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Basic Kirkwood – Buff Theory of Solution Structure and Appropriate Application of Wyman Linkage Equation to Biochemical Phenomena

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Authors' contributions

This work was carried out in collaboration between both authors. Author IIU designed the study, wrote the theoretical section, wrote the protocol and wrote the first draft of the manuscript. Author IIU managed the literature searches, conducted the experiment, analyzed and discussed the result while author AOO supervised the experimental process and advised on the need to prepare the manuscript for publication. Both authors read and approved the final manuscript.

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ABSTRACT

Background: Researchers who have shown interest in the consequence of introducing dry biomolecules or a solution of it into cosolvents generally known as osmolyte, have applied many models for the elucidation of the scientific basis of the results obtained. The Kirkwood and Buff theory (KBT) or its reverse form has been the basis for the interpretation of the effect of the osmolyte. There seems to be no generally acceptable definition of terms in the basic KBT mathematical formalism. There is also error in stated equations describing solution structure and misapplication of Wyman linkage relation. Therefore, the objectives of this research are 1) to show how the equation of preferential interaction parameter is derived based on KBT, 2) to show the

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appropriate way in which Wyman linkage relation can be applied, 3) to apply biochemical approach (using generated data) to the equation of preferential interaction parameter (preferential interaction parameter is symbolised as Γ_{2i}) for its calculation and calculation of parameters linked to KBT derived equations.

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

Results and Discussion: The change in solvation preference upon the ethanol partial denaturation of the enzyme and the corresponding change in preferential interaction parameter were respectively positive and negative in sign. Unexpectedly ethanol was preferentially excluded from the enzyme.

Conclusion: The equations of preferential interaction parameters were derived. The appropriate way is either by calculation or measurement of preferential interaction parameter. Therefore, Γ_{2i} or $\Delta\Gamma_{2i}$ for the change, cannot be a constant (or slope) and an instrumentation–based measurable parameter at the same time. Based on Wyman linkage relation, purely biochemical thermodynamic parameter is linked to preferential interaction parameters which are therefore, thermodynamic parameters.

Keywords: Porcine pancreatic alpha amylase; preferential interaction parameter; change of solvation preference; m – value; Kirkwood-Buff integrals; ethanol.

1. INTRODUCTION

While data generated from experiments may be closer to a near–feature application, a purely theoretical exposition, nevertheless, serves as a very veritable background and insight for feature experimental investigation and likely application. The challenge resulting from the absence of hi-tech instrumentation that bedevil developing institutions experienced by some research students including the leading author of this research should not always deter a prospective researcher if existing theoretical concepts can either be extended, applied, or a new model advanced by such researcher. To this end the work of Timasheff [1] profusely cited in this research has become very instructive and relevant.

There had been objections against the interpretation of intercepts and slope in the derived equation of preferential interaction of solution components with the biomolecule in solution. The concern of biological scientist and medical scientist is the effect of solution components on biomolecules. The effects result from the interaction of the solution components with the biomolecules. Diseases associated with inappropriate folding otherwise called misfolding had been of concern to researchers [2]. According to Sirotkin et al. and cited references by the authors [2] "distinct intermediate protein states, induced by alcohols, ethanol in particular, may be responsible for numerous neurode-generative diseases (Alzheimer's disease, Parkinson's disease, and Huntington's disease)". Apart from genetically induced folding or misfolding, osmolytes in high concentration may also induce such effects. Drug–protein interactions are of interest to the pharmacist in particular [3]. In the papers by Shimizu [4], Timasheff [1], and Zheng [3] are examples of the techniques for the measurement of solution components' (drugs and cosolutes) interaction with biomolecules. The measurement of the interaction parameters is carried out using physical techniques such as ultrafiltration, equilibrium dialysis, fluorescence spectroscopy, capillary electrophoresis, UV−vis spectroscopy, solid-phase microextraction, circular dichroism, surface plasmon resonance, nuclear magnetic resonance spectroscopy, and X-ray crystallo-graphy [3], sedimentation equilibrium [4] and pressure osmometry [1]. Nothing in literature shows that interaction of two different solution components (the cosolutes) with the biomolecule can be measured at the same time by these devices.

The advent of Kirkwood – Buff theory has enabled the formulation of equation that may be applied to the determination of the relative number of solution components interacting with the macromolecule in solution. Water as a solvent has its structure arising from interaction with neighbouring water molecules leading to transient cluster formation due to continuous thermal agitation. Water-water interaction in the structure is called water-self correlation denoted by Kirkwood-Buff integral G_{11} but often ignored. Introduction of a solute into water changes its chemical potential and activity leading to formations such as osmolyte self solvation or osmolation, G_{33} , osmolyte hydration, G_{31} or G_{21}

and G_{23} if a protein, for instance, is in solution, all constituting part of the solution structure. The studies had most often been through biophysical methods as stated earlier. Despite this development advanced by the advocates [1–6] of the theory in their various papers, there is misgiving by other author [1,4] as to the definite definition of terms in the equations emanating from the theory. There seems not be an attempt to reach acceptable position on this issue. There is also a confusing way in the application of Wyman linkage relation for the determination of preferential interaction parameter. The objectives of this research are 1) to show how the equation of preferential interaction parameter is derived based on Kirkwood-Buff theory (KBT), 2) to show the appropriate way in which Wyman linkage relation can be applied, 3) to apply biochemical approach (using generated data) to the equation of Γ_{2i} (*i.e*. preferential interaction parameter) for its calculation and calculation of parameters linked to KBT derived equations.

2. THEORY

In line with information in literature [5], the interpretation of the basic Kirkwood-Buff (KB) solution theory is simply an expression of the thermodynamic properties of an isotropic solution of aqueous biochemical compounds. The thermodynamic properties are connected to the average structure of all solution [5, 6]. The average structure in turn is given by radial distribution functions $g_{2i}(r)$ between species 2 and i (any chemical species referred to as cosolvent) in solution. This definition is the original contribution of Kirkwood and Buff [7] who opined that, the structure of water can be expressed in terms of the average spatial arrangement of molecules in solution, which is given by radial distribution functions. The function, $g_{2i}(r)$ is a measure of the deviation from the random distribution of particles of type i from a central particle (the biomolecule), as a function of the distance (r) from the central particle [5]. The function, $g_{2i}(r)$ can also be described as pair correlation function while the radial distances can also be referred to as sphere diameters [5].

