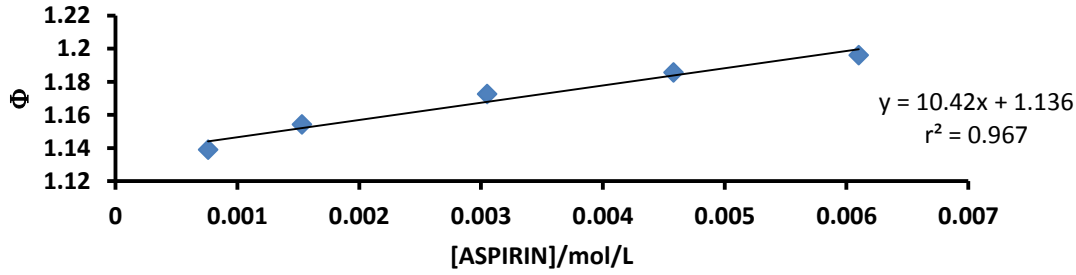

Fig. 3a. A plot for the determination apparent hydrated molar volume as function of [Aspirin]*; the parameter Φ is 1–1/Ina*$_3$.

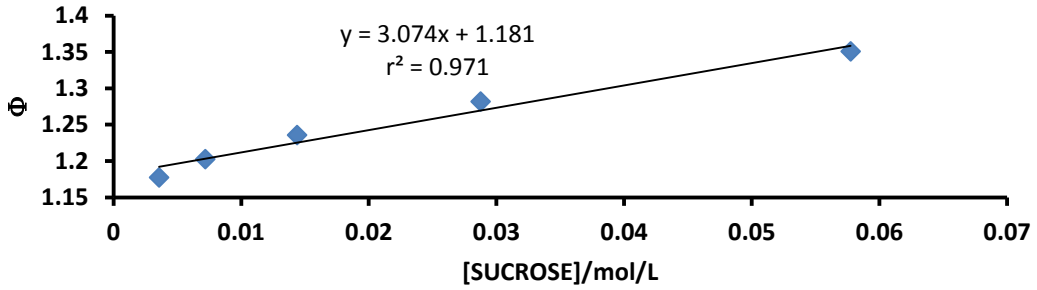

Fig. 3b. A plot for the determination apparent hydrated molar volume as function of [Sucrose]; *the parameter* Φ *is* $1-1/\ln a_3$.

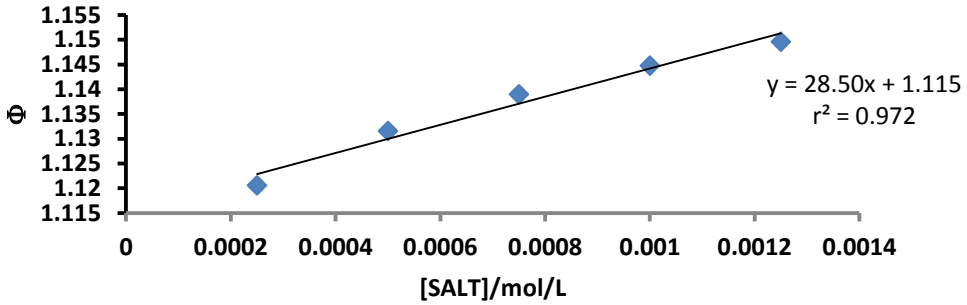

Fig. 3c. A plot for the determination apparent hydrated molar volume as function of [Salt]; *the parameter* Φ *is* $1-1/\ln a_3$.

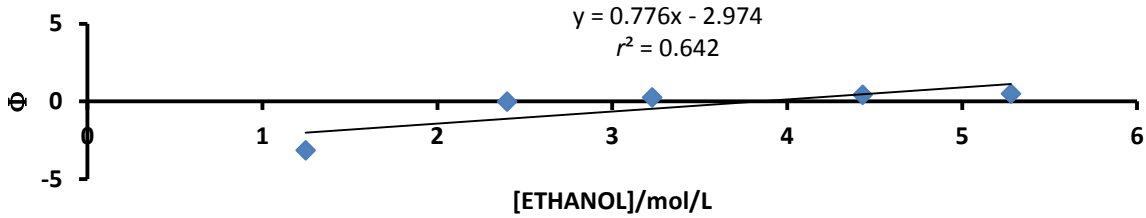

Fig. 3d. A plot for the determination apparent hydrated molar volume as function of [Ethanol]; *the parameter* Φ *is* $1-1/\ln a_3$. *The coefficient of determination* r^2 *(0.642 < 0.900) expresses nonlinearity.*

