SOME STUDIES ON AQUEOUS CONSOLUTE MIXTURES AND ON MICELLES

A THESIS
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DOCTOR OF PHILOSOPHY

BY

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dedicated to

MY MOTHER

AND

MY FATHER

CONTENTS

STATEMENT

I hereby declare that the matter embodied in this thesis is the result of investigations carried out by me as a research student of the School of Chemistry, University of Hyderabad, Hyderabad under the supervision of Professor D. Balasubramanian.

In keeping with the general practice of reporting scientific observations, due acknowledgement has been made wherever the work described is based on the findings of other investigators.

(PARTHASARATHI MITRA)

CERTIFICATE

Certified that the work contained in this thesis, entitled 'SOME STUDIES ON AQUEOUS CONSOLUTE MIXTURES AND ON MICELLES', has been carried out by Parthasarathi Mitra under my supervision and the same has not been submitted elsewhere for a Degree.

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SYNOPSIS

Living things have originated in an aqueous milieu and cannot possibly exist in the absence of water. In spite of the unique role and importance of water, a wide latitude of disagreement exists over the interpretation of several fundamental phenomena occurring in aqueous media. While ionic solutions have been studied extensively and are fairly well understood, the hydrophobic effect -- that is, the unique lack of solvent power of water for many nonpolar substances -- has received less attention and its basis in terms of the theory of solutions and its manifestations in various situations are incompletely understood. Furthermore, aqueous solutions of amphiphilic molecules are even more complex, since they are forced by their inherent duality to adopt unique orientations with respect to the aqueous phase and to form organised structures to optimise their contact with the solvent. Similarly, miscibility and phase separation of aqueous binary mixtures do not follow the simple rules that apply to non-aqueous systems, but are governed by entropic factors associated with water structure.

In this thesis we have tried to understand two different facets of aggregation of 'solute' molecules in aqueous media. One is the special role that the bulk phase water plays in the phenomenon of liquid-liquid phase separation in aqueous binary systems and the features of the hydrophobic effect in these cases that distinguish them from organic consolute pairs. The effect of additives, in particular those that are known to effect water structure, on the critical solution temperatures have also been compared in the two cases. The other is a study of the hydrophobically-driven association of amphiphilic solutes in water to form well-defined aggregates. In particular, we have looked at the extent to which the bulk phase water can 'penetrate' inside such aggregates. This study appears to be appropriate in light of the controversy that has existed in literature on this issue -- and since the problem is of both fundamental interest to the aggregation phenomenon, and specific to the biomolecular amphiphile aggregates that form in aqueous medium. As a prelude, and to set the perspective for these studies, the first chapter reviews the current theories of water structure, hydrophobic effect, liquid-liquid phase separation (coacervation) and amphiphile aggregation.

The second chapter of the thesis, describes a study of the phase separation behaviour of aqueous and non-aqueous binary liquid mixtures and an analysis of the thermal phase separation of some biopolymers in water. Binary liquid mixtures that display partial miscibility exhibit critical solution temperatures (CST) or consolute temperatures. CSTs are of two kinds: upper critical solution temperatures (UCST), above which the liquid pair is completely miscible and below which phase separation occurs, and lower critical solution temperatures (LCST) where the inverse occurs. Two important generalizations applicable to liquid pairs that exhibit CST are: (i) the Prigogine rule which states that a CST will occur at equimolar component compositions; and (ii) the Timmermans rule which describes the effect of an additive on the CST: an additive that is largely soluble in one of the components will decrease the mutual solubility and hence increase UCST and decrease LCST, while an additive that is about equally soluble in both components will behave conversely.

The results highlight the contrasts between organic conjugate liquid pairs and aqueous binaries. Contrary to the Prigogine rule, the CSTs of aqueous binaries occur at high water mole fractions, and contrary to the Timmermans

rule, additives such as a globular protein denaturants and the Hofmeister series of ions solubilize the second liquid in water. Such violations of the rules do not seem to occur in organic liquid pairs. These deviations in water-containing binaries are explained in terms of the hydrophobic effect and the structure of water.

The thermal phase separation, or cloud point phenomenon, that occurs in aqueous solutions of compounds such as Triton X-100, poly(vinylmethyl-oxazolidinone) or poly(L-proline) are shown to be akin to aqueous binary mixtures which display LCST behaviour. The addition of a water-soluble third component which breaks water structure increases the cloud point, and conversely, an additive which is a water structure maker decreases the cloud point. Similarly, the effects of substituting D_2O for water on the LCST of aqueous binary mixtures are paralleled in the case of polymer:water mixtures. Both the preceding cases show a decrease in LCST or cloud point temperatures as would be expected from solvent structure effects. Thus, we believe the cloud point observations made on these polymers belong to the general class of lower consolute behaviour of polymeric solutes and might also be applicable to several other systems including that of the ψ-state of DNA.

In chapter three, we turn to hydrophobic aggregates such as surfactant micelles in aqueous medium. While the classical model, and recent theoretical analyses imply very little water to be present in the interior of micelles and similar aggregates, some recent results using extrinsic probes have suggested the possibility of substantial water penetration in micelles. In light of our interest in the hydrophobic effect, we thought it worthwhile to investigate the role of extrinsic probes used in these studies. Accordingly, the solubilization sites of some extrinsic probes in micelles and the enhancement of surface activity of such solubilizates in these systems have been determined. Several extrinsic optical probes that have been used to monitor the extent of water penetration in micelles are aromatic in nature. The ring current-induced alterations in the NMR spectral signals of the various protons of the surfactant molecules observed on solubilizing some of these probes in micelles are described. The results indicate that such aromatic solubilizates are located near the head group region of the micelles. Hence, these probes would be monitoring the polarity and water content of the headgroup region and not the micellar interior. Similarly, keto compounds that have been used as probes of micellar structure are shown to be inherently surface active. This surface activity is greatly enhanced when these probes are solubilized in systems of micellar dimensions. Also, NMR spectral results indicate that these ketone probes are positioned in the headgroup region of the micelles. Thus, studies using probes which are inherently surface active, and hence interfacially located, do not necessarily prove the existence of water inside micelles and related assemblies. These results have a significant impact on the current debate on the issue of micelle structure and the extent of water penetration into the body of these aggregates. This is relevant in the larger perspective that encompasses the structure and properties of other supramolecular assemblies of amphiphiles and lipids.

CHAPTER I

A DISCUSSION OF WATER STRUCTURE AND AQUEOUS SOLUTIONS

1.1 ABSTRACT

We have reviewed briefly the present status of water structure theories and their ability to explain the various aspects of aqueous solutions. And, the salient features of liquid-liquid phase separation (coacervation) and amphiphile aggregation have been discussed in relation to the hydrophobic effect.

1.2 INTRODUCTION

Living things appear to have originated in an aqueous milieu and the present life forms cannot possibly exist in the absence of water. This unique role and importance of water and the fascinating array of unusual properties that it exhibits, both in its pure form and as a solvent, has inspired physical scientists to find a comprehensive molecular theory for it. Although such attempts have been going on for nearly a century, it is only in the last decade that a satisfactory molecular theory has begun to emerge.

Any successful theory of liquid water structure should be able to explain the notable physical properties of water such as its (i) negative volume of melting, (ii) density maximum in the normal liquid range (at 4°C), (iii) isothermal compressibility minimum in the normal liquid range (at 46°C), (iv) high dielectric constant (v) anomalously high melting, boiling, and critical temperatures for a low-molecular-weight substance that is neither ionic nor metallic, (vi) high-mobility transport for H^+ and OH^- ions and (vii) behaviour towards nonpolar solutes. Ultimately, all the properties of water and aqueous solutions must be explained in terms of the intermolecular forces that are present; that is, using the concept of the hydrogen bond.

In this chapter we briefly review those models of water structure and aqueous solutions that are relevant to the topics covered in Chapters II and III. The comprehensive treatise on 'water' edited by Franks[1] has in-depth reviews of most of the topics discussed here. A recent review by Stillinger[2] discusses the present status of water structure studies -- especially the progress in theoretical modelling. The diversity of the substances that dissolve in water indicates that water structure is sufficiently complex to be able to adopt itself in characteristic manner to different types of solutes -- nonpolar, polar or ionic. In section 1.4 of this chapter we discuss the interesting effects observed when high molecular weight polymers and amphiphilic molecules are dissolved in water. An attempt has been made here to show how the solvent properties of water depend on the interactions between the dissolved substances and H_2O molecules, and effects due to water structure.

1.3 WATER STRUCTURE AND INTERACTIONS

1.3.1 Models of pure liquid water:

The melting of ice to produce liquid water obviously entails basic changes in the way that the molecules are arranged relative to one another. There is a decrease in rigidity and a consequent increase in fluidity. The crystalline periodicity gives way to spatial homogeneity and a much larger diffusion rate and orientation change is possible for the water molecules. The density too rises by about 9%. For any molecular theory of liquid water to be acceptable it must be able to account for the topology and geometry of the hydrogen bond network after it has been altered by the melting process.

The earliest theories of liquid water (reviewed by Chadwell[3]) recognised that water is an associated liquid, but attempted to explain its anomalous properties by postulating the existence of polymers containing very few molecules. For obvious reasons, these theories were not able to account for many of the observed properties — and have been superceded, in recent years, by models which emphasise extensive hydrogen bonding and the formation of spatial networks. This change in point of view was due to the seminal paper of Bernal and Fowler[4]. They proposed a model of the liquid, based on a broken-down ice structure, with most of the hydrogen bonding still in existence. However, they replaced the molecular arrangement found in ice by a more dense, though still tetrahedrally bonded, structure somewhat similar to that of quartz and allowed the hydrogen bonding to break down gradually with increasing temperature, consequently causing the liquid to approach a close-packed structure.

The model of Bernal and Fowler belongs to the class of 'continuum or uniformistic models' of liquid water structure. These types of models treat water as a uniform dielectric medium and when arranged over a large number of molecules, the environment about a particular molecule is considered to be the same as any other molecule so that the behaviour of all the molecules can be considered to be equivalent. Thus, the basic element in the continuum models is that each water molecule is subject to the same kind of intermolecular forces as any other molecule[5,6].

Pople[6] conceived of the possibility of the formation of bent hydrogen bonds, in liquid water, instead of broken ones when it is formed from ice. Bending of hydrogen bonds is regarded as a continuously variable rotation of the hydrogen atom or the lone pair electrons, or both, out of the oxygen-oxygen line of centres. This results in the destruction of the regularly repeating ice-lattice arrangement, leading to the establishment of an irregular arrangement of water molecules beyond a few molecular diameters. The radial distribution and dielectric constants for different temperatures, calculated on the basis of this model, are in good agreement with experimental values[5-9]. But this model is not sufficient to give proper values for the entropy of the liquid as there is very little freedom of rotation allowed in such a structure[10,11]. Furthermore, this model cannot be directly extended to a description of aqueous solutions.

The mixture models of liquid water, especially the 'flickering-cluster' model of Frank and Wen[12], are more useful for statistical mechanical studies[10,11] and have been successfully extended to descriptions of aqueous solutions. Frank and Wen postulate that the formation of hydrogen bonds in the liquid is a cooperative phenomenon; that is, the bonds are not made and broken singly but several at a time. Thus, short-lived 'clusters' of highly hydrogen bonded regions surrounded by non-hydrogen bonded molecules are produced. The formation and dissolution of these 'flickering clusters' is governed by local energy fluctuations.

Nemethy and Scheraga[10,11] have shown that the 'flickering cluster' model can account not only qualitatively but also quantitatively for various experimentally observed quantities such as density, relaxation times in various processes, the structural changes in solution of nonpolar substances, and the subsequent changes in the thermodynamic parameters. They are also of the opinion that Pauling's[13] clathrate model as extended by Frank and Quist[14] is not able to give as good an explanation for the thermodynamics of solution of non-polar substances as the 'flickering cluster' model seems to do. In particular, the 'flickering cluster' model is the best among the earlier proposed models which is able to explain in a quantitative way the thermodynamic properties determining solubility relationships of non-polar substances in water.

During the last few years, it has become apparent that the structural and dynamical correlations in pure water may be profitably studied by postulating rather simple pairwise-additive potentials between rigid water molecules and using Monte Carlo or molecular dynamics techniques on systems of a few hundred molecules[2,15]. The results of these studies indicate that water structure can be thought of as a network of hydrogen bonds. However, as against the ice crystal, these hydrogen bonds are not perfectly formed and the resulting network has to be analyzed and understood in terms of the connectivity and clustering that the imperfectly formed hydrogen bonds engender in the assembly of water molecules.

It is conventional to use pairwise-additive potentials in developing a formal theory of the liquid state and it is justified because of the remarkable simplicity of expressions for thermodynamic energy and pressure that result from this assumption. But, as hydrogen bonding is the important interaction in liquid water and since these interactions are cooperative (that is, nonadditive) it is insufficient for the purpose of computer simulations to use the dimer potential function alone. A procedure has been devised by Stillinger[16] for the selection of a 'best' approximate pair potential which incorporates the average effects of nonadditive potentials. This 'effective pair potential' satisfies a variational principle selected by the requirement that the sum of effective pair interactions preserves local order as far as possible.

In liquid water it is necessary to give a 'definition' of a hydrogen bond even though there is hardly any uncertainty about what constitutes a pair of hydrogen-bonded molecules in ice, since the more diverse set of local arrangements in the liquid can lead to ambiguity on this point. In most computer simulations the potential energy of the interaction between two molecules is used as a criterion to decide whether the pair is hydrogen-bonded or not. If the value of the potential energy V_{HB} is set as an upper bound for the hydrogen bond, then two molecules i, j having $V_{ij} < V_{HB}$ are considered to be bonded, otherwise not. On such an 'energetic' definition of a bond, liquid water appears as a uniform space-filling random network[15].

Although the computer simulation studies of water and aqueous solutions have some characteristic weak points (such as small system size, short time spans, difficulty of including quantum corrections), it is remarkable to see many of the key attributes of water emerge from such studies; e.g. liquid-phase density maximum phenomenon, isothermal compressibility minimum and high heat capacity. Furthermore, the rates of translational and rotational diffusion observed in molecular dynamics simulations stand in moderately good agreement with experiment. Also, the X-ray and neutron diffraction patterns obtained from simulation studies have the correct general appearance for liquid water[17]. Thus one may say that qualitatively a 'water-like' structure has been created by digital computers -- of course, there is still room for quantitative improvements.