At a distance closer than the sum of the two radii (constant distance), steric exclusion operates. However, steric exclusion may have its meaning but not all osmolytes are excluded. Some bind and penetrate the three dimensional (3–D) structures. Exclusion may also be as a result of osmophobic effect [8]. At large distances there is no correlation between particle and the pair correlation function approaches unity ($g_{2i}(r) = 1$) [6]. A positive or negative deviation of g_{2i} from unity, at a certain distance corresponds to an excess or deficit of i at the indicated distance from the biomolecule and is the positive or negative correlation of biomolecule and i at that distance [5]. The overall correlation $g_{2i}(r)$ involving excess or deficit in occupied volume of particles of type α (the biomolecule) around i (or vice versa) is obtained by integrating the deviations from random distribution. The overall correlations as a function of the packing are the KB integrals (KBI) defined by integrating between 0 and ∞ as follows [5,6].

$$\phi_{2i}/N_A = C_i G_{2i} = 4\pi C_i \int_0^\infty (g_{2i}(r) - 1) r^2 \mathrm{d}r \qquad (1)$$

Where C_i and G_{2i} are concentrations of the solution component and KBI respectively; N_A is the Avogadro's number. The end of Eq. (1) is reserved for chemical physicist or biophysical chemist and $N_{2i} = \phi_{2i}/N_A$. Here, N_{2i} is the excess number of component *i* around the biomolecule [4]. The same author [4] sees N_{2i} as a parameter which signifies the change in number of component *i* when biomolecule is introduced into the system. Perhaps, the author seems to refer ΔN_{2i} to the 2nd definition. What may be of interest to the biochemist, is the relation [5].

$$(-)\Gamma_{2i} = C_i(G_{21} - G_{23}) \qquad (2)$$

Where G_{21} and G_{23} are respectively the KBI for hydration and osmolation of any biomolecule; Γ_{2i} is the preferential interaction parameter; $(-)$ means that its absence may refer to preferential hydration such that $\Gamma_{2i} = \Gamma_{21}$. 1, 2, and 3 refer to water, protein (or any biomolecule), and cosolvent otherwise known also as osmolyte. However, Shimizu's [4] definition has been improved upon by Shurr *et al* [9] definition which defines N_{2i} as either N_{21} or N_{32} which respectively denotes the total number of water and osmolyte molecules in a domain of sufficient size surrounding a single isolated macromolecule. The parameter Γ_{2i} which is either Γ_{21} or Γ_{23} "represents the excess water or osmolyte in the vicinity of the macromolecule above the quantity that would be expected from the number of water molecules in that region and the bulk concentration ratio, C_3/C_1"[9]. This is against the view that N_{2i} merely describes experimental results in terms of a model based on site occupancy by water or ligand molecules [10]. Definitions in line with KB theory are also against the view that they are useful descriptive

quantities that sum up all the perturbations by the protein of cosolvent and water molecules, each of which may make only a fractional contribution to N_{23} or N_{21} [1].

Since Γ_{2i} may be directly measurable there is need to state G_{2i} as N_{2i}/C_i. As a result of this the following may hold.

$$G_{21} = N_{21}/C_1 \quad (3)$$

$$G_{23} = N_{23}/C_3 \quad (4)$$

Substituting Eq. (3) and Eq. (4) into $\Gamma_{21} = C_1(G_{21} - G_{23})$ gives after expansion:

$$\Gamma_{21} = N_{21} - \frac{C_1}{C_3}N_{23} \quad (5)$$

Substituting Eq. (3) and Eq. (4) into $-\Gamma_{23} = C_3(G_{21} - G_{23})$ gives:

$$-\Gamma_{23} = \frac{C_3}{C_1}N_{21} - N_{23} \quad (6a)$$

$$\Gamma_{23} = N_{23} - \frac{C_3}{C_1}N_{21} \quad (6b)$$

Meanwhile in line with Wyman linkage relation are the following in literature [1]

$$\left(\frac{\partial \mathrm{In}K}{\partial \mathrm{In}a_1}\right)_{P,T,C_2} = \Delta N_{21} - \frac{C_1}{C_3}\Delta N_{23} = \Delta\Gamma_{21} \quad (7)$$

$$\left(\frac{\partial \mathrm{In}K}{\partial \mathrm{In}a_3}\right)_{P,T,C_2} = \Delta N_{23} - \frac{C_3}{C_1}\Delta N_{21} = \Delta\Gamma_{23} \quad (8a)$$

Where K and a_i are the equilibrium constant for the reaction and activity of the solution component respectively. The views had been that the preferential interactions described by Eq. (5) through Eq. (6b) and change in such interactions described by Eq. (7) and Eq. (8a) are summations over a wide spectrum of interactions, whether attractive or repulsive between the protein and the solvent components [10]. This is apart from the view that they are not real physical number of 1 or 3 [1]. However, inverse KB theory allows for a numerical determination of the KB integrals, G_{2i} (the correlations between solution components) from experimental data [5]. This can be achieved by plotting measured values of Γ_{21} versus $1/C_3$ and Γ_{23} versus C_3 as applicable to Eq. (5) and Eq. (6b) respectively from which the slope or intercept can be substituted into Eq. (3) or Eq. (4) as the case may be for the calculation of KB integrals, G_{2i}. This is contingent upon a linear regression analysis that remains a mere probability. The Kirkwood – Burk theory enables the identification of the relationship between r_{2i} and the structure of the solution at infinite dilution of the biomolecule [4].

Before proceeding further, there is need to reconsider equations (7) and (8a) which contain the equilibrium constant, K; the latter and a_i are dependent on the concentration of the cosolvent. Mathematically, an equation contains a dependent variable and one or more independent variables. Since concentration, C_i on which K and a_i depend is an independent variable, r_{2i} should be the ultimate dependent variable. The suitable equation should be $\left(\frac{\mathrm{In}K}{\mathrm{In}a_i}\right)_{P,T,C_2} = \Gamma_{2i}$. For the purpose of clarity, a simple analogy is the speed (u) recorded after taking measurement of a distance covered in known time (t); if the distance (d) covered increased within the same time, then there must have been acceleration due to an increase in kinetic energy (*KE*). Meanwhile, $u = f(s,t)$. The value of s and t depends on current *KE* (this is as expected of two objects of the same mass moving at different speed because of differences in kinetic energy); longer distance coved within the same time must be at a higher *KE*. A perfect correlation, negative or positive, yields a coefficient of determination $r^2 = 1$. Imperfection or partial deviation from linearity is not precluded, but a slope is expected to be constant. Introduction of a second cosolvent with opposite effect to the first can cause a change in K and a_i and ultimately, a change in Γ_{2i} $\left(\Delta\Gamma_{2i} = \frac{\Delta \mathrm{In}K}{\Delta \mathrm{In}a_i}\right)$. A slope is not final value minus initial value of a variable as it seems to imply given $\Delta\Gamma_{2i}$. Thus Wyman linkage relation needs to be correctly applied.

Another foreseeable dilemma arises from the claim that, two binding parameters, preferential binding and preferential hydration are equivalents. They are linked together as follows [1]: $\Gamma_{21} = -(C_1/C_3)\Gamma_{23}$ and alternatively as may be applicable to reaction $\Delta\Gamma_{21} = -(C_1/C_3)\Delta\Gamma_{23}$. But preferential binding of a ligand ought to precipitate dehydration since water of hydration and preferential interaction may be displaced according to the equation [1]:$\mathrm{P}.nH_2O + \mathrm{L} \rightleftharpoons \mathrm{P}.\mathrm{L} + nH_2O$ where the alphabets, P and L are the protein and ligand that bind. This obviously has nothing to do with preferential exclusion. However, it may be inferred that the source of hydration that arises is due to diffusion along

chemical potential gradient from the bulk to the vicinity of the protein. Within the vicinity of the protein the preferentially binding osmolyte concentration is higher than in the bulk. One may not hastily conclude that this diffusion of water towards the osmolyte-bound protein compensates for the departing water of hydration arising from osmolation. With respect to a protecting polar osmolyte, a different scenario is expected because there may be binding if the dry protein is introduced into such solution unlike what may be expected if the unfolded protein is introduced into the same solution. Exclusion of the protecting osmolyte from the vicinity of the unfolded protein creates osmolyte concentration gradient. In other words the chemical potential of water around the protein is higher than in the bulk. This may constitute the hydration phenomenon. However, based on the concept of translational entropy gain of water molecules [11], water is expected to diffuse along chemical potential gradient towards the bulk, compelling the protein to refold. Tentatively, one may assume that the following equation can serve preferentially excluded osmolyte.