Of particular note is the observed similar values obtained for ethanol based on Eq. (40b) and Eq. (44) (Table 1). This goes to show that concentration regimes seem to create different slopes and, most importantly the derived mathematical equations may not be appropriate unlike Eq. (37b). The values of V_1 based on Eq. (37b) can better serve calculational purpose that gives positive result of other parameters when substituted into

relevant equations in literature [3]. For instance, but for the feature, the values based on Eq. (37b) can be used to determine the change of solvation preference (this is given as $\Delta_N^D(G_{21} - G_{23})$) upon denaturation if the *m*-value is known. It can also be used to determine the chemical potential of osmolyte ($\Delta\mu_3$) given as $RT\ln(C_3/C_3^0)/(1 - C_3V_1)\,G_{23}$, the modified form of Rösgen *et al* [3'] equation. It needs to be stated that this approach is slightly different from conventional methods in literature [22], though it seems to enable the determination of V_1 if $\Delta\mu_3$ is independently determined.

Table 1. Determination of apparent hydrated molar volumes of cosolutes

Equations [Aspirin]	[Ethanol]	[Salt]	[Sucrose]	
		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	10.42
r^2	0.831	0.972	0.970	0.968
Eq. (44)	0.150	3.000 E	3.646 F	18.918 F
r^2	0.832	0.813	0.566	0.749

The parameter V_1 is the apparent hydrated molar volume of cosolutes. The coefficient of determination (r^2) is indicated so as to emphasise the departure from linearity where applicable rather than only the occurrence of outliers arising from imperfection in the assay. E means exp (+6); F means exp (+3).

The capacity of cosolute to force refolding or unfolding, the *m*-value was determined either with a single or multiple cosolute. With ethanol alone unlike with a mixture of the former and sucrose, the *m*-value was positive in sign (Table 2a). With respect to ethanol alone, the positive *m*-value is similar to the result achieved in the past [16]. There has been report that an organic solvent which should have been destabilising may become a stabiliser [23]. To this end, "low water – content ethanol is preferentially excluded from the protein surface" [23]. If this is the case, there may have been positive *m*-value for such solvent,

ethanol as in this research. However, the interest in this research is to use alternative equation to determine the preferential parameters via Eq. (29c) and Eq. (34c).

Table 2a. The *m*-values arising from cosolutes' and aqueous solvent's interactions with the enzyme, in a reaction mixture, containing sucrose and ethanol-RTIn$K_{eq(3)}$ as a 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	0.86	0.97	0.93	0.94	0.99	1.00^z

The data is obtained from the plot of In1/$K_{eq(3)}$ versus [Ethanol] with different concentration of sucrose; the superscript z indicates datum from a straight line of two-data points; (r^2) is the coefficient of determination.

The fact that there were negative *m*-values with a mixture of ethanol and sucrose, points to the possibility that sucrose may either have reduced the solubility of water insoluble native potato starch or has reduced the conformational flexibility of the enzyme needed for function. According to Kurkal *et al* [24] proteins, dynamics otherwise called 'loosening up' facilitates biological function of enzymes. In the same vein, according to Affleck *et al* [25] the increased conformational flexibility due in part, to the reduced interaction of charged and /or polar amino acid residues within the enzyme molecules is caused by water's ability to effect dielectric screening: This prevents unfavourable interactions between charged and /or polar residues within the protein molecule. This explains the residual biological function of the enzyme. It appears therefore, that apart from water – striping effect of ethanol which compromises the role of water as plasticiser, that ought to promote conformational flexibility, the sucrose content may have rigidified the enzyme's three-dimensional structure. But there is an apparent paradox considering the fact that sucrose is known as a folding stabilizer and classified as an additive which shifts the folding equilibrium from the partially unfolded state toward the native state [26]. It seems generally any plot versus folding destabiliser and folding stabiliser should respectively give negative and positive *m*-value.

One may wish to add that, it is the enzyme primary structure that can determine the effectiveness of a cosolute to unfold or rigidify its structure. Without residual biological function of the enzyme, the determination of *m*-value based on kinetics/velocity of biological function will be impossible. There is also the need to add that where there is negative *m*-value there is preferential dehydration [3, 9]. There is a need also to suggest that the presence of sucrose "unusually enhanced the effectiveness of ethanol to act as destabiliser" (this is however, mere speculation) by rather, decreasing the solubility of the substrate. But the plot versus sucrose, due perhaps to the concentration regime, exhibited in all except with lowest concentration of ethanol, the usual positive *m*-values [Table 2b].