1.3.2 Structural differences of D_2O and H_2O:

A comparison of the structure of heavy water with water is interesting, wherever hydrophobic interactions provide a significant contribution in the observed phenomenon, as the substitution of H_2O by D_2O leads to dramatic changes in a similar direction as would be produced by a lowering of the temperature. There is more structural order in liquid D_2O than in liquid H_2O as inferred[18] by the fact that viscosity, melting point, boiling point, temperature of maximum density, and heat capacity of D_2O are higher than those of H_2O. The statistical thermodynamic calculations of Nemethy and Scheraga[18], based on the 'flickering clusters' model are in good agreement with experimental value which indicates that there is a large amount of similarity between the structures of D_2O and H_2O.

Although thermodynamic studies of transfer of nonpolar solutes from light to heavy water[19,20] indicate that D_2O is more structured than H_2O at a given temperature, this view may need reinterpretation as it is not in agreement with the findings of Narten et al.[21] that the X-ray scattering curves for H_2O and D_2O (both at 4°C) are virtually indistinguishable. Also, as their molal volumes are only marginally different it is hard to ascribe more structuredness to D_2O, without an increase in bulkiness usually associated with it. An alternate suggestion of Frank[22] is that the two liquids are closely similar in structure and that it is the greater gain in entropy accompanying structure-breaking in D_2O, because of its larger moment of inertia, which has given the other impression. He has further pointed out that more new insights are likely to result from more systematic comparisons between the properties of H_2O and D_2O.

1.3.3 Ionic hydration:

Aqueous solutions of electrolytes have been studied extensively and are understood to a large extent. But, in the absence of a suitable model of liquid water the understanding of the nature of aqueous solutions remains inadequate. The early explanations of aqueous ionic solutions were attempted by Samilov[23] and Frank and Wen[12]. The latter model is an extension of the cluster model for pure water. It divides the effect of an ion, in water, into three concentric regions.

The region of water closest to the central ion has water molecules tightly bound to the ion by electrostatic forces between the water dipole and the ionic charge. This region of immobilized water molecules is referred to as the hydration of the first kind. The third region is farthest from the ion and contains normal or bulk water having the usual hydrogen-bonded network. The intermediate, or second, region has lesser amount of hydrogen bonding, or structure, than the outermost region consisting of normal water. This lack of regular hydrogen bonding is due to the competition between the orienting effect of the central ion and the hydrogen bonding tendency with the water molecules of the first and third regions.

The effective range of these three regions depends on the charge, size, composition and shape of the central ion. In general, high charge and small size increase the extent of the first region, whereas low charge and large size increase the second region. In this context, 'structure making' refers to the formation of ordered regions with relatively 'rigid' dynamic behaviour of the H_2O molecules, that is, an increase of the first region; this ordering need not obey the rules governing the structure of liquid water as it exists in the third region. On the other hand, 'structure breaking' by solutes means the break-down of the water structure from the 'flickering clusters', <u>via</u> the higher aggregates, to the dimeric and single molecules as it happens in the thermal breakdown of the structure of pure water, and is an increase of the second region. This is probably the basis for the Hofmeister series which is the manifestation of the differences of the power of ions to bind water.

1.3.4 Hydrophobic interactions:

Since the review of Kauzmann[24] describing the role of apolar group interactions in the aqueous medium, or hydrophobic bonding, in maintaining the native structure of proteins the term hydrophobic bond has been used in describing several closely related phenomena. There have been objections to the use of the term 'hydrophobic bond by many (e.g. Hildebrand[25]) and has been replaced by the term hydrophobic interaction[26]. This topic has been extensively reviewed by Franks[27] and more recently by Ben-Naim[28].

Although hydrophobic hydration and solute association are now well-accepted phenomena, and it is well known the very low solubility of hydrocarbons in water is due to entropic effects -- a fact which distinguishes aqueous solutions from other liquid mixtures wherein mutual solubility is determined by enthalpic factors -- it must be emphasized that we still have an incomplete knowledge of the origin of such behaviour. Whereas electrostatic, dipole, dispersion, core repulsion and even hydrogen bonding types of interactions have been described in terms of more or less credible pair potential functions -- relating the potential energy value to the distance and mutual orientation of the interacting molecules -- this has not been successfully achieved for the hydrophobic interaction.

Hydrophobic interaction, which refers to the solvent induced interaction between two or more apolar solute molecules in water, is not directly amenable to experimental study because nonpolar molecules are so sparingly soluble in water. Investigators have tried to infer details of hydrophobic interaction by analyzing solubility data of singly polar, more soluble, substituted hydrocarbons. That is, by studying the phenomenon of hydrophobic hydration, which refers to the structure of water molecules in the immediate neighbourhood of the nonpolar solute molecules, and to the thermodynamic properties of very dilute solutions.

Computer simulation studies offer the best opportunity to understand details of the solvation process, of nonpolar molecules in water, at the molecular level and provide answers that are not otherwise available. Several such studies[29-31] seem to show that insertion of a single space-filling but otherwise essentially inert solute molecule spontaneously causes the hydrogen-bonded network, of water, to rearrange and form a local clathrate-like convex cage. But, the observed structure promotion does not lead to an immobilization of the hydration water as it does in solid structures. The dynamical data show that translation and rotational motions of solvation-sheath water molecules are perceptibly slower (by about 20%) than those in pure bulk water[29]. Some results[30], not consistent with the conventional picture, show hydrophobic interaction occurring between a pair of apolar molecules with a water molecule sitting between the two particles. Further studies are necessary before one may make any definitive conclusions about this type of results. The general result[31] of this type of computer simulation studies is that the molecules in the solvent-shell, around apolar solutes, engage in stronger hydrogen bonding than bulk molecules but there is one less water-water interaction at the short range for the shell molecules.

1.4 MANIFESTATIONS OF THE HYDROPHOBIC EFFECT

It is evident from the above discussion that, as of now, no proper molecular description exists for the hydrophobic effect. This is understandable to some extent since hydrophobic interactions differ from ionic, dipolar, hydrogen bonding or other such cohesive interactions in its generality and nonspecific nature; this makes the writing down of a concise pairwise or other interaction term difficult. Also, the interaction potentials for the cohesive forces, mentioned above, represent the energy term and would be valid in vacuo, appropriately attenuated by a solvent dielectric. The hydrophobic effect however, arises predominantly as an entropy effect and the role of the solvent water is crucial, it is also in essence a reflection of not an attractive influence but of the removal of the solute from the solvent water. Its manifestations in a variety of situations and with molecules of widely different structures are explainable in a thermodynamic sense. This is true, whether it is the solubility of polymers in water, aggregation of surfactants and lipids to produce micelles and membranes, preferences exhibited by several proteins in the aqueous medium to adopt the native globular conformation (with polar residues outside and nonpolar ones buried within) or multi subunit protein aggregation.

The thermodynamic basis of the hydrophobic effect is seen in the dominance of the entropy of interaction between an apolar molecule and water[24,32]. Taking the case of the solubility of n-butane in water at 298 K the ΔG of solubility has been measured to be +5850 cal/mole; which does not prefer solubility. The enthalpy of interaction is favourable (-1000 cal/mole), presumably due to dipole-induced dipole and dispersion forces, but the entropy has a value of -23 e.u. If one looks at the chemical potentials for transfer from a nonpolar phase to water of a series of alkanes, one notes that

$$\Delta\mu_{trans.} = \mu^{o}_{Hc} - \mu^{o}_{H_2O} = - 2436 - 884\ n_c$$

where μ^{o}_{Hc} and $\mu^{o}_{H_2O}$ refer to the chemical potentials in the hydrocarbon and water phases and n_c the number of $-CH_2-$ groups in the alkane. The situation is similar in the case of the transfer of alkanols and alkanoic acids as well. In the former $\Delta\mu^{o}_{trans.} = 4260-825\ n_c$, while in the latter, it is $\Delta\mu^{o}_{trans.} = 833-821\ n_c$

From these, it is seen that the contributions to the transfer free energy term arise independently from the hydrophilic and the nonpolar (i.e. hydrophobic) segments of the molecule, and the latter is characterised by a roughly constant value of about -800 cal/mole of $-CH_2-$ fragment.

Even more illustrative are the values of the enthalpy (ΔH^o), entropy (ΔS^o) and the heat capacity (ΔC^o_p) of transfer from the hydrocarbon phase into water. For ethane at 298 K, $\Delta\mu^{o}_{trans.} = \mu^{o}_{Hc} - \mu^{o}_{H_2O} = -3800$ cal/mole, $\Delta H^o = 1800$ cal/mole, and $\Delta S^o = 19$ e.u. and $\Delta C^o_p = -59$ cal/mole/deg., while the enthalpy term would encourage solubility in water, the ΔS^o term forbids it and ΔC^o_p reflects much the same. It is clear that in hydrophobic interactions, a mutual compensation operates at any temperature between the enthalpy and the entropy terms. Herein lies another characteristic feature of the hydrophobic effect, namely its unusual temperature dependence. The hydrophobic interaction weakens as the temperature is lowered essentially because of a reduction in the value of the $T.\Delta S$ term in the free energy expression. This feature manifests itself particularly in several seemingly disparate instances, when an amphiphilic solute is placed in water. A globular protein chain that is folded in its native conformation in water can be denatured upon heating to 80-90°C, essentially because of the weakening of the cohesive forces that help maintain its structure, e.g. solvation, hydrogen bonding, etc. The same protein can also be unfolded upon supercooling to subzero temperatures, and this has been attributed to a weakening of the hydrophobic interactions between its constituent apolar sidechains upon a reduction in the temperature[33].

The mildly polar compound nicotine shows two critical solution temperatures at any given concentration in aqueous medium; so does the water soluble polymer poly(riboadenylic acid) at appropriate concentrations.

Surfactant aggregates sometimes display critical micellar temperatures. We look at some of these in some detail in later chapters, with a view to study the commonality between them as arising due to possible manifestations of the hydrophobic effect. Yet another manifestation is the ready solubilization exhibited by soap micelles of a wide variety of structurally unrelated, yet partly or totally apolar compounds within them, e.g. other soaps, alkanes, aromatics, lipids, etc.

To the extent that the hydrophobic effect goes hand in hand with the structural features of the solvent water, it is interesting to note that similar 'solvophobic' interactions have been reported with other solvents that also have significant degrees of structuredness in their liquid state. Bello has looked at the case of glycerol and the structure and properties of enzymes in this solvent[34]. Surfactant micellization has been reported in several organic liquids such as N-methyl formamide and dimethyl sulfoxide, which are thought to be intermolecularly structured. Also, addition of third components that preferentially enhance the structure of liquid water (the structure makers) or diminish it (structure breakers) should have marked effects on the stability and the manifestation of the hydrophobic interaction. Compounds such as glucose are known to be structure makers, while urea or $NaClO_4$ are known to be chaotropic; or break water structure. If the variety of disparate phenomena cited above were to arise essentially as manifestation of the hydrophobic effect, an analysis of the effect of third components that make or break water structure should prove worthwhile.

An interesting and well known phenomenon encountered in solution chemistry is the partial miscibility of liquid pairs, also known as binary consolute mixtures, a comprehensive list of which has been provided by Francis[35]. Often such pairs exhibit phase separation into two layers at a particular temperature known as the critical solution temperature (CST) that is characteristic for a given composition. Systems which show phase separation (or precipitate, if one of them is a solid) upon heating are said to have a lower critical solution temperature (LCST) below which they are mutually soluble. Likewise, systems that phase separate upon cooling possess an upper critical solution temperature (UCST) above which the pair is completely miscible. The system water-triethylamine exhibits LCST behaviour, the pair nitrobenzene-hexane displays an UCST, while the pair water-nicotine displays both an LCST and a UCST. Empirical generalizations about the behaviour of a variety of consolute pairs has been presented by Timmermans[38] as early as 1907, while some thermodynamic insight into the phenomenon has been presented by Prigogine and Defay[36] and by Copp and Everett[37].

From such a thermodynamic analysis, it has become known that the LCST phenomenon of phase separation upon heating is principally an entropy-directed process while the opposite process of UCST is predominantly enthalpy-driven. 'The subtle interplay of factors which lead to lower consolute points ... explains why such phenomena are so rare in comparison with upper consolute behaviour[37]. The importance of the excess entropy term, i.e. $S^E < 0$, for an LCST to occur limits the mixtures which may exhibit such behaviour to those that suffer large losses in entropy on formation. Curiously this important clue to which systems might display LCST has not been studied in greater length, though its significance was realised by Kauzmann[24] in connection with hydrophobic interactions. The manifestation of the hydrophobic effect in such aqueous CST pairs is well worth a further study in its own right, and particularly because of some recent observations concerning temperature-dependent 'clouding' or phase separation of some biopolymers from aqueous solutions, and their implications that we discuss in Chapter II. A variety of biologically, and technologically relevant compounds appear to exhibit LCST in water solutions, such as some amphiphiles[39].

Also, recent work by Taylor and Cerankowski[40] has shown that one may be able to tailor-make water soluble polymers to display LCST by adjusting their hydrophile-lipophile balance. The effect of the addition of third components on the CST behaviour is also expected to provide information about the role of solvent structure and the thermodynamic factors governing the phenomenon. In Chapter II, we explore the manifestations of the structure of solvent water and of the hydrophobic effect in the critical solution behaviour of both small molecules and of some biologically relevant polymers in aqueous media.

That amphiphilic soap molecules spontaneously aggregate in aqueous solution into micelles has been known since the dawn of this century. The thermodynamics of micellization has been clarified to a considerable extent as yet another manifestation of the hydrophobic effect[32]. That such micellization is also in effect a phase separation has been considered by many investigators and has been reviewed by Hall and Pethica[41]. Though this treatment suffers from not being a rigorous definition of a phase in connection with an aggregate of nanometer dimensions — and which might not necessarily be homogeneous and uniform within itself — the application of the thermodynamics of small systems[42] to this phase separation approach has led to a good approximation of the micellization process[41]. This phenomenon has also been studied by using the mass action law approach by Hartley and others[43], and two recent reviews by Wennerstron and Lindman[44] summarise the present status of our thermodynamic understanding of micellization. The classical model, based on these arguments, is that of Hartley who regards the micelle as a compact aggregate with its ionic or polar parts 'outside' in contact with water and the nonpolar alkyl tail buried 'inside', somewhat as an oil drop with a polar ionic skin. The compaction and the nonpolar interior are thought to arise as a consequence of the hydrophobic effect and the role of the structure of solvent water. Micellization is known to be a cooperative phenomenon with a well-characterized critical micellar concentration (cmc) above which the aggregate is the predominant species, and in favourable cases a critical micellar temperature as well[41,44]. Analysis of the free energy of micellization term shows the entropy term to be the driving force; and comparison of the free energies of micellization of a homologous series of surfactants reveals a characteristic chemical potential increment of about 800 cal/mole per CH_2 group, as in the cases mentioned earlier in this section. The law of thermodynamic compensation between enthalpy and entropy is seen to hold here as well. Tanford[32] has shown on thermodynamic and packing energy arguments that while surfactant with a single non-polar tail will aggregate as spherical micelles in water, those with multiple tails (but a single headgroup) can aggregate indefinitely to produce bilayers, lamellae, and if the conditions are right, form closed vesicles. Herein lies the thermodynamic basis for the spontaneous formation of micelles, membranes and liposomes in an aqueous milieu, which also appears to suggest minimal amount of water in their body.