$$-\Delta N_{23} + \frac{C_3}{C_1}\Delta N_{21} = -\Delta\Gamma_{23} \quad (8b)$$

The interactions, whether attractive or repulsive between the protein and the solution components [10] may vary in strength. The interaction may vary from strong immobilization to weak momentary perturbations and repulsion that cause these molecules to fluctuate to different degrees with the protein in Brownian motion [1]. This view is very valid considering the fact that the solute of different kinds are under thermal perturbation. The implication is that, interactions based on polar – polar attraction which are much applicable to bulk water, as well as protein water of hydration are subject to such perturbation. Only very strong bonding with water molecule due to formal charge or net charge of a protein for instance, leading to charge – polar attraction that may be resistant to ambient thermal perturbation. It is the existence of net charge in particular that strongly account for hydration. A very stable water of hydration enables consistent hydrogen bonding with surrounding water molecules otherwise steric factor due largely to the size of protein would have partially reduced the solubility of protein. Thus while small molecules like ethanol may possess hydrophobic group it is still very miscible with water because its size cannot permanently overcome the attractive force that yields hydrogen bond. It is well known fact that the solubility of alkanols decreases with increasing size of the alkyl group. A recent research shows that "…increased negative surface charge correlates strongly with increased protein solubility and may be due to strong binding of water by the acidic amino acids" [12].

Changes in physicochemical properties of the biomolecule, protein, and ethanol may occur if the solution of a protein is transferred into a solution of ethanol. If different degree of unfolding occurs, there may be equilibrium state between the subpopulation of native (*N*) and unfolded (*U*) state in a two state model given as $N \rightleftharpoons U$. The absorbance of the molecule is often taken and the equilibrium determined using the equation [13], $U = (A_{\mathrm{N}} - A_{\mathrm{OBS}})/(A_{\mathrm{N}} - A_{\mathrm{D}})$ where A_{N}, A_{OBS}, and A_{D} are the absorbance of the native, the absorbance used to monitor (un) folding, and the absorbance of the fully denatured protein. This equation is then substituted into the equation of equilibrium constant (K) below.

$$K = U/(1-U) \quad (9)$$

This is often the practice and it is essentially biophysical. Kinetic data, the velocity (or maximum velocity) of catalytic action of the enzyme made popular by Baskakov, Wang and Bolen (1998) [14] can also be explored but in a modified form as shown elsewhere [15].

If the hydrolytic activity of an enzyme, alpha amylase, decreases to value < value without ethanol, with increasing concentration of ethanol, then there may have been destabilization of the enzyme. There may also be increasing hydrolytic activity to values < value without the cosolvent, ethanol, with increasing concentration of the latter. Thus there may positive or negative linear correlation of hydrolytic activity with the concentration of the cosolvent. Where there is a decreasing trend, a plot of velocity (v) of hydrolysis versus $1/C_{\mathrm{i}}$ should give an intercept, being an extrapolated velocity ($v_{C_{\mathrm{i}}\to\infty} \to 0$) of hydrolysis as $C_{\mathrm{i}} \to\infty$. It may appear theoretical but that is the essence of this research, a combination of theory and minor experimentation. If on the other hand, there is increasing v with increasing C_{i}, a plot of v versus C_{i} should give an intercept, being an extrapolated velocity ($v_{(C_{\mathrm{i}}=0)}$) of hydrolysis as $C_{\mathrm{i}} \to 0$. A relationship that fits into this scenario needs to be adopted. This can be found in literature [12] and given as follows: $\log v = \log v_{C_{\mathrm{i}}\to\infty} - \beta[C_{\mathrm{i}}]$. Here, a modified form of the latter is applied such

that plot of $\log v$ versus (C_i) is used to determine needed intercept. The equation below may therefore, be relevant if v is increasing with increasing C_i.

$$\log v = \log v_{C_i \to 0} + \beta[C_i] \quad (10)$$

Where there is decreasing trend with increasing C_i the equation below may be the case.

$$\log v = \log v_{C_i \to \infty} + \beta/[C_i] \quad (11)$$

Meanwhile, many destabilizing osmolyte including in particular urea have been studied [5,16]. This entails preferential binding. The preferential binding depends markedly on the chemical nature of the protein surface [2]. Since ethanol is a cosolvent in this research, there is need to examine the theory of its solution structure. First is the dependence of chemical potential (μ_3) of the cosolvent on its concentration, C_i which according to Rösgen et al. [5] is given according to KBT as:

$$\frac{1}{RT}\left(\frac{\partial \mu_3}{\partial C_3}\right)_{T,P} = \frac{1}{C_3} + \frac{V_1}{1 - V_1 C_3} \quad (12)$$

Where, *T*, *R*, and *P* refer to the thermodynamic temperature, gas constant, and standard pressure respectively.

Meanwhile,

$$V_1 = G_{13} - G_{33} \quad (13)$$

In Eq. (13) V_1 is defined as apparent molar hydrated volume and it is seen as a constant in this first-order expression (Eq. (12)) for the chemical potential of the cosolvent. The integrated form given by integrating the derivative with respect to C_3 in Eq. (12) gives,

$$\mu_3 = \mu_3^0 + RT\mathrm{In}\left(\frac{C_3}{1 - V_1 C_3}\right) \quad (14a)$$

Equation (14a) is important because it shows that thermodynamic property of any solution expressed via chemical potential has the potential to influence the solvent that might ultimately influence the solution properties of the biomolecules. For calculational purpose, Eq. (14a) may be useful for the determination of V_1 if $\Delta\mu_3$ can be independently determined. Thus,

$$V_1 = \frac{1 - \exp\left(\mathrm{In}C_3 - (\mu_3 - \mu_3^0)/RT\right)}{C_3} \quad (14b)$$

The view is that V_1 is a constant and dependent on C_3 (which may remain a theoretical speculation) and the factor $1 - V_1 C_3$ modulates (up or down) the sensitivity of the protein chemical potential with respect to the concentration of the osmolyte [5]. Moving away from binary solution containing 1 and 3 to ternary solution containing 1, 2, and 3 may alter the chemical potential environment of all solution components. This takes one to protein osmolation that has effect on its stability and solution structure. For dilute protein solution as it is often the case in an *in vitro* assay, the chemical potential (μ_2) of the protein (enzyme for instance) depends according to many researchers [5,17–18] on C_3 through the relation:

$$\frac{1}{RT}\left(\frac{\partial \mu_2}{\partial C_3}\right)_{T,P} = \frac{G_{21} - G_{23}}{1 - C_3 V_1} \quad (15)$$

As usual integrating the derivative in Eq. (15) with respect to C_3 for calculational purpose, reechoes the issue of Γ_{23} as a dependent parameter given that $\Gamma_{23} = -C_3(G_{21} - G_{23})$. The result of integration gives:

$$\Delta\mu_2 = \frac{RT(G_{21} - G_{23})}{V_1}\mathrm{In}(1 - C_3 V_1) \quad (16)$$

The result which seems to be ignored in literature shows that $\Delta\mu_2$ is the only dependent variable given, albeit speculatively, that V_1 is constant. The implication is that, the slope is given as:

$$S_{\mathrm{lope}} = \frac{RT(G_{21} - G_{23})}{V_1} \quad (17)$$

Re-emphasising the fact that Γ_{23} may not be a constant under a given condition, leads one to assume that what should be a constant is G_{21}; this may not preclude the fact that given different concentration range of an osmolyte, different slopes may be obtained. What is very certain is that given different concentration of the osmolyte, G_{21} assumed to be constant, the results of $-C_3(G_{21} - G_{23})$ should be different from one osmolyte concentration to another. Again this seems speculative otherwise using physical methods, Γ_{23} may be directly measured at different C_3, so that its division by C_3, should be seen to be constant. This is however, a speculation until proved experimentally. Substitution of $-\Gamma_{23}/C_3$ into Eq. (16) and rearrangement makes Γ_{23} subject of the formula to give

$$-\Gamma_{23} = \frac{\Delta\mu_2 V_1 C_3}{RT\mathrm{In}(1 - C_3 V_1)} \quad (18)$$

Looking at Eq. (18) one sees that Γ_{23} and $\Delta\mu_2$ are both function of C_3. Therefore, $-\Gamma_{23}/\Delta\mu_2$ may be

the dependent variable if information about V_1 is known. There is need not to shy from the question of whether or not mathematical procedure is valid considering however, that the same procedure was applied in deriving Eq. (14a) which appears meaningful.