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

[Ethanol] (mol/L)	~1.25	~3.22	~5.28
m-value (kJL/mol^2)	– 27.93	28.55	276.69
r^2	0.87	0.53	0.96

The data is obtained from the plot of In1/ $K_{eq(3)}$ versus [sucrose] with different concentration of ethanol; (r^2) is the coefficient of determination.

Unlike ethanol, aspirin showed what it may be, a folding destabiliser, having no effect on substrate solubility which is unexpected considering the fact that while ethanol is a solvent, aspirin is not. The *m*-values generated from the plot versus [Aspirin] with and without sucrose yielded negative *m*-values (Table 3a). It thus, appears that aspirin is a folding destabiliser to the enzyme, porcine pancreatic alpha-amylase. Therefore, as explained by **Singh et al [27],** the critical factor is the partitioning between water and osmolyte (in this case aspirin) at solvent-exposed surfaces of a protein whereby denaturing cosolute accumulate or bind at the surface and promote unfolding as applicable to the effect of aspirin on the enzyme.

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

[Sucrose] (mol/L)	0.00	7.19	14.38	28.76	57.75
m-value (kJL/mol^2)	– 188.55	– 3754.56	– 4177.46	28.76	– 2174.34
r^2	0.87	1.00^z	1.00^z	0.99	0.99

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

With a mixture of aspirin and sucrose the *m*-values from the plot versus [Sucrose] were all positive (Table 3b) in line with the view that stabilizing osmolytes have an overwhelming tendency to be excluded from the protein surface, forcing the polypeptide to adopt a compactly folded structure with a minimum of exposed surface area. On this issue of *m*-values, it is pertinent to note that it may not be unusual that sucrose was unable to totally refold rather than over-rigidify because it has been observed that similar observation was made in respect of chymotrypsin, chymotrypsin, and ribonuclease [28].

Table 3b. The *m*-values arising from cosolutes' and aqueous solvent's interactions with the enzyme, in a reaction mixture, containing sucrose and aspirin-RTln$K_{eq(3)}$ as a 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	0.74	0.80	1.00^z

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

Next is the issue of preferential solvation, hydration and osmolation, which has been described as a thermodynamic quantity that describes the protein occupancy by the cosolvent/water molecules [23]. The results in this research are based on either Eq. (29b) or Eq. (34c) which shows direct link between the *m*-value and change in preferential interaction parameter (PIP). With ethanol alone, the PIP values were unexpectedly negative (Table 4a).

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

[Ethanol] (mol/L)	1.25	~2.4	~3.23	~4.31	~5.28
$\Delta_N^D\Gamma_{23}$	– 0.78	–1.49	–2.01	– 2.68	– 3.28

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

This has been observed for chymotrypsin elsewhere [23]; but with the presence of sucrose as part of ternary mixture of cosolutes, PIP values as a function of [Ethanol], showed positive sign (Table 4b) because, *ab initio* the *m*-values were negative in sign. This is as expected if the known effect of ethanol is taking into account. Such effect includes the change in the protein-water interactions and consequently, the modulation of the protein stability. The stripping of weakly bound water [9, 29] due to the binding of ethanol is inevitable, thereby leading to altered function of the enzyme. However, the PIP values as a function of [Sucrose], gave in all, except with lowest [Ethanol], negative values of PIP (Table 4c). This may be as a result of the greater solubilising effect of a higher concentration of ethanol on the insoluble raw starch.

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

	[Sucrose]/mmol/L				
	3.57	7.19	14.38	28.76	57.75
	$\Delta_N^D\Gamma_{23}$				
[Ethanol] mol/L)					
~1.25	~0.86	–1.46	0.81	0.33	0.2
~3.23	~2.22	3.79	2.09	0.86	0.55
~5.28	3.64	6.20	3.42	1.40	0.90

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

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

	[Ethanol]/mmol/L		
	~1.25	~3.23	~5.28
	$\Delta_N^D\Gamma_{23}$		
[Sucrose]/mmol/L			
3.57	0.04	– 0.04	–3.83
7.19	0.08	– 0.08	–0.77
14.38	0.16	– 0.16	–1.54
28.76	0.31	– 0.32	–3.09
57.75	0.63	– 0.64	–6.20

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

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

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

[Aspirin] (mmol/L)	1.247	~2.398	~3.228	4.311	5.279
$\Delta_N^D\Gamma_{23}$	0.556	~2.398	~3.228	4.311	5.279