Yet, several recent experimental results, notably by Menger and associates[45], on surfactant micelles have been interpreted to mean that micelles are not dry, compact assemblies but are indeed 'porous clusters' with considerable water contents in them. In light of what has been described above, it becomes important to study this controversy and to gain further insight into the polarity, water content, and the interior environmental features of surfactant aggregates. With our interest in the hydrophobic effect and its manifestations in a variety of seemingly disparate situations, we have spent some effort on the issue of the water content in micelles and describe our contributions in this area in Chapter III.

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CHAPTER II*

CRITICAL SOLUTION TEMPERATURE OF LIQUID MIXTURES AND THE HYDROPHOBIC EFFECT

2.1 ABSTRACT

Two important generalizations applicable to liquid pairs that exhibit critical solution temperatures (CST) are the Prigogine rule and Timmermans rule. We have shown that, while these rules are obeyed by organic liquid pairs, they are violated by aqueous binary systems. Contrary to the Prigogine rule, the CSTs of aqueous binaries occur at high water mole fractions, and contrary to the Timmermans rule, globular protein denaturants and Hofmeister series ions solubilize the second component in water. We trace these violations to the structure

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of water and the hydrophobic effect. Also, the phenomenon of thermal phase separation of some biopolymers in water is shown to belong to the general class of lower consolute behaviour. Further, a comparison of the effect of substituting D_2O for H_2O on CST has been made.

2.2 INTRODUCTION

There has been renewed interest in binary liquid mixtures that exhibit critical solution behaviour, particularly with reference to studying the universality of the behaviour of several properties around the critical point. Our interest in this chapter is to show their utility in studying aspects of hydrophobic interactions and in exploring the similarity of the features of the phenomenon of heat precipitation, or cloud point, of biopolymers with those of partially miscible binary liquid mixtures.

In Chapter I we have discussed, in general about binary liquid mixtures that display partial miscibility referred to as consolute pairs. A detailed thermodynamic analysis of consolute binaries has been done by Prigogine and Defay[1,2] using the concepts of regular solution theory and excess functions, which explains the empirical observations reported earlier by Timmermans[3]. Two important generalizations that have emerged from these studies[1] are: (i) the critical solution temperature of consolute liquid pair will occur at equimolar composition of the two components (we have termed this as the Prigogine rule), and (ii) an additive that is preferentially soluble in one of the components of the binary mixture will decrease their mutual solubility i.e. increase upper critical solution temperature (UCST) or decrease lower critical solution temperature (LCST), while an additive that is equally soluble in both components will increase their mutual solubility (decrease UCST or increase LCST). This may be referred to as the Timmermans rule.

Our interest here is to highlight the contrasts between organic consolute liquid pairs and those that contain water as a component. Aqueous binaries offer a convenient model for a study of hydrophobic interactions that occur in aqueous solutions. Indeed, Kauzmann[4] has referred to the CST behaviour of liquid aliphatic derivatives and water in this connection. Even earlier, Copp and Everett[5], in an elegant and prophetic paper, had hinted on the important roles of the structure of water and hydrocarbon-water interactions in the LCST behaviour of water-amine mixtures. We explore these points further in this chapter, and look at the effects of additives that alter liquid water structure on the CST behaviour of both aqueous and nonaqueous consolute solutions. The specific additives of interest are water structure breakers such as urea, guanidinium chloride (GuCl) and water structure enhancers such as sugars, and also the lyotropic or Hofmeister series of inorganic salts. In many ways, conceptual insight that is obtained from conventional experiments on solubility measurements of nonpolar compounds in water is more conveniently obtained by following the CST behaviour of aqueous binaries.

We present results to show that while the Prigogine and Timmermans rules are obeyed in general by consolute liquid mixtures where both the components are organic liquids, these rules are violated in water-organic binaries. Agents that are known to disrupt liquid water structure solubilize the organic component into water in aqueous binary mixtures, and water structure makers decrease the mutual solubility in violation of the Timmermans rule. Such violation is not observed while these compounds are added to non-aqueous binary mixtures.

These studies are expected to be of interest in providing an insight into the mode of action of globular protein denaturants and renaturants.

Similarly, the occurrence of UCST of polymer solutions is known and Freeman and Rowlinson[6] have shown that most polymer solutions undergo phase separation at a LCST at high temperatures; in the vicinity of the gas-liquid critical temperature of the small molecule solvent. But, the thermal phase separation, or cloud point phenomenon, that occurs in aqueous solutions of compounds such as Triton X-100, poly(vinylmethyl-oxazolidinone) or poly(L-proline) are akin to aqueous binary mixtures which display LCST behaviour. The addition of a water-soluble third component which breaks water structure increases the cloud point, and conversely, an additive which is a water-structure maker decreases the cloud point. Also, the effects of substituting D_2O for water on the LCST of aqueous binary mixtures are paralleled in the case of polymer:water mixtures. Both the preceding cases show a decrease in LCST or cloud point temperatures as would be expected from solvent structure effects.

2.3 EXPERIMENTAL SECTION

The reagents used were the best analytical grades commercially available. Poly(L-proline) was obtained from Sigma Chemical Co., U.S.A. (Lot No.37C 5052, mol.wt. 12000). The experimental method for the measurement of CST was similar to that of Hales, Bertrand and Hepler[7]. The binary mixtures were placed in a suitable tube, which in turn was submerged in a beaker containing water whose temperature could be changed slowly by heating or cooling. The bath water and mixture were both stirred manually; at times a magnetic stirrer was used to stir the bath liquid. The temperature of phase separation of the mixtures were determined visually with the temperature both increasing and decreasing. The temperatures could be read with an accuracy of ± 0.05°C in most cases. For some of the higher temperatures (>60°C) the accuracy was ± 0.2°C. The worst disagreement in duplicate readings of temperature were 0.1°C, and 0.5°C for the higher (>60°C) temperatures. The heating or cooling rates were of the order of 0.5 to 1°C per minute. In the case of biopolymers the temperature change was controlled to 1 to 2°C per hour. Experiments on methanol-containing systems were conducted using sealed tubes in order to avoid moisture contamination and evaporation losses.

Data exists in literature on the effect of added salts on the CST of some aqueous and organic binaries; but not on the effects of denaturants and renaturants. Typical denaturants chosen were urea, thiourea and guanidinium chloride. The renaturants were sugars and glycerol. The lyotropic series of ions were chosen as suggested in the review by von Hippel and Schleich[8]. Since these compounds are sparingly soluble in several organic solvents, we have had to choose such liquids where these dissolve to some extent. Accordingly, the organic consolute pairs chosen were: benzyl alcohol-hexane, ethylene glycol-methyl acetate, methanol-hexane, and acetone-hexadecane.

2.4 RESULTS AND DISCUSSION

2.4.1 The Prigogine and Timmermans rules:

It has been shown[1] from thermodynamic arguments that the condition with respect to diffusion of a homogeneous phase consisting of two components 1 and 2 is

$$\mu_{11} > 0; \mu_{22} > 0; \mu_{21} < 0 \qquad \ldots \qquad (1)$$

where $\mu_{ij} = \partial\mu_i/\partial x_j$. Here μ_i is the chemical potential of component i and x_j the number of moles of j. The conditions of interest are:

$$\frac{\partial \mu_1}{\partial x_2} < 0 \quad \text{and} \quad \frac{\partial \mu_2}{\partial x_1} < 0 \qquad \ldots \qquad (2)$$

Now, consider the liquid mixture as a regular solution characterized by the activity coefficient relationships

$$RT\ln \gamma_1 = \alpha_{12}\, x_2^2 \quad \text{and} \quad RT\ln \gamma_2 = \alpha_{12}(1-x_2)^2 \ \ldots \qquad (3)$$

where γ_i is the activity coefficient of component i, and α_{12} represents the interaction free energy between 1 and 2. We may write

$$\mu_1 = \mu_1^o(T,P) + RT\ln(1-x_2) + \alpha_{12}\, x_2^2$$

$$\mu_2 = \mu_2^o(T,P) + RT\ln x_2 + \alpha_{12}(1-x_2)^2 \qquad \ldots \qquad (4)$$

If the system splits into two phases, μ_1^o (and μ_2^o) will have the sam· value in the two layers. Then

$$\frac{\partial \mu_1}{\partial x_2} = \frac{-RT}{1-x_2} + 2\,\alpha_{12}x_2 \qquad \ldots \qquad (5)$$

If the system is to be in stable equilibrium with respect to phase separation, we invoke condition (2) and write

$$\frac{2\alpha_{12}}{RT} < \frac{1}{x_2(1-x_2)} \qquad \ldots \qquad (6)$$

If α_{12}/RT is large and positive, this inequality cannot be satisfied for all x_2; for $x_2(1-x_2)$ has a maximum value of 0.25. Hence for values of $2\alpha_{12}/RT > 4$, there will be a range of x_2 where the system will separate into two phases. At the critical point T_c, then

$$(x_2)_c = 0.5 \quad \text{and} \quad T_c = \frac{\alpha_{12}}{2\ R} \qquad \ldots \qquad (7)$$

Regular solution activity coefficient relationships can thus be seen to lead to the equimolar composition rule of Prigogine. The T_c mentioned here is UCST due to the condition (5); if we write the alternate possibility of (5) i.e. $x_2(1-x_2)\ RT < 2\ \alpha_{12}$, the corresponding T_c in equation (7) will be LCST.

The behaviour of the excess thermodynamic functions G^E, H^E, S^E, may be written, if these functions have the same sign for all x_2 at a given T_c, as:

at the UCST: $G^E > 0$; $H^E > 0$ and $S^E \gtrless 0$ ... (8)

at the LCST: $G^E > 0$; $H^E \leqslant 0$ and $S^E < 0$ ... (9)

UCST's are thus related to positive deviations of the excess enthalpy of the system from ideality, while LCST's are related to sufficiently large negative deviations of the excess entropy from ideality[5]. It is also easy to see from the expressions for G^E of the type $G^E = K(T)x_2(1-x_2)$ that around equimolar compositions, G^E will be a symmetric function.

When a third component is added to the binary system, we may write the activity coefficients again in terms of the inter-component interaction free energies, α_{ij}, and by a treatment similar to what has been outlined earlier, the effect of the third component on the CST of the 1-2 pair can be derived as

$$\left(\frac{\partial T}{\partial x_3}\right)_c = -\frac{1}{2R}\cdot\frac{(\alpha_{12}-\alpha_{13}+\alpha_{23})(\alpha_{12}-\alpha_{23}+\alpha_{13})}{\alpha_{12}} \qquad (10)$$

In equation (10), we have the basis of the <u>Timmermans rule</u>: (a) if component 3 is more soluble in 2 than in 1, then $\alpha_{23} \gg \alpha_{13}$ and $\alpha_{23} \gg \alpha_{12}$, and equation (10) simplifies as

$$\left(\frac{\partial T}{\partial x_3}\right)_c = \frac{1}{2R} \cdot \frac{\alpha_{23}^2}{\alpha_{12}} > 0 \qquad \ldots \qquad (11)$$

and if 3 is more soluble in 1 than in 2, $\alpha_{13} \gg \alpha_{23}$ and $\alpha_{13} \gg \alpha_{12}$ and we have

$$\left(\frac{\partial T}{\partial x_3}\right)_c = \frac{1}{2R} \cdot \frac{\alpha_{13}^2}{\alpha_{12}} > 0 \qquad \ldots \qquad (12)$$

In other words, addition of component 3 which is preferentially soluble in one component of the binary mixture will always decrease mutual solubility, i.e. raise the UCST; (b) if 3 is about equally soluble in 1 and 2, $\alpha_{13} \cong \alpha_{23}$ and equation (10) reduces to

$$\left(\frac{\partial T}{\partial x_3}\right)_c = -\frac{\alpha_{12}}{2\ R} < 0 \qquad \ldots \qquad (13)$$

An additive that is equally soluble in 1 and 2 will increase their mutual solubility, or depress UCST.

In the case of a liquid binary that exhibits LCST, equations (10)-(13) are interpreted to mean that the LCST will be depressed upon the addition of a substance that preferentially dissolves in one of the components, while the LCST will be raised by additives that are equally soluble in both 1 and 2.