Further examination of Eq. (5) and Eq. (6b) reminds one that under a defined condition of temperature, pressure, and *p*H, the parameters, N_{23} and N_{21} are either a slope, part of a slope or intercept. Thus considering the relations $G_{21}\ C_1$ i.e. N_{21} and $G_{23}\ C_3$ *i.e.* N_{23}, in the light of Eq. (5) and Eq. (6b) respectively, the following analysis could reveal that the only constant KB integral is the KB integral for preferential hydration. Beginning from Eq. (5), the slope $\frac{\partial \Gamma_{21}}{\partial C_3^{-1}}$ and intercept $\left(\Gamma_{21(C_3\to\infty)}\right)$ are $C_1 N_{23}$ and $N_{21}^{C_3\to\infty}$ respectively. Therefore,

$$\frac{\partial \Gamma_{21}}{\partial C_3^{-1}} = C_1 N_{23} \tag{19}$$

Thus, N_{23} may be seen as constant since the slope and C_1 are constant quantities given defined conditions stated earlier. But, from Eq. (19) $\frac{\partial \Gamma_{21}}{C_1 \partial C_3^{-1}} = N_{23}$ and division by C_3 yields

$$\frac{N_{23}}{C_3} = \frac{\partial \Gamma_{21}}{C_3 C_1 \partial C_3^{-1}} = G_{23} \tag{20}$$

Since C_3 is the only variable in Eq. (20), G_{23} cannot be a constant with any C_3. From the intercept is the following, $N_{21}^{C_3\to\infty}$ (where the superscript denotes the value of Γ_{21} as $C_3 \to \infty$) and division by C_1 gives

$$\frac{N_{21}^{C_3\to\infty}}{C_1} = G_{21} \tag{21}$$

One sees that in Eq. (21), both denominator and nominator are constant, giving the informed impression that, G_{21} , the KB integral for hydration, is a constant. From Eq. (6b) is the slope, $\frac{\partial \Gamma_{23}}{\partial C_3} = N_{21}/C_1$. Therefore,

$$N_{21} = C_1 \frac{\partial \Gamma_{23}}{\partial C_3} \tag{22a}$$

Division of Eq. (22a) by C_1 gives

$$\frac{N_{21}}{C_1} = \frac{\partial \Gamma_{23}}{\partial C_3} = G_{21} \tag{22b}$$

Equation (22b) shows that the variation of Γ_{23} with C_3 is a constant denoted by KB integral for hydration thereby suggesting that G_{21} is always a constant parameter. From the intercept, $\Gamma_{23(C_3\to 0)}$ is $N_{23}^{C_3\to 0}$ in which the superscript, $C_3 \to 0$ denotes value of Γ_{23} when $C_3 \to 0$. Division of intercept by C_3 gives

$$\frac{\Gamma_{23(C_3\to\infty)}}{C_3} = \frac{N_{23}^{C_3\to 0}}{C_3} = G_{23} \tag{23}$$

Equation (23) again shows that KB integral for osmolation cannot be a constant with every C_3. With this scenario, Eq. (16) as in literature needs to be modified. Accepting the fact that $\Gamma_{23} = -C_3(G_{21} - G_{23})$, and that G_{21} appears to be a constant then, Γ_{23} can be restated as:

$$\Gamma_{23} = -C_3\left(G_{21} - \frac{N_{23}^{C_3\to 0}}{C_3}\right) \tag{24a}$$

$$G_{21} - \frac{N_{23}^{C_3\to 0}}{C_3} = \frac{-\Gamma_{23}}{C_3} \tag{24b}$$

Substitution of Eq. (24b) into Eq. (15) gives

$$\frac{1}{RT}\left(\frac{\partial \mu_2}{\partial C_3}\right)_{T,P} = \frac{\left(G_{21} - N_{23}^{C_3\to 0}/C_3\right)}{1 - C_3 V_1} \tag{25a}$$

Expansion of Eq. (25a) gives

$$\frac{1}{RT}\left(\frac{\partial \mu_2}{\partial C_3}\right)_{T,P} = \frac{G_{21}}{1 - C_3 V_1} - \frac{N_{23}^{C_3\to 0}/C_3}{1 - C_3 V_1} \tag{25b}$$

Further rearrangement gives

$$\frac{1}{RT}\left(\frac{\partial \mu_2}{\partial C_3}\right)_{T,P} = \frac{G_{21}}{1 - C_3 V_1} - \frac{N_{23}^{C_3\to 0}}{C_3(1 - C_3 V_1)} \tag{25c}$$

Integrating the derivative, Eq. (25), for calculational purpose (if V_1 is known), gives

$$(\Delta\mu_2)_{T,P} = \frac{G_{21}}{V_1} \mathrm{In} \frac{1}{(1 - C_3 V_1)} - N_{23}^{C_3\to 0} \int_{C_3\to 0}^{C_3\to\infty} \frac{\partial C_3}{C_3(1 - C_3 V_1)} \tag{26a}$$

The alternatives to Eq. (26a) in terms of only $N_{2\mathrm{i}}$ and only $G_{2\mathrm{i}}$ are respectively

$$(\Delta\mu_2)_{T,P} = \frac{N_{21}}{C_1 V_1} \mathrm{In} \frac{1}{(1 - C_3 V_1)} - N_{23}^{C_3\to 0} \int_{C_3\to 0}^{C_3\to\infty} \frac{\partial C_3}{C_3(1 - C_3 V_1)} \tag{26b}$$

$$(\Delta\mu_2)_{T,P} = \frac{G_{21}}{V_1} \mathrm{In} \frac{1}{(1 - C_3 V_1)} - \frac{G_{23}^{C_3\to 0}}{V_1} \mathrm{In} \frac{1}{(1 - C_3 V_1)} \tag{26c}$$

Being another form of Eq. (26a), Eq. (26c) makes further derivation easier since, $G_{23}^{C_3\to 0}$ as $C_3 \to 0$ does not nullify the fact that $G_{23}^{C_3\to 0}$ is not constant and can be replaced with $N_{23}^{C_3\to 0}/C_3$. One should also recall too, that, $\frac{\partial \Gamma_{21}}{C_1 \partial C_3^{-1}} = N_{23}$ *i.e.* slope divide

by solvent concentration, C_1 as expected from Eq. (5). Thus replacing G_{21} and $G_{23}^{C_3\to 0}$ respectively with $\frac{N_{21}}{C_1}$ and $N_{23}^{C_3\to 0}/C_3$ in Eq. (26c) gives a more convenient equation as follows:

$$(\Delta\mu_2)_{T,P} = \frac{N_{21}}{C_1V_1}\ln\frac{1}{(1-C_3V_1)} - \frac{N_{23}^{C_3\to 0}}{C_3V_1}\ln\frac{1}{(1-C_3V_1)} \quad (26d)$$

There should be a way of calculating V_1 so that the dependent parameter that is mainly a function of C_3 can be calculated.