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

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

	[Sucrose]/mmol/L			
	7.19	14.38	28.76	57.75
	$\Delta_N^D\Gamma_{23}$			
[Aspirin]/mmol/L				
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

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

The PIP values as a function of [Sucrose] with different [Aspirin] were negative (Table 5c); this again conforms to the conventional behaviour of sucrose as a stabilising osmolyte. This seems to suggest that the concentration regime of sucrose is sufficient to cancel the initial effect of aspirin if the enzyme was incubated in an aqueous solution of aspirin. Meanwhile, there are theories of preferential interaction which are Kirkwood-Buff, cavity formation, solvophobic/solvophilic, surface tension theories *etc* with which to elucidate the results. By being excluded from the peptide back bone as to imply solvophobic effect,

784 sucrose unlike ethanol and aspirin, is able to force protein to fold, leaving, as a
785 consequence, excess of the cosolute in the bulk solution. Here, according to Rösgen *et al* [3]
786 the Kirkwood-Buff theory comes into relevance. Thus an enrichment or relative excess of
787 water around protein corresponds to a positive G_{21} (positive correlation resulting from
788 exclusion), whereas a depletion of water around protein corresponds to a negative G_{21}
789 (negative correlation which is due to preferential binding) [7].

790 There is a recent theory implicating Lifshitz's dispersion forces which are inextricably
791 involved in solute-induced stabilization/destabilization of globular proteins [11]. The positive
792 and/or negative electrodynamic pressure generated by the solute–protein interaction
793 (perhaps as implied in Lifshitz's dispersion forces) across the water medium seems to be the
794 fundamental mechanism by which solutes affect protein stability [8] as against preferential
795 hydration or exclusion of cosolute.

796 As stated elsewhere [18] another aspect of the effect of sucrose is the energy cost
797 of cavity formation in order to accommodate the expanded conformation of the unfolded
798 enzymes. The free energy needed to accommodate the expanded form in the presence of
799 sucrose is high. Therefore, in line with Lech atelier principle, there was a shift towards the
800 direction of less expanded or more compacted species within native state ensemble [28, 30].
801 This may be as a result of exclusion of sucrose from the enzyme due to increase in surface
802 tension of water occasioned by sucrose in a manner dependent on the proteins' surface area
803 [28, 30]. The increase in surface tension may explain the increase in the free energy cost for
804 cavity formation for the accommodation of the unfolded protein [28, 30]. If the case of
805 glycerol is a general one [31] then sucrose, may have achieved partial refolding of the
806 enzyme by strengthening hydrophobic interaction and by overcoming the unfavourable
807 electrostatic interaction between charged residues [31]. Since destabilisers and stabilisers
808 have opposing effects, one may conjecture that unlike sucrose, ethanol and aspirin which
809 bind may be decreasing the surface tension, reducing the energy cost for cavity formation for
810 the accommodation of the expanded unfolded enzyme.

Table 5c. Preferential interaction parameters in a reaction mixture containing aspirin 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

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

In summary, it is pertinent to state that lack of details occasioned by what may have been considered as basic principles requiring less attention results in perceived technical or conceptual error in well-intended research papers in literature. Although a dimensionally consistent equation may be the case, it does not necessarily imply that the equation/model is suitable for the qualitative and quantitative analysis of issues being addressed. On the other hand the issue/concept being addressed may be clear, the theoretical background, both qualitative and mathematical may become invalid if in particular, the mathematical models, give results that are dimensionally inconsistent with the parameters to be determined. This is the hallmark of various observations in literature that motivated this research. The contentious issue was precipitated by the observation in Eq. (19a), as found in literature, which shows that the left hand side is dimensionless while the right hand side is not (unit is litre/mol.). The appearance of $\left(\frac{\partial \mu_3}{\partial \mathrm{In} C_3}\right)_{\mathrm{T,P}}$ and $\frac{\partial \mu_3}{\partial C_3}$ in some equations in literature is one such evidence of inconsistence making the value of hydrated molar volume of cosolute contentious. Both parameters can be dimensionless if the mole fractions were to be the case otherwise, some of the equations where they appear, become invalid. For instance Eq. (21) and Eq. (23) are dimensionally inaccurate.

