Original Research Article

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

ABSTRACT

Background: Researchers interested in the effect 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 bases 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 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 (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 of solvation preference upon the ethanol partial denaturation of the enzyme and the corresponding change in preferential interaction parameter were 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 parameter/coefficient. 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 of 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 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 follow 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 neurodegenerative 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 also of concern and 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 crystallography [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 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 self – correlation defined by the term 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_{13} 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 seem to be 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 preferential interaction parameter (Γ_{2i}) for its calculation and calculation of parameters linked to KBT derived equations.

2 Theory

Similar to the presentation in literature [5] **t**he interpretation of the basics tenet of Kirkwood-Buff (KB) solution theory or theory of solution structure is simply an expression of the thermodynamic properties of an isotropic solution (isotropy is a physical property that is uniformly distributed in all direction; consequently, it is one that is independent of direction) of aqueous biochemical compounds, in terms of 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 point is 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 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) structure. Steric factor may not be the only reason why osmolyte are excluded. It 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 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 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].

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

Where C_i and G_{2i} are concentrations of the solution component and KBI respectively; N_A is the Avogadro's number. The far 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 imply ΔN_{2i} for the 2nd definition. What may be of interest to the biochemist, is the relation [5].

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

Where G_{21} and G_{23} are respectively the KBI for hydration and osmolation of any biomolecule and Γ_{2i} is the preferential interaction parameter and $(-)$ 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_{12} 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]. Definition in line with KB theory is 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}.

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.

$$G_{21} = N_{21}/C_1 \tag{3}$$

$$G_{23} = N_{23}/C_3 \tag{4}$$

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

$$\Gamma_{21} = N_{21} - \frac{C_1}{C_3}N_{23} \tag{5}$$

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

$$-\Gamma_{23} = \frac{C_3}{C_1}N_{21} - N_{23} \tag{6a}$$

$$\Gamma_{23} = N_{23} - \frac{C_3}{C_1}N_{21} \tag{6b}$$

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

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

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

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 $G_{2\mathrm{i}}$ (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 $G_{2\mathrm{i}}$. This is contingent upon a linear regression analysis that remains a mere probability. The Kirkwood – Burk theory enables the identification of the relationship between $\mathrm{r}_{2\mathrm{i}}$ 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, $\mathrm{r}_{2\mathrm{i}}$ should be the ultimate dependent variable. The suitable equation should be $\left(\frac{\mathrm{ln}K}{\mathrm{ln}a_{\mathrm{i}}}\right)_{P,T,C_2} = \Gamma_{2\mathrm{i}}$. To further drive the point home 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 $\Gamma_{2\mathrm{i}}$ $\left(\Delta\Gamma_{2\mathrm{i}} = \frac{\Delta \mathrm{In}K}{\Delta \mathrm{In}a_{\mathrm{i}}}\right)$. A slope is not final value minus initial value of a variable as it seems to imply given $\Delta\Gamma_{2\mathrm{i}}$. 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, being 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]:$\mathrm{P}.n\mathrm{H_2O} + \mathrm{L} \rightleftharpoons \mathrm{P}.\mathrm{L} + n\mathrm{H_2O}$ where the alphabets, P and L are the protein and ligand that binds. 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 where 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 following 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 depleting the vicinity of the protein of such osmolyte leads to higher concentration of it in the bulk. The vicinity of the unfolded protein becomes dilute with respect to osmolyte concentration. 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.

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

The interactions, whether attractive or repulsive between the protein and the solution components [10] is determined by the strength of the interaction with the protein, which may vary from strong immobilization to weak momentary perturbations, to 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 such that interactions based on polar – polar attraction which are much applicable to bulk water, let alone, hydrogen bond with water of protein 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 which is very stable and 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 due to hydrogen bond. It is well known fact that the solubility of alkanols decreases with increasing size of the alkyl group. A recent result 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].

If the solution of the protein is transferred into a solution of ethanol changes in physicochemical properties of the protein and ethanol may occur. 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 [Pace], $U = (A_{\mathrm{N}} - A_{\mathrm{OBS}})/(A_{\mathrm{N}} - A_{\mathrm{D}})$ 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.

$$K = U/(1 - U) \tag{9}$$

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 not as common as biophysical method. The relevant equation from first principle is $K = (SA_{\mathrm{MAX}} - SA_{\mathrm{OBS}})/(SA - SA_{\mathrm{MIN}})$. As shown elsewhere [15], $U = (SA - SA_{\mathrm{OBS}})/(SA - SA_{\mathrm{MIN}})$ if catalytic activity of the enzyme (SA) without additive or cosolvent is < the catalytic activity (SA_{OBS}) of the observed treated enzyme, and SA_{MIN} is the catalytic activity (which may → 0) of the denatured enzyme.