2.1 The Determination of Apparent Hydrated Molar Volume of the Osmolyte in Terms of *m* – value

First is the relationship between the *m*-value and KB integral for hydration and osmolation [5].

$$-\left(\frac{\partial \ln K}{\partial C_3}\right)_{T,P} = \frac{m}{RT} = \frac{\Delta_N^D(G_{21}) - \Delta_N^D(G_{23})}{1-C_3V_1} \quad (27)$$

Where, $V_1 = G_{13} - G_{33}$ and G_{13} and G_{33} are the KBI for osmolyte hydration and osmolyte self osmolation (correlation) respectively; *m* for short denotes the *m* – value and the change in solvation preference upon unfolding is $\Delta_N^D(G_{21} - G_{23})$; but elsewhere in the text the author [5] used $\Delta_N^D(G_{21)} - \Delta_N^D(G_{23})$ while explaining why the former may be zero. Thus "both $\Delta_N^D(G_{21)}$ and $\Delta_N^D(G_{23})$ approach the partial molar volume of the protein given as $-\Delta_N^D\nabla_2$ at high C_3 and their difference $\Delta_N^D(G_{21)} - \Delta_N^D(G_{23})$ converges to zero, *i.e.* $\Delta_N^D(G_{21} - G_{23})$→zero" [5]. As applied earlier in the text, Eq. (27) can be re-stated as:

$$\frac{m}{RT} = \frac{\frac{\Delta N_{21}}{C_1} - \frac{\Delta N_{23}}{C_3}}{1-C_3V_1} \quad (28)$$

The apparent hydrated molar volume of the protein is therefore, given as:

$$V_1 = \frac{1}{C_3}\left(1 - \frac{RT}{m}\left(\frac{C_3\Delta N_{21} - C_1\Delta N_{23}}{C_1C_3}\right)\right) \quad (29)$$

Once again given different values of C_3 it is rather not certain how V_1 can remain constant for every value of C_3.

Equation (14b) and Eq. (29) can be combined. Thus,

$$V_1 = \frac{1-\exp(\ln C_3 - (\mu_3 - \mu_3^0)/RT)}{C_3} = \frac{1}{C_3}\left(1 - \frac{RT}{m}\left(\frac{C_3\Delta N_{21} - C_1\Delta N_{23}}{C_1C_3}\right)\right) \quad (30)$$

Simplification and rearrangement gives first:

$$\exp(\ln C_3 - (\mu_3 - \mu_3^0)/RT) = \frac{RT}{m}\left(\frac{C_3\Delta N_{21} - C_1\Delta N_{23}}{C_1C_3}\right) \quad (31a)$$

Further rearrangement gives

$$\frac{m}{RT} = \frac{-\Delta_N^D\Gamma_{23}}{C_3\exp\left(\ln C_3 - \frac{\mu_3 - \mu_3^0}{RT}\right)} \quad (31b)$$

Equation (31b) results from the substitution of $C_1\Delta G_{21}$ and $C_3\Delta G_{23}$ for ΔN_{21} and ΔN_{23} respectively in Eq. (31a) to give after simplification $\frac{RT}{m}(\Delta G_{21} - \Delta G_{23})$ on the right hand side. But according to Rösgen et al. [5], $-\Delta_N^D\Gamma_{23} = C_3(\Delta G_{21} - \Delta G_{23})$. Thus substitution of $-\Delta_N^D\Gamma_{23}$ into Eq. (31a) after rearrangement gave Eq. (31b).

2.2 A Method for the Theoretical Determination of the Density of Aqueous Solution of Ethanol with Known Concentration (in % (V/V)) of Ethanol

Although hi-tech equipment such as Anton Paar (Graz, Austria) DMA 38 vibrating U-tube densitometer [16] may be available for the measurement of the densities of solvent and solution of osmolytes, the challenge of not having readily available equipment for experimental research is inexcusable. An equation for the determination of the density of aqueous solution of ethanol and any other cosolvent with known concentration in % (V/V) is hereby derived. This is part of the theoretical presentation.

$$\rho_3^0 = \frac{m_3^0 + \rho_1 v_1}{100} \quad (32)$$

Where, m_3^0 and ρ_1 are respectively the mass of cosolvent (ethanol) in the pure stock solution as produced by the manufacturer and the density of pure water; v_1 and ρ_3^0 are respectively the volume of the solvent, pure water, in the solution and the initial density of the pure solution of the cosolvent, ethanol (as stock), with known concentration (95% (*V*/*V*)) as specified by the manufacturer.

$$m_3^0 = 100\rho_3^0 - \rho_1 v_1 \quad (33)$$

The volume contribution to the total volume of the pure commercial ethanol (95% (*V*/*V*)) by ethanol is

$$v_3^0 = \frac{m_3^0}{\rho_3} \quad (34)$$

The volume of ethanol, in diluted stock solution of ethanol is

$$v_3^{\mathrm{dil}} = \frac{p_{\%}}{100}\frac{m_3^0}{\rho_3} \tag{35}$$

Where v_3^{dil} and $p_{\%}$ are the volume of ethanol in its diluted stock solution and its concentration in % (V/V) respectively. Therefore, the volume (v_1^{dil}) of water in diluted solution of ethanol is

$$v_1^{\mathrm{dil}} = 100 - \frac{p_{\%}}{100}\frac{m_3^0}{\rho_3} \tag{36}$$

The masses of water and ethanol in the diluted stock solution of ethanol are $\frac{p_{\%}}{100}m_3^0$ and $\left(100 - \frac{p_{\%}}{100}\frac{m_3^0}{\rho_3}\right)\rho_1$ respectively. Thus, the density (ρ_{sol}) of the diluted stock solution is

$$\rho_{\mathrm{sol}} = \frac{\frac{p_{\%}}{100}m_3^0 + \left(100 - \frac{p_{\%}m_3^0}{100\rho_3}\right)\rho_1}{100} \tag{37a}$$

If solution density is known, the concentration in % (*V*/*V*) can be given as

$$p_{\%} = \frac{10^4(\rho_{\mathrm{sol}} - \rho_1)}{m_3^0 - m_3^0\rho_1/\rho_3} \tag{37b}$$

The value of $p_{\%}^{C_3 \to 0}$ at infinite dilution can be obtained by substituting $\rho_{\mathrm{sol}}^{C_3 \to 0}$ as C_3 tend to 0 from the plot of density of cosolvent solution versus weight fraction of cosolvent into Eq. (37b).

3. MATERIALS AND METHODS

3.1 Materials

The chemicals used were: Soluble potato starch from Sigma Chemicals Co, USA; ethanol, hydrochloric acid, and sodium chloride from BDH Chemical Ltd, Poole England; 3, 5-dinitrosalicyclic acid (DNA) from Lab Tech Chemicals India; Tris from Kiran Light Laboratories and BSA from Sigma USA; porcine pancreatic alpha amylase (PPA) (EC 3.2.1.1) from Sigma, Aldrich, US. All other chemicals were of analytical grade and solutions were made in distilled water.