2.4.2 Violation of the Equimolar rule:

In Table 2.1 we have collected a random list of consolute binary liquid mixtures, both aqueous and organic, that exhibit CST behaviour; Francis[9] lists over 6,000 conjugate binaries in his monograph. It can be seen from the Table that most of the organic binaries, items (a) to (d) indeed obey the Prigogine rule. Deviations of a minor nature are seen in the case of items (e) to (m), where certain alcohols and amide form a component of the mixture. But large deviations from the equimolar rule are seen in every case where water is a component (items (n) to (r)) and these deviations occur both in the UCST and LCST situations. Copp and Everett[5], over two decades ago, had concerned themselves with such deviations in H_2O:aliphatic amine pairs exhibiting LCST and closed solubility loops, and concluded that LCST behaviour in these systems might be related to (i) mutual hydrogen bonding between the amine group and H_2O, (ii) the enthalpy, entropy and heat capacity effects arising from the interaction of the inert hydrocarbon part of the second component with water structure. Everett[10] has also pointed out that the critical value of G^E/RT may exceed the regular solution value of 0.5 to as high as 0.60 for associated liquids or to the limit

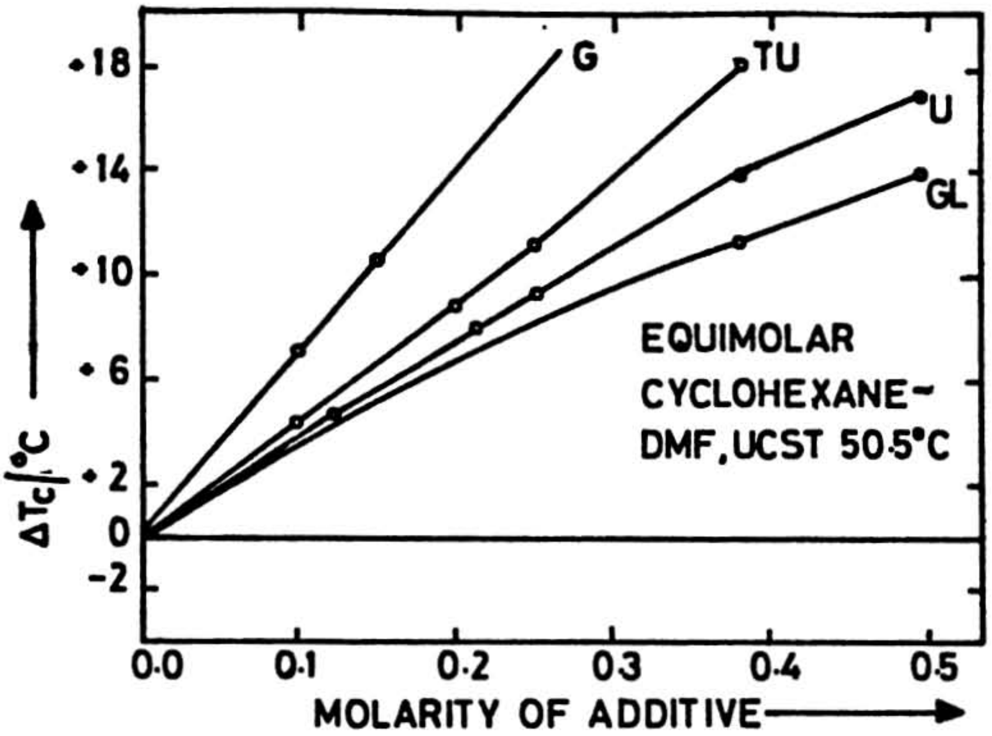
Table 2.1 Data on some consolute liquid pairs.

Item	Liquid A	Liquid B	Moles of A	Moles of B	C.S.T., °C
a	Nitrobenzene	n-Hexane	0.5	0.5	19.0
b	Aniline	n-Hexane	0.55	0.55	59.6
c	Benzyl Alcohol	n-Hexane	0.5	0.5	50.5
d	Nitroethane	3-Methyl pentane	0.5	0.5	20.0
e	Dimethyl formamide	Cyclohexane	0.5	0.5	50.5
f	Phenol	n-Heptane	0.53	0.52	52.9
g	Phenol	n-Hexane	0.5	0.6	52.6
h	Methanol	Cyclohexane	0.9	0.85	49.1
i	Methanol	Carbon disulfide	0.6	1.0	40.5
j	Methanol	n-Hexane	0.63	0.93	42.0
k	Ethanol	Paraffin (Decane)	0.28	0.61	33.5
l	Ethylene glycol	Methyl acetate	0.8	0.7	21.4
m	Formamide	Nitrobenzene	0.88	0.63	108.2
n	Water	Phenol	3.66	0.3	65.9
o	Water	Triethylamine	0.92	0.08	18.5
p	Water	Diethylamine	0.70	0.30	140.0
q	Water	Triton X-100	0.997	0.003	66.0
r	Water	n-Butanol	3.8	0.43	124.4

of 0.69. While these arguments have been particularly for LCST systems, the implication of the structure of liquid water is noteworthy. We believe that the deviations from the Prigogine rule seen in Table 2.1 with the alcohols, amides and water owe their origin to the associated nature of these liquids[2]. The former two are mildly structured in the liquid phase, while liquid water is highly structured. This polymeric association of liquid water would be expected to make aqueous binary liquid mixtures deviate from regular solution behaviour and lead to violation of the equimolar consolute rule. It is also clear that in aqueous binaries, we are encountering examples of hydrophobic interactions, and in the case of LCST mixtures, hydrophobic hydration as well (C_p^E is expected to be positive). It is also interesting to notice that among the many LCST mixtures that exist, a large proportion of them involve water as a component and occasionally glycerol. The latter solvent has been described by Bello et al.[11] as 'water-like, or perhaps super-water.' This is due to the ability of 99 % glycerol to increase the denaturation temperature, i.e. T_m, of a typical globular protein such as RNase; and other such water-like effects. The driving force in binaries exhibiting LCST is, as pointed out in section 2.4.1, the excess entropy term, which is particularly significant in aqueous binary mixtures due to the structure of liquid water and the hydrophobic effect.

2.4.3 Violation of the Timmermans rule in aqueous systems:

If the deviations from the Prigogine rule seen in aqueous systems were to be due to the structure of water and hydrophobic effect, one would expect these features to play a role in the effects of additives on the mutual solubility of aqueous binaries as well. We show in this section the effects on CST of certain additives that are known to affect the structure of liquid water or alter the strengths of hydrophobic interactions. The compounds chosen are: (a) urea, thiourea and guanidinium chloride, which disrupt the structure of water efficiently[13] and weaken hydrophobic associations between nonpolar moieties[8], and (b) polyhydroxy compounds such as sugars and glycerol, which show the opposite behaviour[14]. The compounds cited were used as the third component in four nonaqueous liquid pairs, i.e. benzyl alcohol-hexane (item c of Tables 2.1), dimethyl formamide-cyclohexane (item e), methanol-hexane (item j), and ethylene glycol-methyl acetate (item l), and in several aqueous binary mixtures as well. In Figures 2.1 and 2.2 we present the



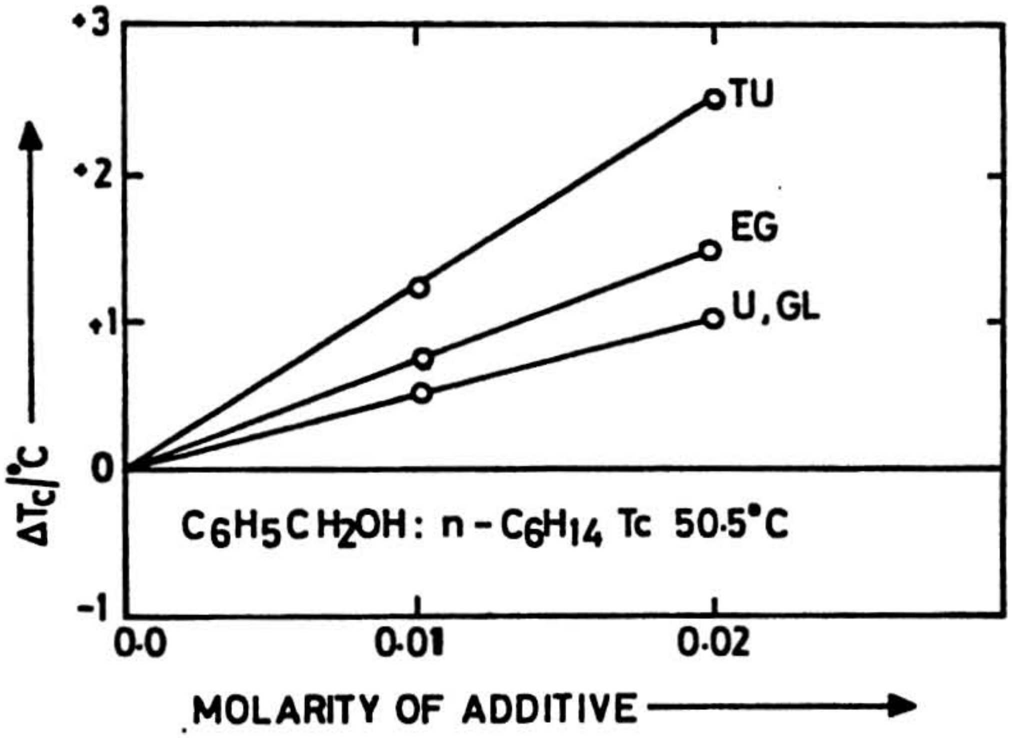


Figure 2.1 Upper figure: effect of additives on the CST of cyclohexane:dimethylformamide consolute pair. Lower figure: changes in the CST of benzyl alcohol: hexane consolute pair brought about by additives. TU = thiourea, U = urea, GL = glycerol, G = glucose, and EG = ethylene glycol.

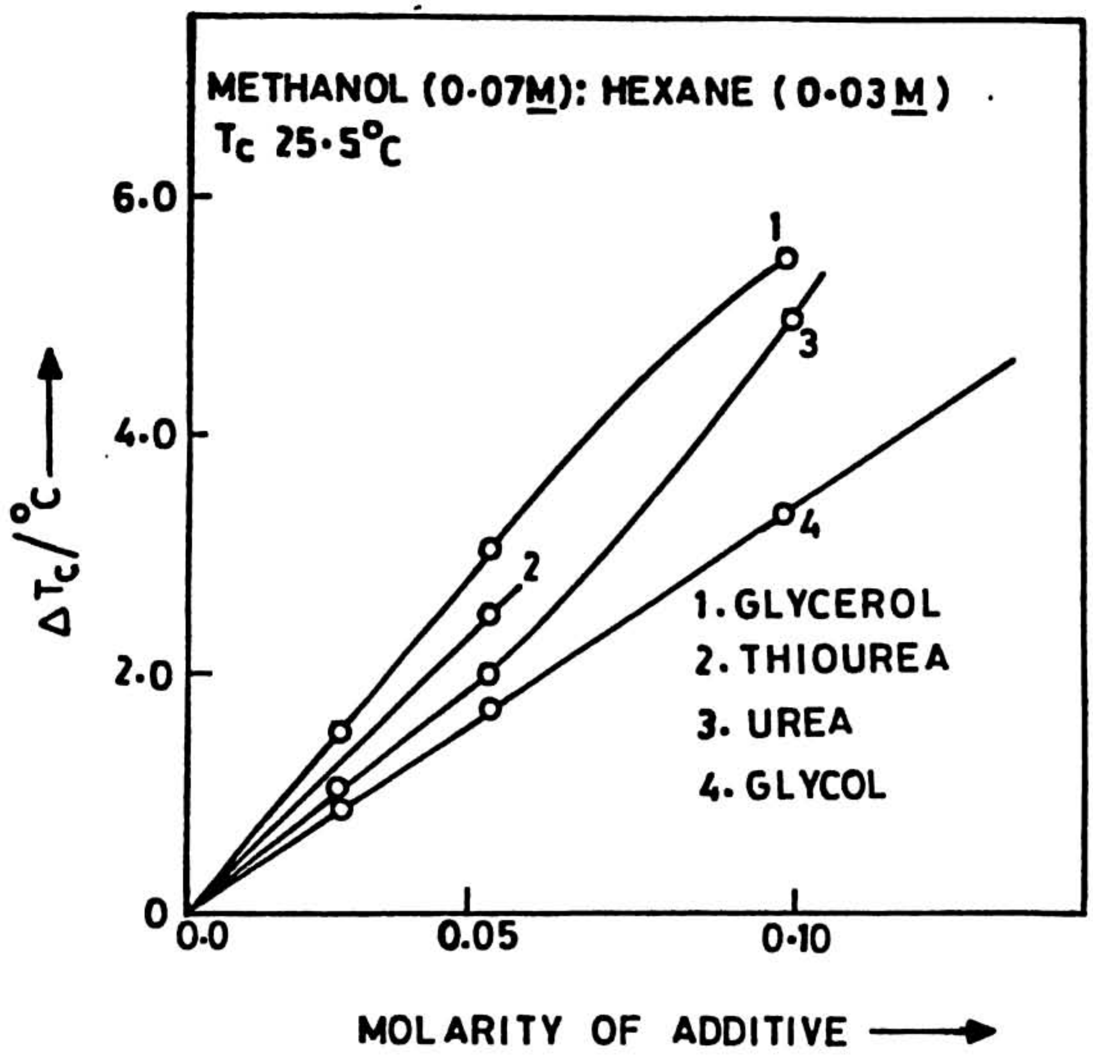


Figure 2.2 Changes in the CST of methanol: hexane consolute pair brought about by protein structural perturbants: (1) glycerol, (2) thiourea, (3) urea, and (4) ethylene glycol.

effect of the additives cited on the CST of three organic binary mixtures. In the absence of detailed information about the nature of G^E curve of the binaries, and with a view to assess the molarity dependent variation of the change in the CST (ΔT_c), we have focussed on the measurements at the consolute compositions only. The solubilities of the additives in benzyl alcohol, ethylene glycol, dimethyl formamide and methanol being limited (they are essentially insoluble in hexane, cyclohexane and methyl acetate), ΔT_c measurements could be run only over a limited concentration range. Furthermore, the additives chosen were, as far as possible, common to both organic and aqueous mixtures so as to make a direct comparison possible.

The data illustrated in Figures 2.1 and 2.2 on the organic liquid pairs reveal that in these instances, the Timmermans rule (equations 11 or 12) is obeyed by these additives, which are preferentially soluble in one of the components. We have also studied the effects of some of these compounds on the mutual solubility of the pair ethylene glycol:methyl acetate and found obedience of the Timmermans rule. (0.02 M GuCl increases the UCST of this binary by 2.2° while 0.02 M glycerol increase it by 0.4°. The other additives cited above did not dissolve in sufficient amounts).

The situation of interest is when one turns to the effect of these additives on the CST of aqueous binary systems. In Figures 2.3 and 2.4, we highlight their effects on the water-phenol UCST pair, and the water-Triton X-100 LCST liquid pair. It is noteworthy that in these cases, despite the fact that the third component added is preferentially soluble in water, their effects are not uniform or similar, as was seen in the case of the organic binaries. While the Timmermans rule dictates that all these additives should decrease the mutual solubility of the aqueous binary mixture, we see that urea, thiourea, GuCl and acetone increase the mutual solubility. Sugars and polyhydroxy compounds studied are seen to behave in obedience of equation (11). What we are observing here is formally similar to the increase in the solubility of non-polar organic compounds in water brought about by the addition of urea, noted by Wetlaufer et al.[15], or the postponement of the cmc of a surfactant in aqueous urea and in aqueous acetone seen by Bruning and Holtzer[16]. This observation reiterates the belief that urea and related globular protein denaturants weaken hydrophobic interactions that occur between the nonpolar sidechains of amino acids in water, while polyhydroxy compounds such as sugars strengthen such hydrophobic interactions.