Combining Timasheff equation (Eq. (2)) with derived equation (Eq. (27a)) results in a different slope and consequently the value of $G_{21} - G_{23}$which appeared as a reciprocal

equal to the slope is also different from what is expected from Eq. (28a). Also, the introduction of apparent molar volume, V_1 into Eq. (41) for instance creates, *ab initio*, a dimensionally consistent equation, including the derived equation for the determination of V_1. The introduction of $RT \ln a_3$ into Eq. (39) and V_1 into Eq. (41) gives after integration equations which are dimensionally valid but not necessarily suitable equations for the determination of V_1. Taking $1\text{-}1/\gamma_3$ as a function of C_3 gives a better correlation, where V_1 is a slope. The equation of unfolding has also been revisited, and deriving in the process, alternative equations that are suitable for different situations in which velocity of amylolysis as observed is either greater or less than the velocity for native untreated enzyme, with a caveat that the observed velocity of hydrolysis for the treated enzyme is greater than for the unfolded enzyme. The concept of preferential interaction and *m*-value were investigated by treating the enzyme with three cosolutes, ethanol, aspirin, and sucrose.

This summary is imperatively terminated with following comment. The fact that ethanol has been implicated in the aetiology of distinct intermediate protein states responsible for numerous neurodegenerative diseases such as Alzheimer's disease, Parkinson's disease, and Huntington's disease [23] should motivate the need for appropriate models that can be used to quantify the physico-chemical and biophysical effect of ethanol so as to establish a standard. This does not rule out improvisation as was the case in the thesis that generated the data; but the truth needs to be told as to the degree of precision of instrumentation. Stating otherwise to gain acceptance or evade censorship render quantitative result invalid and below standard in the light of the wishes of Strenda and what is expected of high precision instrumentation.

CONCLUSION

A major theoretical investigation was carried out on the issue of solution structure with a conclusion that it 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 preferential interaction coefficient or parameter remains thermodynamically an extensive

parameter. Some of the derived equations may remain dimensionally invalid if standard reference concentration/activity is not substituted into such equations. All derived equations based on speculation or assumption except the equation derived from first principle may be useful for the determination of ($G_{13} - G_{33}$), the apparent hydrated molar volume of the osmolyte/cosolute. As with ethanol unlike aspirin, the *m*-values exhibit positivity contrary to the usual; the cognate preferential interaction coefficient has sign other than the usual with ethanol unlike with aspirin alone and with sucrose. In the light of earlier comment, it is hereby recommended that for feature research, scholars or researchers should against the backdrop of the theoretical exposition in this research carry out experiment with a-state-of-the-act high precision instrumentation so as generate very high quality data.

COMPETING INTERESTS DISCLAIMER:

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

COMPETING INTERESTS

There is no competing interest..

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SUPPLEMENTARY TEXT

1. Apparent hydrated molar volume based on alternate equations to Eq. (19a)

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

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

Equation (38) expresses accurate dimension because C_3V_1 is dimensionless. The value of V_1, in this case is taken at a fixed concentration of the osmolyte at varying temperature which expectedly affects the chemical potential of the osmolyte at constant pressure. The concentration of the osmolyte can also be affected because, the density of the aqueous solvent changes with temperature. But at a fixed thermodynamic temperature and pressure,

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

There is a deduction from Eq. (39) which is the issue of generalisation to both ideal and nonideal solution of the osmolyte. The variable $\left(\frac{\Delta\mu_3}{RT}\right)_{\mathrm{P,T}}$ may be equal to InC_3 or Ina_3, if ideal or nonideal case is applicable. However, in line with Levine [22], it seems more appropriate to use In X_3 (for the idea case solution) and In γ_3X_3 (for the nonideal case). Nonetheless, rearrangement of Eq. (39) gives equation which shows clearly again that V_1 can only be seen as constant quantity if obtained as a slope. The equation is

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

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

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

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

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

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

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

Next, one integrates as follows:

$$\int \partial \mathrm{In}a_3 = \int \frac{V_1}{1-C_3V_1}\partial C_3 \tag{43a}$$

$$\mathrm{In}a_3 = -\mathrm{In}(1-C_3V_1) \tag{43b}$$

$$1-\frac{1}{a_3} = C_3V_1 \tag{44}$$

Nonetheless Eq. (44) remains conjectural until firmly proven by the core chemical physicist. The slope of the plot of left-hand side versus right-hand side will always yield a positive slope. The implication is that the apparent hydrated molar volume yielded from such plot can be exceptionally large for very dilute solution of the cosolute given that for ideal solution $a_3 \cong C_3$ unlike nonideal solution. The same is applicable, but to a greater extent, if mole fraction is taken in place of a_3. Having used C_3 directly and $\mathrm{In}C_3$ where applicable and having seen a clear dimensional inconsistency, there is need to consider the use of mole fraction of solution component as in literature [22]. Doing so is very likely to give very large slope as the apparent hydrated molar volume for the dilute solutions well above the values obtained using a_3 and γ_3.