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

The research from inception is mainly theoretical but with minor experiment in other to examine by quantification some parameters analysed in the theoretical section; the determination of $\Delta G_{2\mathrm{i}}$, or $N_{2\mathrm{i}}$, is according to equations 5 through 8 and equilibrium constant, K is according to Eq. (9). The calculation of chemical potential needed the determination of osmotic pressure and the partial molar volume of solution components which were carried out using theoretical method originally cited by Tardieu et al. [17] and Stothart [18] respectively. The determination of solution density at infinite dilution by extrapolation is according to the method by Millero et al. [19] and the determination of K is according to modified [20] Baskakov et al. method [14] (Eq. (9)).

Osmotic Pressure (Π) is:

$$\mathrm{Log}\Pi = 2.75 + 1.03W^{0.383} \tag{38}$$

Where W > 10% g/g.

$$\mathrm{Log}\Pi = 2.48 + 1.03W^{0.416} \tag{39}$$

Where W < 10% g/g. The osmotic pressure at infinite dilution of the stock solution of ethanol is obtained by plotting $\mathrm{In}\Pi$ versus C_3 (%(W/W)). The intercept from the plot gives the value of Π at $C_{3\to 0}$.

The velocity of hydrolysis of the polysaccharide at infinite dilution was extrapolated from the plot of $\mathrm{Log}v$ versus C_3 (Eq. (10)). The equation for the determination of solution density at infinite dilution is:

$$\rho_{\mathrm{sol}} = \rho_0 + AX_{\mathrm{i}} \tag{40}$$

Where ρ_0 is solvent density at infinite dilution; *A* and X_{i} are temperature dependent parameter and mass (weight) fraction of solution component respectively and ρ_{sol} is solution density. Densities of solution were plotted as a function of X_{i}. The apparent partial specific volume, is according citation by Stothart [18] given as

$$\phi = \frac{\left(1 - \frac{\left(\rho_{\mathrm{sol}} - \rho_{\mathrm{sol}}^{C_3\to 0}\right)}{C_3}\right)}{\rho_{\mathrm{sol}}^{C_3\to 0}} \tag{41}$$

Where C_3 and ϕ are the concentration of solute and its partial specific volume respectively. The density of the solution given concentration in % (V/V) is according to Eq. (37a); given molar concentration of the cosolvent, the density of the former is $\frac{1}{100}\left(\frac{C_3 M_3 m_3^0}{100\rho_3^0}+\left(100-\frac{C_3 M_3}{\rho_3}\right)\rho_1\right)$ where $\rho_1, \rho_3, m_3^0, C_3, \text{and } \rho_3^0$ are as defined earlier in the text and M_3 is the molar mass of the cosolvent. This takes into account different density of solvent and cosolvent at different thermodynamic temperature. It can serve a routine but a serious preliminary test for a theory or even hypothesis so as to establish probable pattern or trend pending full blown use of state – of – the – art facility for experimentation at higher cost. The product of molar mass of ethanol and result from Eq. (41) gives the apparent molar specific volume used for the determination of preferential interaction parameter. The Wyman equation for the determination of preferential interaction, osmolation, either positive or negative, to be specific is given as:

$$-RT\frac{\text{In}K}{\Delta\Pi\nabla_3}=\Delta\Gamma_{23} \quad (42)$$

Where $\Delta\Pi \text{ and } \nabla_3$ denote the difference in osmotic pressure between $C_3 \to 0$ and $C_3 > 0$ and apparent molar specific volume respectively. If K is < 1, $\Delta\Gamma_{23}$should be positive and if K>1, $\Delta\Gamma_{23}$ should be negative signifying preferential binding and preferential exclusion respectively. The value of Π as $C_3 \to 0$ is obtained as explained earlier. The calculated values of $\Delta\Gamma_{23}$ were plotted versus C_3 according to Eq. (8b). The mathematical expression of Wyman linkage relation in terms of cosolvent chemical activity implies that in Eq. (42),$-\Delta\Pi\nabla_3 = RT\text{In}a_3$.

The independent variables were various concentrations of osmolyte, ethanol, a human xenobiotic cosolvent, thermodynamic temperature (310.15 K), and *p*H (7.4). The control reaction mixtures were without xenobiotic osmolyte - ethanol. Assay of alpha-amylase for the determination of the effect of ethanol was according to Bernfeld (dinitrosalicylic acid) method [21]. A mixture of water and raw potato starch whose manufacturer labeled it as soluble (but indeed, it was seen to be far from being soluble), was the substrate. 0.01 g of PPA was dissolved in 20ml of distilled water to give 500 µg/mL while potato starch solution was prepared by mixing 1g in tris-HCl(aq) buffer (90 mL), 5 mL 6% (W/W) NaCl(aq) and 5 mL distilled water to give 1 g/100 mL. The enzyme, PPA, was mixed with different concentration of aqueous solution of ethanol and assayed for 5 min without any separate incubation of the enzyme in ethanol before assay. Spectrophotometric readings were taken at 540 nm with extinction coefficient equal to 181.1 $M^{-1}\text{cm}^{-1}$.

3.4 Statistical Analysis

The velocities of hydrolysis were determined in triplicates. The mean values were used to determine the equilibrium constant. Microsoft Excel (2007) was used to plot the dependent variable versus independent variable.

4. RESULTS AND DISCUSSION

There are two aspects of this research, theoretical and experimental aspects. The theoretical section examined claims in literature with the view to eliminate errors arising from misconception and ultimately produce data generated from the mathematical models connected to the structure of reaction mixture solution and thermodynamic properties. Notably, the views of Rösgen et al. [5], and Timasheff [1] were reexamined. The fact that a thermodynamic parameter, preferential interaction coefficient or parameter is strictly a dependent variable and as such should not be seen as a slope was emphatically established. It cannot be a measurable quantity and at the same time be a constant quantity implied in being a slope. It is either what has been seen to be the only measurable parameter by means of dialysis equilibrium and pressure osmometry is measured or calculated given the independent or other dependent variables that also dependent on an independent variable, the osmolyte concentration for instance.

The alphabets N_{12} and N_{32} (as originally cited by Schurr et al. [9]) with the corresponding defining subscripts are not exactly N_{21} and N_{23}. They are however, used without clear motivation. They denote the total number of water and osmolyte molecules, respectively, in a domain of sufficient size surrounding a single isolated macromolecule, and C_1 and C_3 denote the respective bulk concentrations in an exterior domain, no part of which is near any macromolecule. $\Gamma^{\text{m}}_{\mu_1,\mu_3}$ which in the usual notation is Γ_{23} can be regarded as the excess number of osmolyte molecules in the vicinity of the macromolecule above the quantity that would be expected from the number of water molecules in that region and the bulk concentration ratio, C_3/C_1 [9]: The interest in these definitions

lies in their clarity and simplicity serving as such as a good background for the presentation of small results and discussion.