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}.

$$\log v = \log v_{C_{\mathrm{i}}\to 0} + \beta[C_{\mathrm{i}}] \tag{10}$$

Where there is decreasing trend with increasing C_{i} the equation below may be the case.

$$\log v = \log v_{C_{\mathrm{i}}\to\infty} + \beta/[C_{\mathrm{i}}] \tag{11}$$

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]. Citing other authors Sirotkin and Kuchieskaya [2] posit that, 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). 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:

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

Where, *T* and *P* refer to the thermodynamic temperature and standard pressure respectively. Meanwhile,

$$V_1 = G_{13} - G_{33} \tag{13}$$

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,

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

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,

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

The view is that V_1 is a constant and dependent on C_3 (which may remain a theoretical speculation) and the factor $1 - V_1C_3$ 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 [5, 17 – 18] on C_3 through the relation:

$$\frac{1}{RT}\left(\frac{\partial\mu_2}{\partial C_3}\right)_{T,P} = \frac{G_{21} - G_{23}}{1 - C_3V_1} \tag{15}$$

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:

$$\Delta\mu_2 = \frac{RT(G_{21} - G_{23})}{V_1}\mathrm{In}(1 - \mathrm{C}_3V_1) \tag{16}$$

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:

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

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

$$-\Gamma_{23} = \frac{\Delta\mu_2 V_1 C_3}{RT\mathrm{In}(1-C_3V_1)} \tag{18}$$

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 and 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_1N_{23} and $N_{21}^{C_3\to\infty}$ respectively. Therefore,

$$\frac{\partial\Gamma_{21}}{\partial C_3^{-1}} = C_1N_{23} \tag{19}$$

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

$$\frac{N_{23}}{C_3} = \frac{\partial\Gamma_{21}}{C_3C_1\partial C_3^{-1}} = G_{23} \tag{20}$$

Since C_3 is the only variable in Eq. (20), G_{23} cannot be a constant with any C_3. From the intercept are 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

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

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

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

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

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:

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

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].

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

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*. $\Delta_N^D(G_{21} - G_{23})\to$zero"[5]. As applied earlier in the text, Eq. (27) can be re-stated as:

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

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

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

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,

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

Simplification and rearrangement gives first:

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

Further rearrangement gives

$$\frac{m}{RT} = \frac{\Delta_N^D \Gamma_3}{C_3 \exp\left(\mathrm{In}C_3 - \frac{\mu_3^0 - \mu_3}{RT}\right)} \quad (31b)$$

2.2 A method for the theoretical determination of the density of the 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 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 the mass and density of cosolvent in the pure stock solution as produced by the manufacturer and pure water respectively; v_1 and ρ_3^0 are 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 in diluted stock solution of ethanol is

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

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

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

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

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

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

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

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

3.0 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.

3.2 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 ΔG_{2i}, or N_{2i}, 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 substituting the concentration in % (*V*/*V*) (converted to % (*W*/*W*) and <10% g/g) obtained from Eq. (37b) into Eq. (39).

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 d_{o} 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

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

Where C_3 and ϕ and are the concentration of solute and 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 ρ_1, ρ_3, m_3^0, C_3, and ρ_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 temperature which takes into account different density of solvent and cosolvent at different thermodynamic temperature, and, again, 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 independent variables were various concentrations of osmolyte, ethanol, a human xenobiotic cosolvent, and thermodynamic temperature and *p*H of assay were 310.15 K and 7.4 respectively. 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 $M^{-1}\mathrm{cm}^{-1}$.

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.

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.

Although the alphabets N_{12} and N_{32} (as originally cited by Schurr *et al* [9]) with the corresponding defining subscripts are used without clear motivation as they are not exactly N_{21} and N_{23}; they however,

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. $\Gamma^{m}_{\mu_1,\mu_3}$ 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) arises 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. 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 aqueous solution of cosolvent produced from the stock solution of the cosolvent which 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 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).

Table 1 Reaction mixture solution structure thermodynamic parameters.

$\Delta_{\mathrm{N}}^{\mathrm{D}}(G_{21}-G_{23})$	$\Delta_{\mathrm{N}}^{\mathrm{D}}\Gamma_{23}$	Residual activity as % of control
–0.123	–0.165	36.118
–0.525	–1.261	36.779
–0.704	–2.273	50.641
–0.878	–3.786	55.545
–0.999	–5.275	57.620

$\Delta_{\mathrm{N}}^{\mathrm{D}}(G_{21}-G_{23})$, is the change of solvation preference upon the ethanol partial denaturation of the enzyme; $\Delta_{\mathrm{N}}^{\mathrm{D}}\Gamma_{23}$ 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 $(\Delta\Gamma_{23}^{C_3\rightarrow 0})$ from the same plot is = – 4670. The latter is value as $C_3\rightarrow 0$; 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.

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 the activity was 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 residual activity shows that preferential binding for which ethanol is known was not total. This presupposes incidents of preferential exclusion ($-\Delta_{\mathrm{N}}^{\mathrm{D}}\Gamma_{23}$) as results in Table (1) shows. The same table also shows that the change of solvation preference upon the ethanol partial denaturation of the enzyme and the *m* – value are negative in sign. This presupposes a destabilising effect where the *m* – value is negative if the position of Rösgen *et. al*. [5] to the contrary with respect to the implication of positive *m* – value is taken into account**.** 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. This is similar to the report for alpha chymotrypsin which retained significant (50 %) residual activity in water – poor ethanol leading to the submission 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 interact with the interior hydrophobic core leading to fluidization and

concomitant partial unfolding. Perhaps, the observed increase in 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 definition of Timasheff [1], it seems the perturbation of the chemical potential, $\left(\frac{\partial \mu_L}{\partial m_P}\right)_{m_L}$, is positive given that the interaction between the cosolvent, ethanol, and the protein is unfavourable, a negative preferential interaction otherwise called preferential exclusion which promotes preferential hydration. 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.

CONCLUSION

In other to achieve better insight to the tenets 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/coefficient. 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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