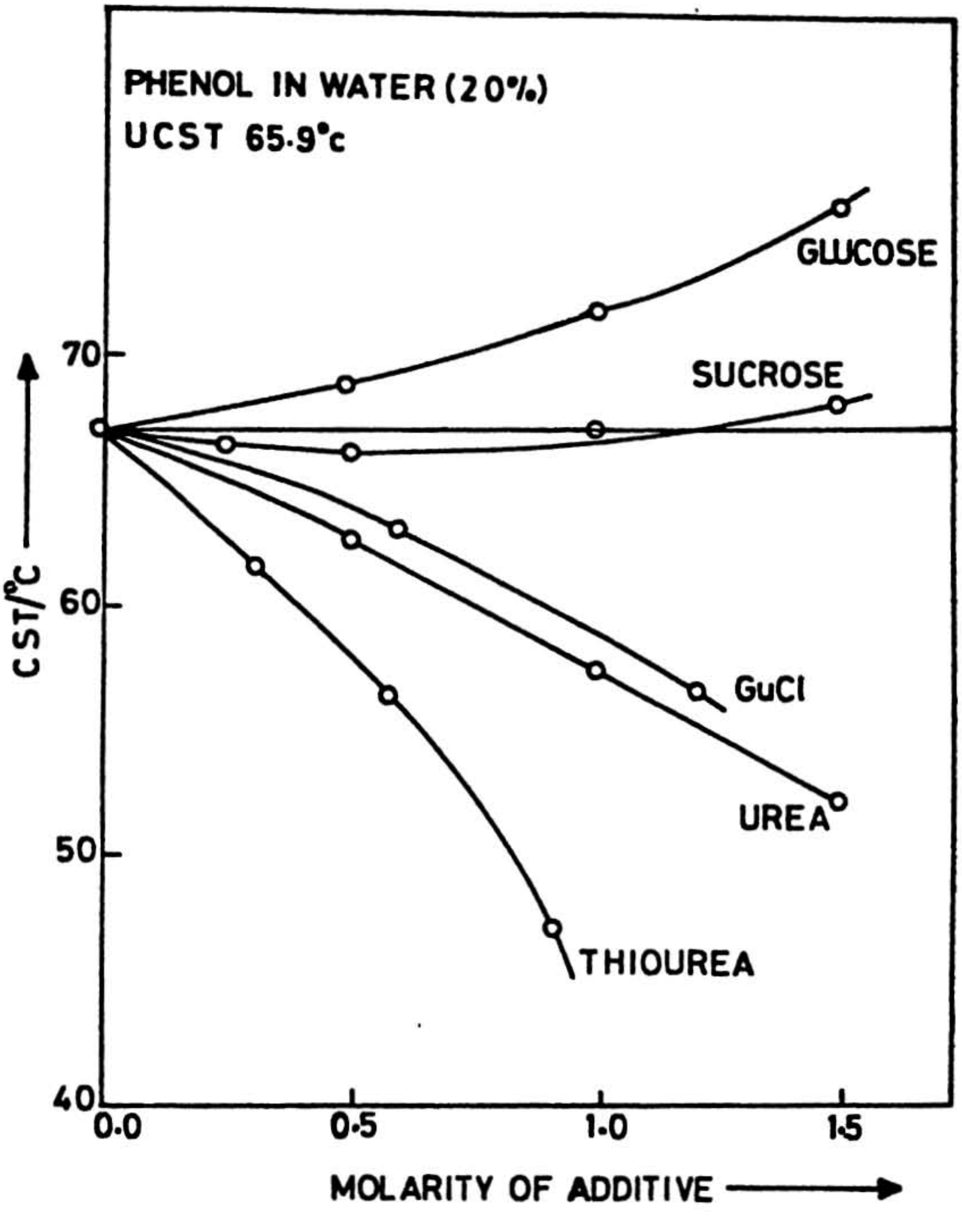


Figure 2.3 Effect of protein structural perturbants on the CST of phenol:water system (20% v/v phenol).

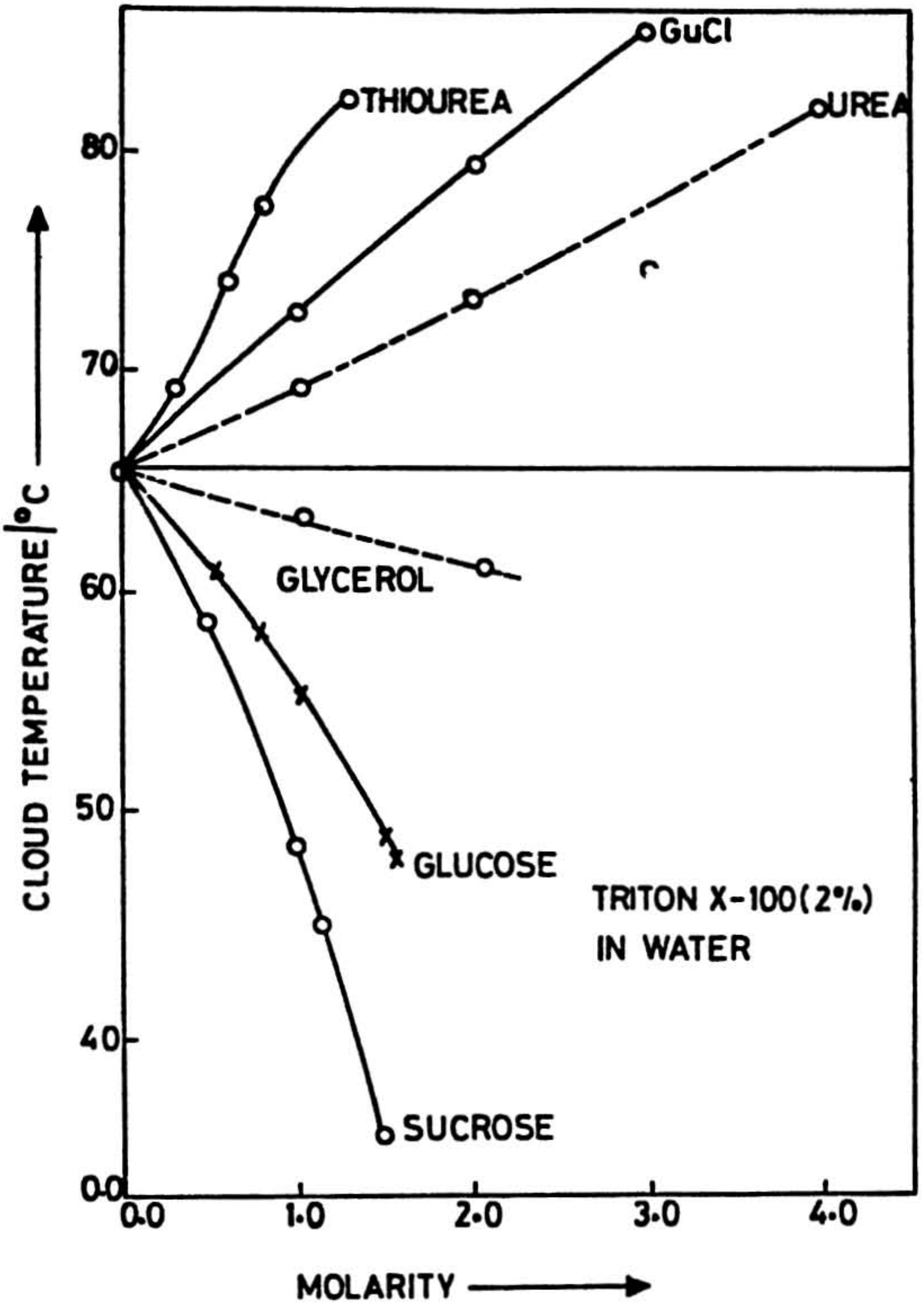


Figure 2.4 The effect of additives which are protein-structure perturbants on the cloud point of 2% (v/v) Triton X-100 in water.

2.4.4 The Hofmeister Series:

In Figures 2.5, 2.6 and 2.7 we show the effects of several salts of the lyotropic or Hofmeister series on the CST of two aqueous consolute pairs (water-phenol, and water-Triton X-100) and a nonaqueous pair (ethanol: paraffin) respectively. The latter figure is drawn from the data reported by Howard and Patterson[17]. All the salts (component 3) used are preferentially soluble in only one of the components (component 1) of each of the binaries studied (water in the first two, and ethanol in the third system), and according to the Timmermans rule (equation (12)), one should expect a salting out effect in a sequence that should reflect the α_{13} values. While this is observed in the case ethanol-paraffin, KI and KSCN salt in Triton X-100 into water, in violation of the Timmermans rule. The efficiency of salting out by the additives is in the order $SO_4^{2-} > Cl^- > I^- > SCN^-$ in the aqueous binaries. This is indeed the Hofmeister series[8], observed in the cases of salting out of globular proteins, surfactants and colloids from aqueous solutions. The sequence is, however, anamolous or reversed in the case of the organic liquid pair, viz. $I^- > Br^- > Cl^-$. Such anamolies in the lyotropic series

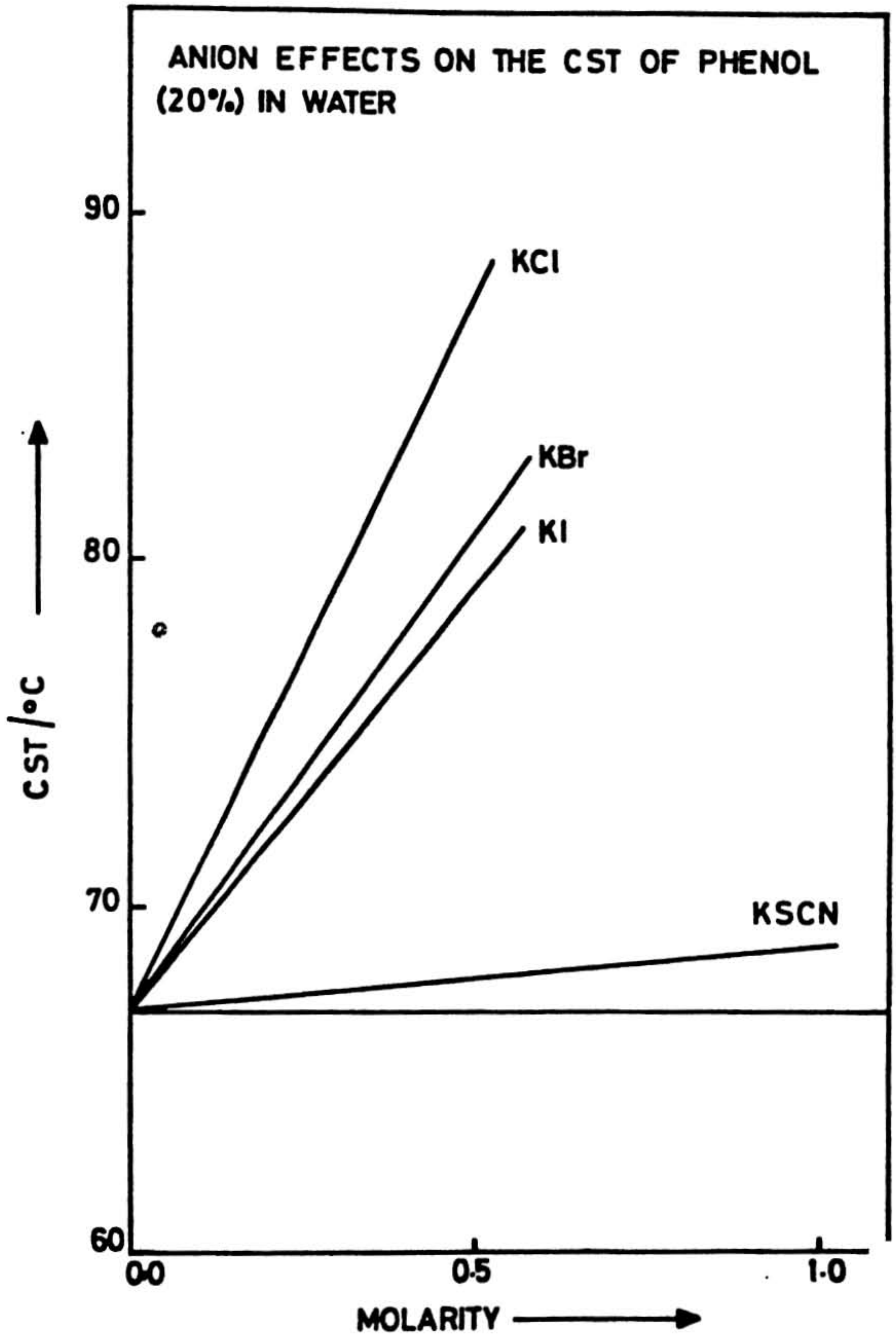


Figure 2.5a Effect of the Hofmeister series o anions on the CST of (20% v/v) phenol:water system.

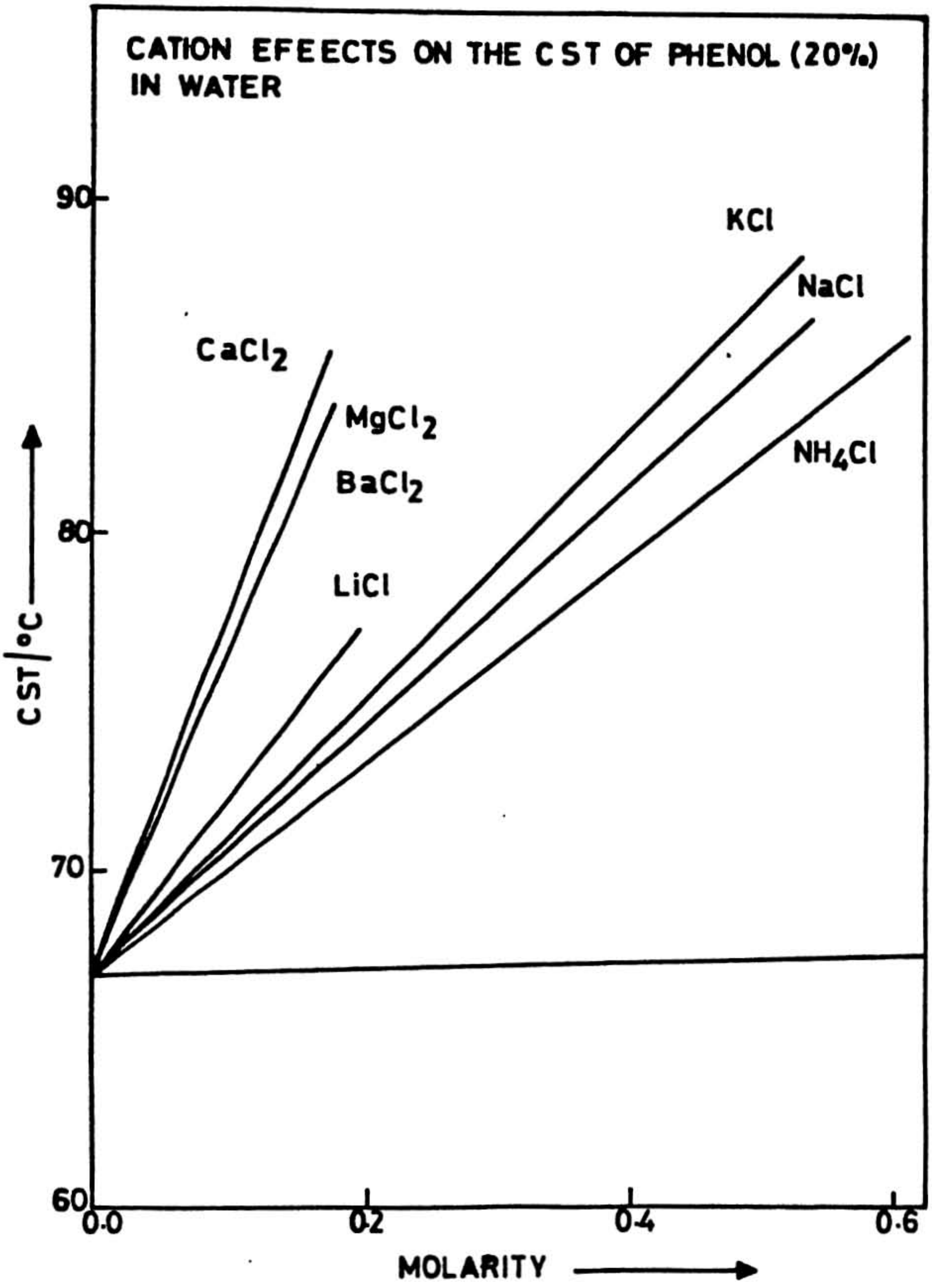


Figure 2.5b Effect of the Hofmeister series of cations on the CST of (20% v/v) phenol:water system. The series shows: $Ca^{2+} > Mg^{2+} \cong Ba^{2+} > Li^{+} > K^{+} > Na^{+} > NH_4^{+}$.