The origin of the equations linking the excess number of water and osmolyte to the total number of water and osmolyte molecules is illustrated. These quantities are linked to the KBI and all the equations (Eqs. 3, 4, 5, 6b, 7, 8a, and 8b) are shown in theory section. Equations (7) and (8a) arise when there are changes arising from reaction which may be conformational change due to the presence of cosolvent and Eq.(8a) is always applicable to binding interaction expected to be positive. But, there are instances in which it may be negative if preferential exclusion is the case [1,5]: This intuitively led to the suggestion for alternative equation, for such situation, as implied in Eq. (8b) in this research. Most importantly, is the need for the appropriate use of Wyman linkage equation which from this research serves calculational purpose given other relevant dependent parameters such that Γ_{23} (or in the case of change, $\Delta\Gamma_{23}$) cannot be regarded as a slope and a devise–based measurable parameter. The Wyman linkage equation can be seen as an equation of thermodynamic parameters in which changes in the physicochemical property of the biomolecule, enzyme for instance, can be linked or related to the structural properties of the ternary reaction mixture components. The structural characteristic is described by the KB integral. The structural properties of the ternary solution can influence the thermodynamic property. This research shows that the KB integral for binding and exclusion can be determined either from the slope or intercept as the case may. This can be illustrated with Eq. (5), Eq. (6b), Eq. (7), Eq. (8a), and Eq. (8b). A plot of the measurable or calculable parameter versus either C_3 or $1/C_3$, as the case may be, provides the appropriate slope or intercept for this purpose. Under Table 1 are the following. The slope from the plot of $-\Delta\Gamma_{23}$ versus C_3 is $(\Delta N_{21}/C_1)$ = 1071≡ ΔG_{21} . The intercept from the same plot (not shown) gave ΔN_{23} $(\text{or } \Delta G_{23}.C_3)$ = – 4670; division of the latter by C_3 gives various values of KB integral for osmolation, ΔG_{23}. The *m* – value at 310.15 K is −1549.787 J L/mol.

A theoretical method has also been formulated for the determination of the density of diluted aqueous solution of cosolvent. The stock solution of the cosolvent from the producer's warehouse may be < 100% pure as in this research in which the stock solution of ethanol is 95% (V/V). The equations may be useful for preliminary investigation and opens opportunity for feature research that may be needed to confirm the equations.

Assay of the enzyme with and without ethanol yielded results, residual hydrolytic activity, which was recorded as percentage of the control without ethanol (Table 1). The hydrolytic activities of the ethanol–treated enzyme were lower than ethanol–free enzyme. What appeared to be a paradox, considering the known effect of ethanol, is the increasing trend in the residual amylolytic activity of the enzyme with increasing concentration of the former. Interpretation based on KB theory is inevitable. But before this, there is need to examine literature. Onyesom and Erude [22] have shown that alpha – amylase activity in saliva and plasma was significantly higher in habitual alcohol drinkers than in non-alcohol controls. It is possible that as a physiological response to the presence of ethanol in the gastrointestinal tract and plasma, the transcriptional and translational apparatus may have been activated to produce such enzyme even though ethanol is not a substrate. This view may be speculative but alcohol is poor in calorie such that its consumption in place of higher calorie food may trigger a sense of starvation leading to the mobilization of carbohydrate reserve. This is to say that synthesis of the enzyme is different from the direct effect of the alcohol on the enzyme in a test tube. Nonetheless report by the authors [22] does not agree with the residual amylolytic activity of a direct ethanol – treated enzyme reported for PPA as in supervised thesis [15] (Table 1).

The reduction in the enzyme activity may be due to lower water activity around the enzyme. This shows that decrease in water activity may reduce the activity of the enzyme. This scenario has been reported for the enzyme lysozyme which in the presence of higher water content has a higher affinity for water than for acetonitrile and a concomitant residual enzyme activity values are close to 100% [23]. The presence of higher water content ought to promote higher activity of PPA but on the contrary there were residual activities which were increasing with increasing values of C_3. Although ethanol is not acetonitrile, both are organic solvents, cosolvents to be technically precise. The decrease in the amylolytic activity suggests that ethanol may have caused conformational instability of the enzyme. The

Table 1. Reaction mixture solution structure, thermodynamic parameters, and residual activity as percentage of control

$\Delta_N^D(G_{21}-G_{23})$(From Eq. of *m* - value)	$\Delta_N^D\Gamma_{23}$(From Eq. of *m* - value)	Calculated $\Delta\Gamma_{23}$(From Wyman relation)	Residual activity as % of control
0.7494	0.934	– 5002.968	36.118
~ 0.6009	1.441	– 558.574	36.779
~ 0.6008	1.939	– 103.677	50.641
0.6007	2.590	– 31.608	55.545
0.6004	3.169	– 11.906	57.620

$\Delta_N^D(G_{21}-G_{23})$, is the change of solvation preference upon the ethanol partial denaturation of the enzyme; $\Delta_N^D\Gamma_{23}$ is the corresponding preferential interaction parameter. The slope$(\Delta N_{21}/C_1)$ from the plot of $-\Delta\Gamma_{23}$ versus C_3 is $= 1071 = \Delta G_{21}$; Intercept ($\Delta\Gamma_{23}^{C_3\to 0}$) from the same plot is = – 4670. The latter is value as $C_3\to 0$; division by different values of C_3 including value at infinite dilution gives different values of ΔG_{23} showing the fact that ΔG_{23} is never a constant. The m – value at 310.15 K is –1549.787 J L/mol

residual activity shows that preferential binding for which ethanol is known was not total. This presupposes incidents of preferential exclusion ($-\Delta\Gamma_{23}$) as results in Table 1 show. The magnitude of $-\Delta\Gamma_{23}$ calculated on the basis of Wyman relation decreased with increase in C_3. This simply means that with increasing C_3, there is a decrease in preferential exclusion. The same table also shows that the change of solvation preference, $\Delta_N^D(G_{21}-G_{23})$, upon the ethanol partial denaturation of the enzyme and the *m* – value are respectively positive and negative in sign. Before proceeding further, it is necessary to restate that *m* – value is the capacity of an osmolyte to either force a protein to unfold or to refold. In doing so, the preferential interaction parameter changes due to unfolding or refolding as the case may be. In this research as shown in Table 1, there was partial unfolding resulting in residual activity. In this case the purpose of *m* – value equation differs from the purpose of Wyman linkage relation. The results of calculation using the equations are different. Meanwhile the negative *m* – value presupposes a destabilising effect if the position of Rösgen et al. [5] is taken into account. This is contrary to the implication of positive *m* – value**.** It means that a known destabilizer can also be excluded as if it is a stabilizing osmolyte. This may no longer be strange since it has also been shown that at the lowest water content, the organic solvent, acetonitrile molecules, are preferentially excluded from the dried lysozyme, resulting in the preferential hydration [23]. This seems to imply that ethanol may be more preferentially excluded from the enzyme at much lower water content (or higher C_3) with concomitant hydration. But this cannot be conclusive because decreasing magnitude of preferential exclusion parameter (Table 1) is a tendency to less hydration with increasing C_3 . However, it is similar to the report for alpha chymotrypsin which retained significant (50%) residual activity in water – poor ethanol leading to the conclusion that protein hydration level is one of the critical factors that govern the stability of protein – water – monohydric alcohol system [2].

Another issue that may be in support of the observed effect of ethanol is the notion of negative effect of excessive rigidity [24]; perhaps the negative $\Delta\Gamma_{23}$, which implies that there was folding may have promoted rigidification, reducing conformational flexibility needed for catalytic function. But ethanol being a known denaturant has the capacity to penetrate the protein interior 3 – D structure. Taking advantage of its size and hydrophobic alkyl group it interacts with the interior hydrophobic core leading to fluidization and concomitant partial unfolding. Perhaps, the observed increase in residual activity of PAA with increasing concentration of ethanol and with the increasing hydrophobic environment, may be due to the promotion of lower local relative permittivity [25] leading to partial enhancement in activity.