The place of standard reference molar concentration or activity has general implication. It is necessary to note that $\Delta\mu_3 = RT\mathrm{In}C_\mathrm{i} \neq RT\mathrm{In}x_\mathrm{i}$ where, x_i is the mole fraction of the solution component given as $n_\mathrm{i}/(n_1+n_\mathrm{i})$ where respectively, n_i and n_1 are the number of moles of any solution components and water (usually $\cong$ 55.5556). For reason

stated elsewhere [15], the equation, $\Delta\Gamma_{2i} = \frac{\ln K_{eq}}{\ln a_i}$ may need to be rewritten as $\Delta\Gamma_{2i} = \frac{\ln K_{eq}}{\ln a_i/a_i^0}$ which is never equal to $\Delta\Gamma_{2i} = \frac{\ln K_{eq}}{\ln(\gamma_i x_i)}$.

2. The reexamination and derivation of the model equations for the determination of the equilibrium constant, for the transition from folded to unfolded protein.

The reexamination and derivation of equations for the determination of equilibrium constant for the transition from folded to unfolded protein is the purpose of this subsection. Meanwhile, there is the need to make further modification of Baskakov and Bolen equation [32]. The equation seems to suggest that the equilibrium constant for folding-unfolding transition may be increasing with increasing concentration of the protecting osmolyte in particular in the presence of a known destabilizing cosolute. This is against the backdrop of the fact that the specific activity of the enzyme may be increasing with increasing concentration of the protecting osmolyte. The paradox is that $[U]/[F] > 1$ for such a case. Here, *U* and *F* are respectively the unfolded and folded protein. The conformational adjustment by partial unfolding does not amount to instability. The issue of conformational flexibility for function dictated by the environment is well studied [33, 34]. The 2nd paradox is that the *m*-value should also be negative even if the specific activity of the enzyme is > the native activity.

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

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

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

Another scenario is the specific activity which may be > unfolded enzyme specific activity but < the native enzyme activity even with increasing concentration of the protecting osmolyte in the presence of strong destabiliser. The specific activities may also be increasing but < native activity. In this case, the original Baskakov and Bolen equation [32] cannot apply. While the equations that are to be derived shortly may not be sacrosanct, the preceding issues cannot easily be ignored.

The equilibrium constant (K_{eq}) for the process folded (*F*)→unfolded (*U*) is adapted from Pace equation [35] and modified Baskakov and Bolen equation [32] as in previous publications [15-16, 18]. 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

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

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.

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

Therefore,

$$[N]=1-[U] \quad (48a)$$

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

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

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

$$K_{\mathrm{eq}}=\frac{V_{\mathrm{N}}-V_{\mathrm{OBS}}}{V_{\mathrm{OBS}}-V_{\mathrm{MIN}}} \quad (49)$$

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

Next is the equation for the assay in which the catalytic velocity of the enzyme is increasing with increasing concentration of the protecting osmolyte. Such velocities may be > velocity of the native enzyme in a reaction mixture containing destabilising cosolute and increasing concentration of the protecting osmolyte. The equation is also relevant to the case in which the velocities are increasing for the treated enzyme with increasing concentration of the osmolyte. Such velocities should also be > the velocity of the untreated native enzyme. To begin with it is imperative to realise that the original equation by Pace [35]

concerns the unfolding enzyme. It can be adapted for the refolding case leading to hydrodynamic radius equal to or less than the radius of the native enzyme (if there is extra-rigidification that is not very common).

$$[N] = \frac{A_{\mathrm{MAX}} - A_{\mathrm{N}}}{A_{\mathrm{MAX}} - A_{\mathrm{MIN}}} \tag{50}$$

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

$$[N] = \frac{V_{\mathrm{MAX}} - V_{\mathrm{N}}}{V_{\mathrm{MAX}} - V_{\mathrm{MIN}}} \tag{51}$$

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

$$[U] = \frac{V_{\mathrm{N}} - V_{\mathrm{MIN}}}{V_{\mathrm{MAX}} - V_{\mathrm{MIN}}} \tag{52}$$

Therefore, the equilibrium equation should be

$$K_{\mathrm{eq}} = \frac{V_{\mathrm{N}} - V_{\mathrm{MIN}}}{V_{\mathrm{MAX}} - V_{\mathrm{N}}} \tag{53}$$

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