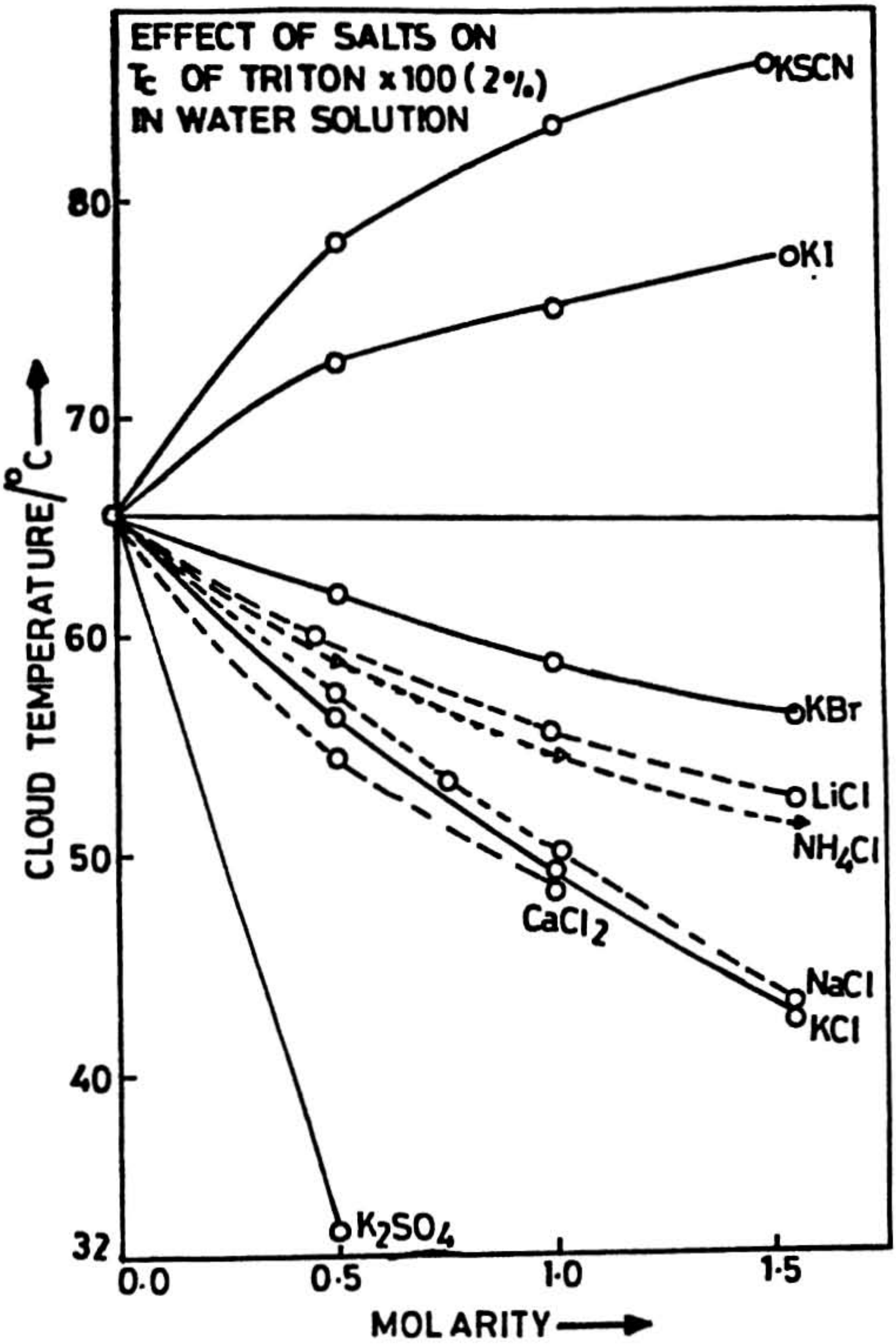


Figure 2.6 Effect of the Hofmeister series of salts on the cloud point of 2% (v/v) Triton X-100. The anion series shows: $SO_4^{2-} > Cl^- > Br^- > I^- > SCN^-$. And, the cation series is: $Ca^{2+} > K^+ \cong Na^+ > NH_4^+ > Li^+$.

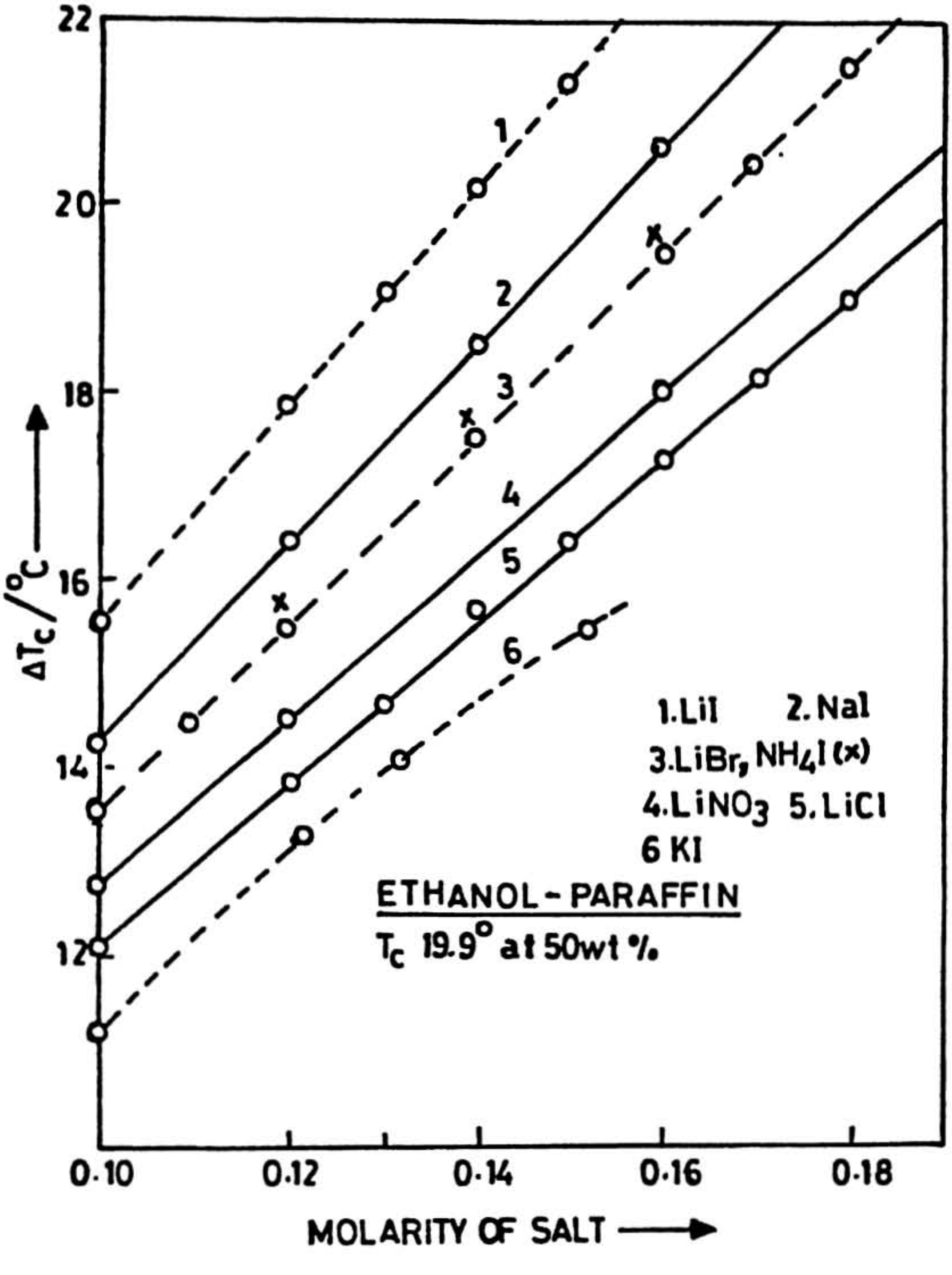


Figure 2.7 The effect of the Hofmeister series of salts on the CST of the non-aqueous binary mixture Ethanol:Decane. Plotted from data reported by Howard and Patterson, ref. 17.

have been observed earlier in literature in other organic systems, e.g. on the salting out of the consolute binaries methanol-hexane[17] and methanol-cyclohexane[18], and on the solubility of CS_2 in alcohol[19].

The effects of ions on a liquid mixture may be considered from the point of view of 'solvent sorting by ions' and the contributions of various interactions to the term α_{13} of equation (12) above to the salting out process[2,20]. Ion-solvent interactions are governed by the charge/size ratio and the polarizability of the ion and the polarity and nature of association of the solvent molecules. Ions with high polarizability increase the internal pressure of the solvent in a binary liquid mixture, leading to efficient salting out effects[20,21], through what has been classically termed as the ion-polarization power[22]. This is an enthalpic effect that dominates α_{13}, and the salting out process from solvents that possess little internal structure, and salting out efficiency follows the sequence $SCN^- > I^- > Br^- > Cl^-$. In the case of highly associated solvents such as water, the enthalpic contribution to α_{13} is attenuated, while the entropic contributions due to the charge/size ratio and polarizability of the ion predominate, i.e. the solvent structure-alteration feature becomes important. This leads to the lyotropic sequence of salting out $SO_4^{2-} > Cl^- > Br^- > I^- > SCN^-$ and $Ca^{2+} > Li^+ > Na^+ > K^+$

2.4.5 Thermal phase separation of some biopolymers in water:

The thermal precipitation of poly(adenylic acid) in water has been studied by Eisenberg and Felsenfeld[23]. More recently, a detailed study has been made on the polypeptides poly(L-proline)[24-31] and poly(L-hydroxyproline)[32] in aqueous solutions. Several interesting features that have emerged from these studies are: (1) the precipitation is not accompanied by any changes in the conformation of the polymer, (2) the effect of agents such as urea and LiBr is to inhibit precipitation, i.e. to solubilize the polymer in water, and (3) there are indications that such a phenomenon may be exhibited by several other biopolymers in aqueous solutions, e.g. cellulose derivatives[33], poly(L-glutamic acid)[34], and DNA[35].

In this section we show the similarity of the features of such heat precipitation, or cloud point, phenomenon with those of aqueous binary liquid mixtures (vide supra). In Figure 2.8 is shown the behaviour of the H_2O-poly(vinylmethyl-oxazolidinone) (PVMO) pair; a system initially studied by Klotz[36]. The effect of additives which are water structure breakers, such as urea or guanidinium chloride, is to increase the cloud point -- i.e. solubilize PVMO in water. On the other hand, water-structure makers such as sugars precipitate the polymer at lower temperatures; i.e. decrease the cloud point.