Going by the view of Timasheff [1], it seems there is always a mutual perturbation of the chemical potential of both osmolyte and protein whenever a solution of the latter is introduced to the former giving rise to the equation, $\left(\frac{\partial \mu_3}{\partial m_2}\right)_{T,P,m_3} = \left(\frac{\partial \mu_2}{\partial m_3}\right)_{T,P,m_2}$. A positive preferential interaction is the case if there is binding of cosolvent with the protein leading to increase in the chemical potential of the cosolvent around the surface domain of the protein. Interaction between the cosolvent, ethanol, and the protein is unfavourable, if a negative preferential interaction otherwise called preferential exclusion which promotes preferential hydration is the

case. These have effect on the function of the enzyme which as a result of conformational change may lead to upward or downward trend in its catalytic function. However, it was an increasing trend in residual activity (Table 1). There is need to add too, that "the effect of a neutral osmolyte, like ethanol, on the water activity of aqueous compartments in equilibrium with a protein depends on the degree to which, it is excluded from the protein associated water" [26]. Alcohol and water exist preferentially in the solvation layer of the protein. When a protein is placed into a water-alcohol mixture, its properties are altered as a function of the solvent composition. The preferential solvation/hydration process accounts for the augmentation or depletion of the alcohol/water molecules at the protein surface [2]. The preferential binding depends markedly on the chemical nature of the protein surface. According to Sirotkin and Kuchierskaya and references made by the authors [2] protein unfolding may be induced by the preferential binding to specific regions on the protein (peptide groups in the case of urea and guanidinium hydrochloride or hydrophobic regions in the case of alcohols). While admitting that the $(\partial g_1/\partial g_2)_{T,\mu_1\mu_2}$values are positive at low water content it is also supportive of the earlier view regarding the effect of preferential exclusion; this is to admit that due to the reduced conformational flexibility in organic solvents with low water content, the enzymes remain in the active conformation [23] even if residual activity was observed.

5. CONCLUSION

In other to achieve better insight to the basic theory of Kirkwood and Burk as may easily be applicable to processes in biochemistry, the equation of preferential interaction parameters were derived. This does not distract from the linkage between thermodynamic parameters, equilibrium constant and activity of cosolvents as implied in Wyman linkage relation. The appropriate way is either by calculation or measurement of preferential parameter. Therefore, Γ_{2i} or $\Delta\Gamma_{2i}$ for the change, cannot be a constant (or slope) and an instrumentation–based measurable parameter at the same time. Based on Wyman linkage relation, purely biochemical thermodynamic parameter is linked to preferential interaction parameters which are therefore, thermodynamic parameters. Since this research is mainly theoretical, it is hereby recommended for feature research that, with state–of–the–art equipment, a detailed experiment needs to be carried out so as to re–evaluate the equations formulated in this research.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

REFERENCES

1. Timasheff SN. Protein solvent preferential interaction, protein hydration, and the modulation of biochemical reactions by solvent components. Biochemistry. 2002; 99(15):9721-9726.
2. Sirotkin VA, Kuchierskaya AA. Alpha-chymotrypsin in water-ethanol mixtures: Effect of preferential interactions Chem. Phys. Lett. 2017;689:156–161.
3. Zheng X, Li Z, Podariu MI, Hage DS. Determination of rate constants and equilibrium constants for solution-phase drug−protein interactions by ultrafast affinity extraction Anal. Chem. 2004;86: 6454–6460.
4. Shimizu S. Estimating hydration changes upon bimolecular reactions from osmotic stress, high pressure, and preferential hydration experiments. Proc. Natl. Acad. Sci. U.S.A. 2004;101:1155–1199.
5. Rösgen J, Pettit MB, Bolen DW. Structure of solutions of aqueous biochemical compounds-pair correlations Biophys. J. 2005;89:2988–2997.
6. Harries D, Rösgen J. Use of macroscopic properties of solution to derive microscope structural information. Methods Cell Biol. 2008;84:680–730.
7. Kirkwood JG, Buff FP. The statistical mechanical theory of solutions. J. Chem. Phys. 1951;19:774–777.
8. Bolen DW, Baskakov IV. The osmophobic effect: Natural selection of a thermo-dynamic force in protein folding. J. Mol. Biol. 2001;310(5):955–963.
9. Schurr JM, Rangel, DP, Aragon SR. A contribution to the theory of preferential interaction coefficients Proc. Natl. Acad. Sci. USA. 2005;89:2258–2276.

10. Timasheff SN. In disperse solution "osmotic stress" is a restricted case of preferential interactions. Biochemistry. 1998;95(13):7363–7367.
11. Harano Y, Kinoshita M. Translational-entropy gain of the solvent upon protein folding. Biophys. J. 2005;89:2701-2710.
12. Kramer RM, Shende VR, Motl N, Pace N, Scholtz JM. Toward a molecular understanding of protein solubility: Increased negative surface charge correlates with increased solubility. Biophys. J. 2012;102: 1907–1915.
13. Pace CN. Measuring and increasing protein stability. Trends Biotechnol. 1990; 8:93–98.
14. Baskakov I, Wang A, Bolen DW. Trimethlamine – N – oxide counteracts urea effects on rabbit muscle lactate dehydrogenase function: A test of the counteraction hypothesis. Biophys. J. 1998;74:2666–2673.
15. Udema II. *In vitro* investigation into the effects of ethanol, aspirin, and stabilizers on mesophilic alpha amylase. Ambrose Alli University, Ekpoma; 2013
16. Sibiya PN, Deenadayalu N. Excess molar volumes and partial molar volumes of binary systems (ionic liquid + methanol or ethanol or 1-propanol) at *t* = (298.15,303.15 and 313.15) k. S. Afr. J. Chem. 2009;62:20–25.
17. Tardieu A, Veretout F, Krop B, Slingsby C. Protein interactions in the calf eye lens: Interactions between β - crystallins are repulsive whereas in γ – crystallins they are attractive Eur. Biophys. J. 1992;21:1-12.
18. Stothart PH. Determination of partial specific volume and absolute concentration by densimetry. Biochem. J. 1984;219: 1049–1052.
19. Millero FJ, Ward GK, Chetirkin P. Partial specific volume, expansibility, compressibility, and heat capacity of aqueous lysozyme solutions J. Biol. Chem. 1976;251(13):4001–4004.
20. Udema II. The effect of additives and temperature on the velocity of hydrolysis of raw starch with human salivary alpha – amylase. Int. J. Biochem. Res. Rev. 2016; 10(2):1–17.
21. Bernfeld P. Amylases, alpha and beta. Methods. Enzymol. 1955;1:149–152.
22. Onyesom I, Erude HO. Alpha - Amylase activity in the saliva and plasma of habitual alcohol drinkers Biokemistri. 2004;16(1): 11–14.
23. Sirotkin VA, Kuchierskaya AA. Lysozyme in water – acetonitrile mixtures: Preferential solvation at the inner edge of excess hydration. J. Chem. Phys. 2017; 146:215101–215108.
24. Ovando SL, Waliszewski KN, Pardio VT. The effect of hydration time and ethanol concentration on the rate of hydrolysis of extracted vanilla beans by commercial cellulose preparations (A): Int. J Food Sci. amp Technol. 2005;40(9):1011–1018.
25. Csermely P. Water and cellular folding process. Cellular Mol. Biol. 2001;47(5):1-9.
26. Mitchell DC, Lawrence JTR, Litman BJ. Primary alcohols modulate the activation of the G. protein-coupled receptor rhodopson by a lipid-mediated mechanism. J. Biol. Chem. 1996;271(32):19033-19036.

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