We believe that these observations may explain the increase in the precipitation temperature (T_p) of aqueous solutions of poly(L-proline), seen upon the addition of urea or LiBr[24] and the decrease in T_p in the presence of KF and KCl[27]. The former two additives are known to be chaotropic while the latter two are mild structure-makers of water. Our own experiments reveal that the addition of a water-structure promoter, glucose (1 M), causes the cloud point of poly(L-proline) to decrease from 55°C to 50°C. The striking similarity between the effect of additives on helical poly(L-proline) and on the presumably randomly coiled PVMO suggests that the effect of these additives on the solubility of poly(L-proline) is not through any conformational change that they may have been brought about in this polymer, but rather

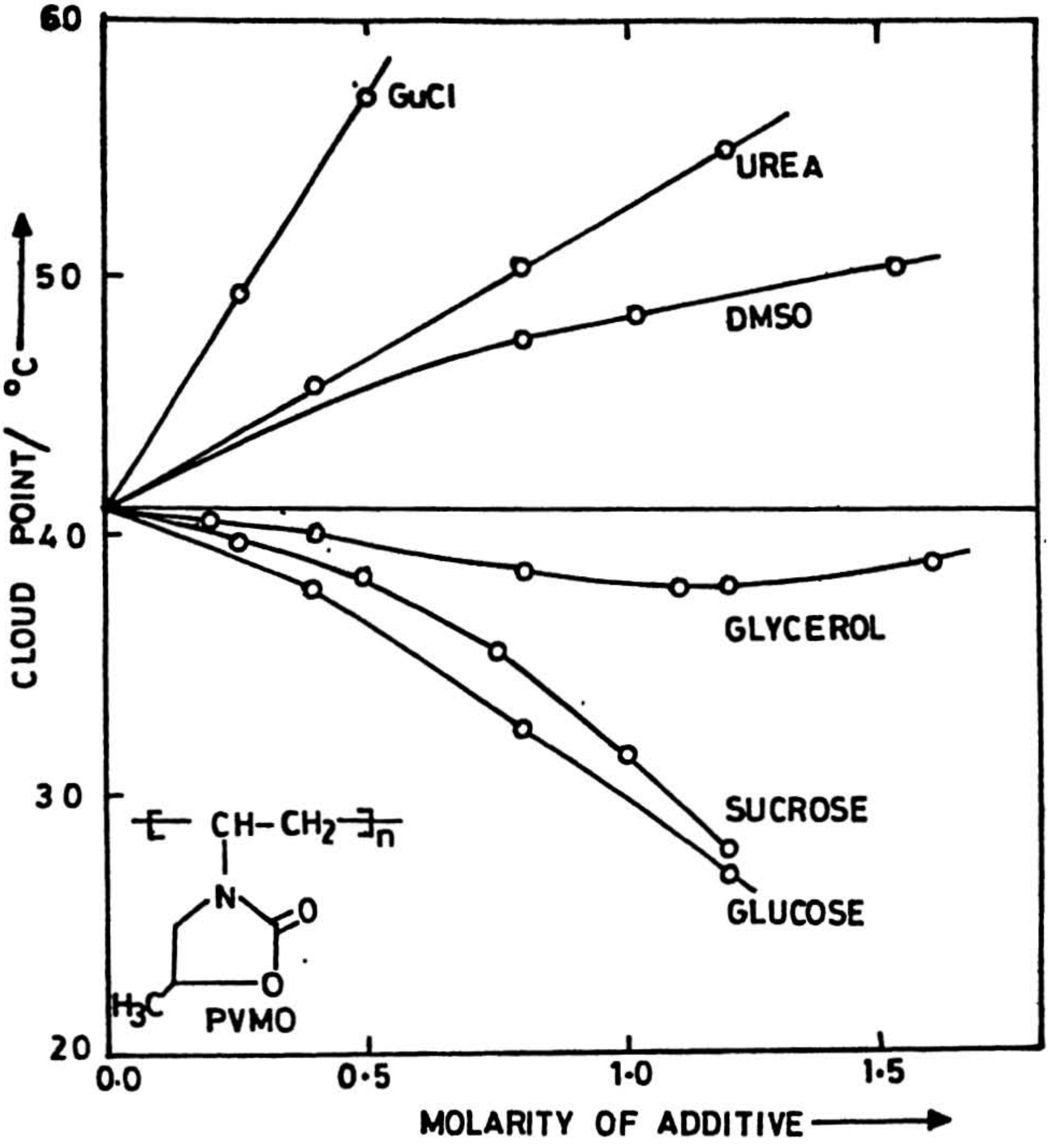


Figure 2.8 The effect of additives which are protein structure perturbants on the cloud point of 0.2 w % poly(vinylmethyl-oxazolidinone).

through their effect on the structure of the solvent water. In order to test this point, we have studied the effects of urea, thiourea, GuCl, glucose and glycerol on the CD spectrum of poly(L-proline) in water, and found their effects to be negligible; in other words, these agents do not perturb the helical conformation of poly(L-proline)II helix in water at room temperature. This observation, taken in conjunction with those of Clark et al.[32] suggest that no conformational changes occur in poly(L-proline) or in poly(L-hydroxyproline) near the cloud point region. Incidentally, this ineffectiveness of these globular protein conformational perturbants towards the poly(L-proline)II helix is in keeping with the belief that these perturbants act by altering the strengths of inter-segment hydrophobic interactions, that occur in such biopolymeric systems, through their effect on the structure of solvent water[8,36].

2.4.6 Effect of substituting D_2O for H_2O on CST:

Our experiments on isotropic substitution were motivated by the fact that liquid D_2O is thought[37,38] to be somewhat more structured than liquid H_2O. In the present context the use of D_2O in place of H_2O as the solvent phase should be equivalent to the effect of the addition of a water-structure promoter, such as glucose, in H_2O — i.e. a raising of the UCST and lowering of the LCST of aqueous binaries. Such an expectation has indeed been borne out. The thermodynamic properties of non-electrolyte solutions prepared with either H_2O or D_2O as one of the components have been previously studied[39], as well as the effects of isotropic substitution on phase relationships in binary solutions. The substitution of D_2O for H_2O produces, in all cases studied, a sizable increase in the UCST of a binary aqueous solution[40]. The UCST for the phenol-water system was reported[41] to increase by 13°C when D_2O is substituted for H_2O.

Similarly, the LCST of several aqueous binary mixtures[40], and also the cloud points of several compounds in water, decrease when H_2O is replaced by D_2O. The LCST of triethylamine-D_2O is 14.5°C, compared to 18.5°C in water, and LCST of nicotine in D_2O is 7.0°C lower than the value found in water[9]. We have found the cloud point of 2% Triton X-100 to decrease from 65.5° to 62.0°C upon replacing H_2O by D_2O, and the cloud point of PVMO in D_2O is found to be 36.5° compared to 41.0°C in H_2O. Interestingly, we observed that the precipitation temperature of our sample of poly(L-proline) decreases from 55°C in H_2O to a value of 50°C in D_2O. This decrease in cloud point is similar to the 9°C decrease seen in the precipitation temperature of poly(L-hydroxyproline) when H_2O is substituted by D_2O.

2.5 CONCLUSIONS

The results obtained indicate that the aqueous binary liquid mixtures are different from the usual organic consolute pairs, and we may conclude that the effects of water structure, i.e. the excess entropy of interaction, predominate in these cases. The violation of the Timmermans rule by additives, which are globular protein-structure perturbants, clearly indicate that their effect on CST is through the effect on water structure -- as renaturants and denaturants show opposite changes although they are both preferentially soluble only in the aqueous phase. Similarly, the Hofmeister series of ions can be seen as a sequence of agents that are increasingly effective in disrupting water structure and weakening hydrophobic interactions. In other words, the greater the capacity of an ion to disrupt the structure of water, the greater is its salting in ability, and _vice versa_. This is a fact that has been intuitively accepted[42,43], and which is gratifyingly illustrated from our studies on salt effects on consolute temperatures.

The cloud point observations made on some biopolymer solutions in water, may be said to belong to the general class of lower consolute behaviour of quasi-polar polymeric solutes, and might also apply to several other systems mentioned earlier, including that of the ψ-state of DNA seen in aqueous solutions containing certain inert polymer co-solutes[35]. The general features of such polymer phase separations have been highlighted by Koningsveld[44]; in the case of aqueous solutions, certain features of the solvent structure appear to be manifested. The effects on CST observed on substituting D_2O for H_2O also substantiates the fact that solvent structure plays a dominant role in these cases.

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CHAPTER III*

CRITIQUE OF WATER PENETRATION STUDIES IN MICELLES USING EXTRINSIC PROBES

3.1 ABSTRACT

The sites of solubilization of some classes of 'water penetration probes' in micelles have been studied. These probes, which invariably contain polar or aromatic groups in them, are shown to be inherently interfacially active. The effect of such an activity is seen to be enhanced when the probes are solubilized in systems of large surface area-to-volume ratios such as amphiphile aggregates. NMR results reveal that these 'water penetration probes' are located largely in the head group

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region of micelles. They would thus be expected to monitor the polarity and water content of this region rather than those of the micellar interior. Thus, studies using such probes do not necessarily prove the existence of water inside micelles and related assemblies.

3.2 INTRODUCTION

There is considerable current interest on the structural organization of supramolecular assemblies of amphiphilic compounds and on the extent of water penetration into their interiors. The implications of a given structural model are significant not only for micelles in water but for related assemblies including biomembranes as well, since the general principles of organization of these assemblies are thought to be common[1-4]. As has been discussed in Chapter I of this thesis, the Hartley model[5] of a micelle is 'an oil drop with an ionic or polar coat' and requires the micellar interior to be anhydrous, while Menger[6] has described micelles as 'porous cluster' aggregates with considerable amounts of water in their interiors.

The presence of significant amounts of water inside micelles would imply similar situations in bilayers, and in artifical and natural membranes as well. Yet one finds the rates of self-diffusion of water in micelles, and of water diffusion among reverse micelles and across lipid bilayers to be rather low[7]. The permeability of water across artificial lipid membranes, in the absence of pores, channels, or carriers, is also quite low[8]. These results agree for the presence of very little water in the main body of these aggregates. Thus the question of which model best describes micellar structure becomes highly relevant.

Structural investigations on amphiphile aggregates often make use of external additives as solubilized probes that monitor the polarity, microviscosity and similar microenvironmental features. Since such molecules report on the properties of their 'cybotactic' regions[9], it is important to establish the actual site of occupation of the added probe in the host aggregate. A probe that is largely located in the core of a micelle would be expected to report on the properties of the core, while one that is positioned in the headgroup region would largely monitor the properties of this region. It is our belief that much of the conflicting interpretations about the structures of micelles, based on results using external probes, is due to a lack of appreciation of this point.

Table 3.1 lists several extrinsic probes that have been recently used in studies on micelle structure. It is worthwhile to note that all these molecules contain polar groups or conjugated π-electron systems. There is evidence in literature that benzene and higher arenes are located largely in the headgroup region of ionic micelles[10-16]. Mukerjee and co-workers[10,11] have shown that benzene is mildly interfacially active, and have suggested that the mild surface activity of the aromatic or polar solubilizates tends to be greatly amplified when placed in micelles and related assemblies whose surface area per unit volume (cm^3) would be as large as 10^6-10^7 cm^2, leading to a large fraction of the solubilized molecules accumulating at interfacial sites.

In light of these observations, it becomes important to establish where the water penetration probes are located when they are solubilized in micelles. Are these probes inherently surface active? Do they position themselves near the headgroup region of micelles, or are they located in the micellar core? Do the polarity and the water content that they sense reflect those of the micellar surface, core, or an average of the two environments? How far do the properties reflect the dynamic features -- such as probe translocation between sites, and differential

Table 3.1 Some typical 'water penetra
recent literature.

Probe	Method	Ref.
1. <u>Aromatic probes</u>		
acridine	fluorescence quantum yield	18
1-methyl indole	fluorescence quantum yield and lifetime	19
pyrene and derivatives, related arenes	fluorescence hetero excimer emission	20
methyl viologen (acceptor); 3-methyl indole, pyrene, benzyl-1,4-dihydronicotinamide (donors).	charge transfer spectra	21
2. <u>Keto probes</u>		
Octanal, 1-naphthaldehyde, dihexyl ketone	^{13}C NMR	22
8-ketohexadecyltrimethylammonium bromide	''	22
ω-keto dodecanoates	''	23
(+) trans-2-chloro-5-methyl cyclohexanone; 17α-hydroxy-5α-pregnan 20-one	ORD	6c
Steroid enones	reduction by aq. BH_4^-	6d

segmental protrusions as suggested by Aniansson -- of surfactant monomers in the micelles? In this chapter we describe some experiments that attempt to provide answers to some of these questions. First, we have looked at the issue of the sites of solubilization of some representative aromatic optical water penetration probes by an NMR method. Second, we have measured the interfacial activity of some ketone probes by tensiometry and NMR methods and located their solubilization sites in micellar aggregates. Based on these results an alternate explanation, to that of Menger and Bonicamp[6d], for the chemical reduction of micelle solubilized steroid enones by aqueous phase $NaBH_4$ has been given.

3.3 EXPERIMENTAL SECTION

The surfactants used were cetyltrimethylammonium bromide (CTAB), dodecyltrimethylammonium bromide (DTAB), sodium dodecyl sulphate (SDS), sodium dodecyl benzene sulphonate (SDBS), cetyl pyridinium chloride (CPC), polyethylene glycol (20) cetyl ether (Brij 58) and octyl glucoside (OG) and their purity checked and found satisfactory. Acridine was purified by sublimation under reduced pressure. 1-Methyl indole was prepared from indole by methylation and purified by chromatography. Benzophenone, testosterone and 4-cholestene-3-one were purified by recrystallization. All other reagents were of best analytical grade available and used as such.

The NMR spectra were recorded using JEOL (FX 90, 100 and 270 MHz) and Bruker (WH 270 MHz) spectrometers at ambient probe temperature (30°C). The aromatic probes were solubilized at various concentrations (6,9, 12 and 15 mM) in micelles of the detergents (concentration 50 mM) in D_2O for recording proton NMR spectra. Interfacial tensions were measured using a White model OS torsion balance with a platinum ring of 4 cm circumference. All measurements were done at 24 ± 1°C, in CCl_4:H_2O interfaces - the solute concentrations in CCl_4 were sufficiently low in order to avoid interfacial crystallization.

The borohydride reduction of the ketonic probes was performed similar to the method of Menger and Bonicamp. Fresh solutions were prepared for each run and $NaBH_4$ was stabilized in 0.1 M NaOH. The detergent concentrations were 50 mM for CTAB and SDS and 10 mM for OG. For experiments in water medium, a saturated solution of the enone was prepared and diluted to a suitable value of absorbance at 250 nm (OD around 0.4). The absorbance changes at 250 nm were recorded using Beckman 26 or 5260 model UV-Vis recording spectrophotometers.

3.4 RESULTS AND DISCUSSIONS

3.4.1 Solubilization sites of aromatic optical probes:

The method used to establish the sites of solubilization was basically the same as adopted earlier[12-16], wherein the ring-current induced shielding and deshielding effects produced by the added aromatic probe on the NMR signal of the protons in various segments of the surfactant molecules is followed in the micellar phase. Acridine, which has been used as a water penetration probe by Wolff[18], and 1-methyl indole which has been used by Turro et al.[19] for a similar purpose were used as representative solubilizates in cationic (CTAB, DTAB), anionic (SDS) and nonionic (Brij 58, OG) micelles. Benzophenone was also studied for comparison. The results of the study are as follows.

The signals due to the α-, β-CH_2 and ω-CH_3(and N-CH_3) protons in the various surfactants are easily assigned. Excepting the case of OG, further resolution of the γ-CH_2

(assignment following Zachariasse[25]) and two other CH_2 groups down the chain (as yet unassigned) has also been possible. The interior ω-CH_2 protons resolve as a composite sharp manifold. Figure 3.1 summarizes the probe-induced changes in the chemical shifts of these specific segments of the surfactants in four different micelles. Such changes are attributable to the ring-current induced shielding (and deshielding) effects of the aromatic solubilizates on the surfactant protons. With acridine and benzophenone, such an effect is maximum for the CH_2 proton that are γ- to the headgroup in the ionic micelles. The effect follows the order γ- > β- > α- > ω-CH_2. With 1-methyl indole as the probe, however, the maximal shielding is experienced by the β-CH_2 groups in the same micelles. In the case of the nonionic micelle Brij 58, maximal perturbations were seen on the signals of the α-CH_2 group of the cetyl chain that is directly attached to the polyoxyethylene segment. (Results that we have obtained with these probes dissolved in the other nonionic micelle of OG requires definite assignment of the signals to the various protons before further analysis and interpretation can be done.)

In all these cases, the effects observed on the ω-CH_2 and ω-CH_3 signals were smaller than those on the

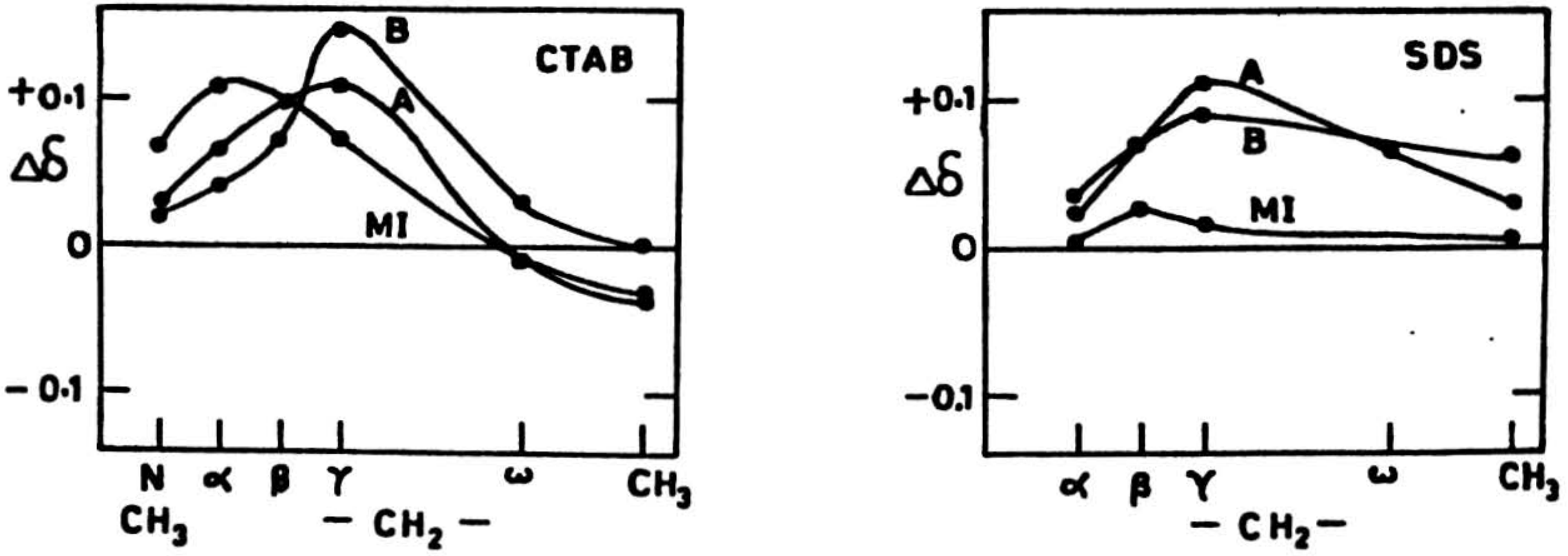

Figure 3.1a Shifts in the NMR signals of various surfactant protons caused by the ring-current effects of the added probe. Δδ, in ppm, is the difference between the chemical shift value of the same set of protons with and without the added probe. '+' refers to an upfield shift and '-' to a downfield shift. The abscisa shows the assignment of the signals to the various protons of the surfactant chain. The probes are A = acridine, B = benzophenone, and MI = 1-methyl indole. CTAB = cetyltrimethylammonium bromide and SDS = sodium dodecyl sulphate.

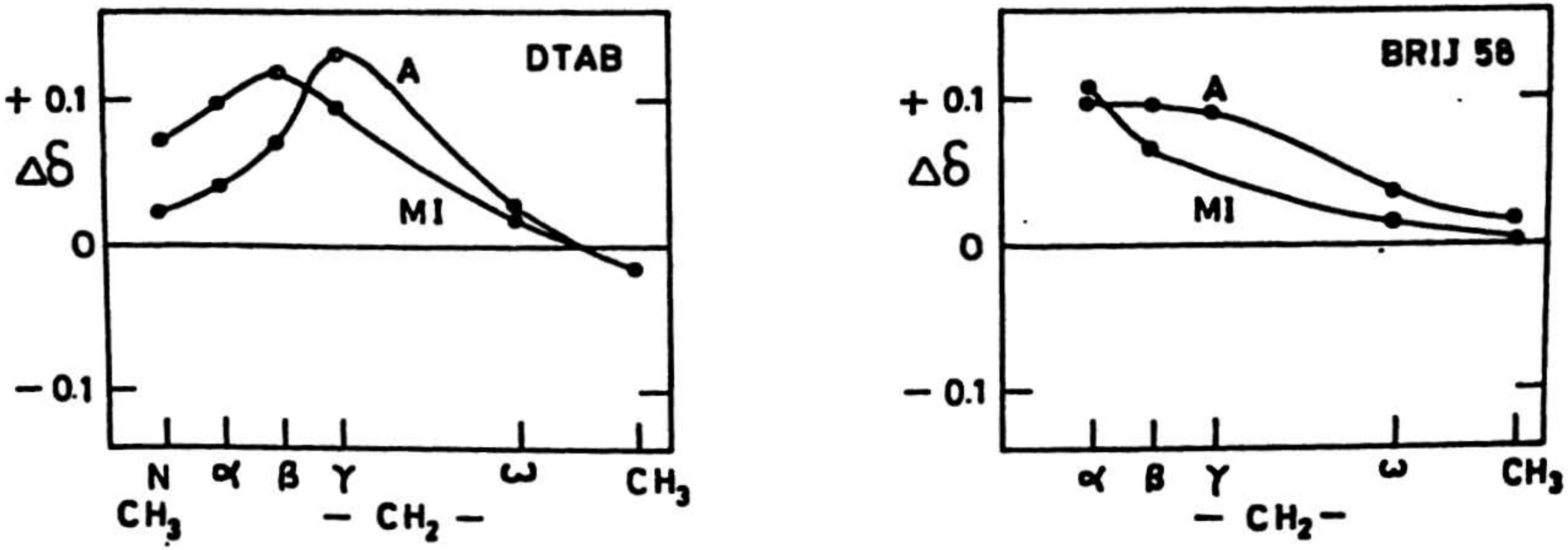

Figure 3.1b Probe induced changes in the NMR chemical shifts, $\Delta\delta$ in ppm, of the various protons in the hydrocarbon tails of dodecyltrimethylammonium bromide (DTAB) and polyethylene glycol (20) cetyl ether (Brij 58) micelles. A = acridine and MI = 1-methyl indole; '+' refers to upfield shift and '-' refers to downfield shift. (For further details see legend of Figure 3.1a).

other protons. Also, with acridine and 1-methyl indole as solubilizates in CTAB micelles, these signals showed downfield shifts, indicating deshielding effects (Gratzel <u>et al</u>.[14] had observed similar deshielding of these protons with pyrene solubilized in CTAB micelles). In DTAB micelles, these probes induced downfield shifts of the ω-CH_3 signals as in CTAB, but small upfield shifts of the ω-CH_2 protons in contrast.

It was also thought desirable to study the variations in such probe-induced effects as a function of the probe/detergent mole ratio, since some reports[10,12] have suggested that at high mole ratios, the solubilized molecules might move (translocate) from the headgroup region of the micelle to the interior. The representative results shown in Figure 3.2 reveal that as this mole ratio is increased from 1:10 to 1:3, the induced effects on all the signals display a monotonic undramatic variation. In addition, however, in the particular case of the ω-CH_2 signals, the peak profile was found to alter with an increase in the probe mole ratio. The ω-CH_2 manifold, discernible separately as a 20-proton peak and 4-proton peak in the pure CTAB micelle, underwent a splitting of the 20-proton signal into further subgroups upon the addition of the probe; such an alteration of this peak

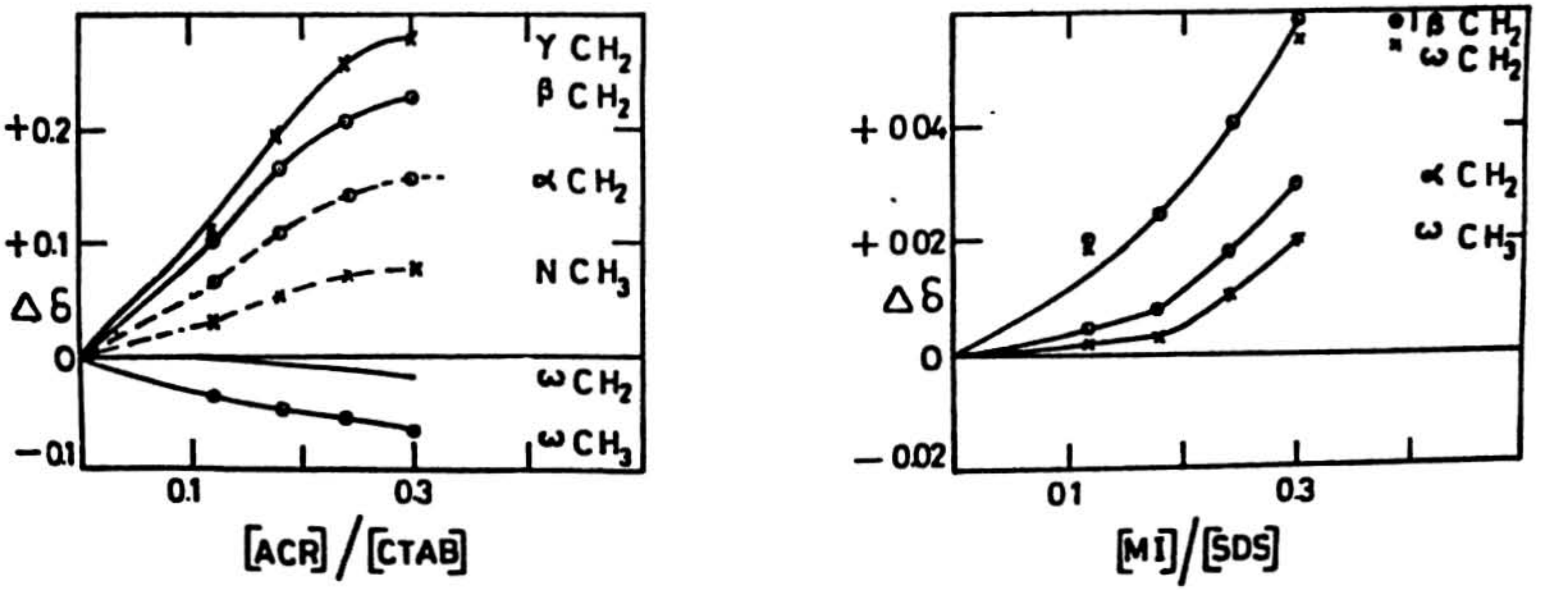

Figure 3.2 Variations in the chemical shift difference, $\Delta\delta$ in ppm, of the various surfactant protons with the increase in probe:detergent mole ratio. '+' indicates upfield shift and '−' indicates downfield shift. ACR = acridine and MI = 1-methyl indole. The surfactants are: cetyltrimethylammonium bromide (CTAB) and sodium dodecyl sulphate (SDS).

profile has been seen earlier in CTAB micelles with methyl naphthalene as the solubilizate[15].

The results that we have obtained are consistent with the interpretation that all the probes studied here are located in the headgroup region of the micelles of CTAB, DTAB and SDS. In the case of Brij 58 micelles, the probes are positioned around the α-CH_2 group of what is referred to as the palisade region -- largely at the junction of the polyoxyethylene segment and the cetyl chain. In all the cases, the probes induce only a shift of the existing peaks and do not give rise to multiple signals for the same set of protons. This would suggest that the probe exchanges its location between the headgroup region and the interior at a rate faster than the NMR time scale, yet with the average residence time weighted in favour of the headgroup region; or that the surfactant monomers in the micelles exhibit differential protrusions into the bulk water phase, as suggested by Aniansson[17], which results in differential exposure of the various surfactant segments to the ring-current effects of the probe. The present results cannot distinguish between these possible processes. Thus the relatively larger shielding effects seen on the ω-CH_2 and ω-CH_3 protons of SDS and Brij 58 micelles might suggest either a greater mobility of the probes within, and/or more facile protrusions of the monomers in these micelles than in those of CTAB, DTAB or OG.

The fact that these induced effects do not display any notable changes upon increasing the probe/surfactant mole ratio (from 1:10 to 1:3) suggests that there is no perceptible translocation of the solubilized species in this concentration range. Eriksson and Gillberg[12] had found such probe translocations at a probe/detergent mole ratio of 1:1, and the data of Ulmius et al.[15] suggest a possible movement of the probe 1-methyl naphthalene from the headgroup region into the interior at mole ratios around 1:1. At such high concentrations, the possibility of the added probe perturbing the structure of the surfactant micelle exists; the changes in the peak profile and the splitting of the ω-CH_2 signals seen at high probe mole ratios has been thought to reflect probe orientation effects and the microdynamics of the aggregate[15]; we have seen similar effects in the present systems at high probe mole ratios (vide supra). It thus appears safer to work with as low a probe concentration as possible in such NMR experiments in order to minimize any perturbations in the micellar structure, and we have thus restricted most of our studies to the (presumably safer) probe/surfactant mole ratio of 1:8.

3.4.2 Interfacial activity of ketone probes:

Figure 3.3 shows the effects of several ketones on the interfacial tension between water and CCl_4 and the results indicate that they are indeed amphipathic. While this was expected of the aromatic compounds studied, in light of the earlier results on benzene itself[10], it is interesting to observe that testosterone and 4-cholestene-3-one are surface active as well, with magnitudes similar to those of the biomembrane component, cholesterol. (While our measurements on several different recrystallized samples of 4-cholestene-3-one show it to be even more surface active than testosterone, HPLC analysis of these samples revealed minor amounts of a close moving contaminant that was not separable despite repeated efforts. Hence the data are not shown in Figure 3.3. This problem was not encountered with other compounds studied; they could be purified easily.) This interfacial activity of such alicyclic enones is likely to originate in their more favourable interaction with water than with the nonpolar organic solvent; such activities would suggest that a significant proportion of these probes might position themselves at the interfacial zones of the micelles in which they are solubilized. That benzophenone is largely located in the headgroup region of micelles has been shown

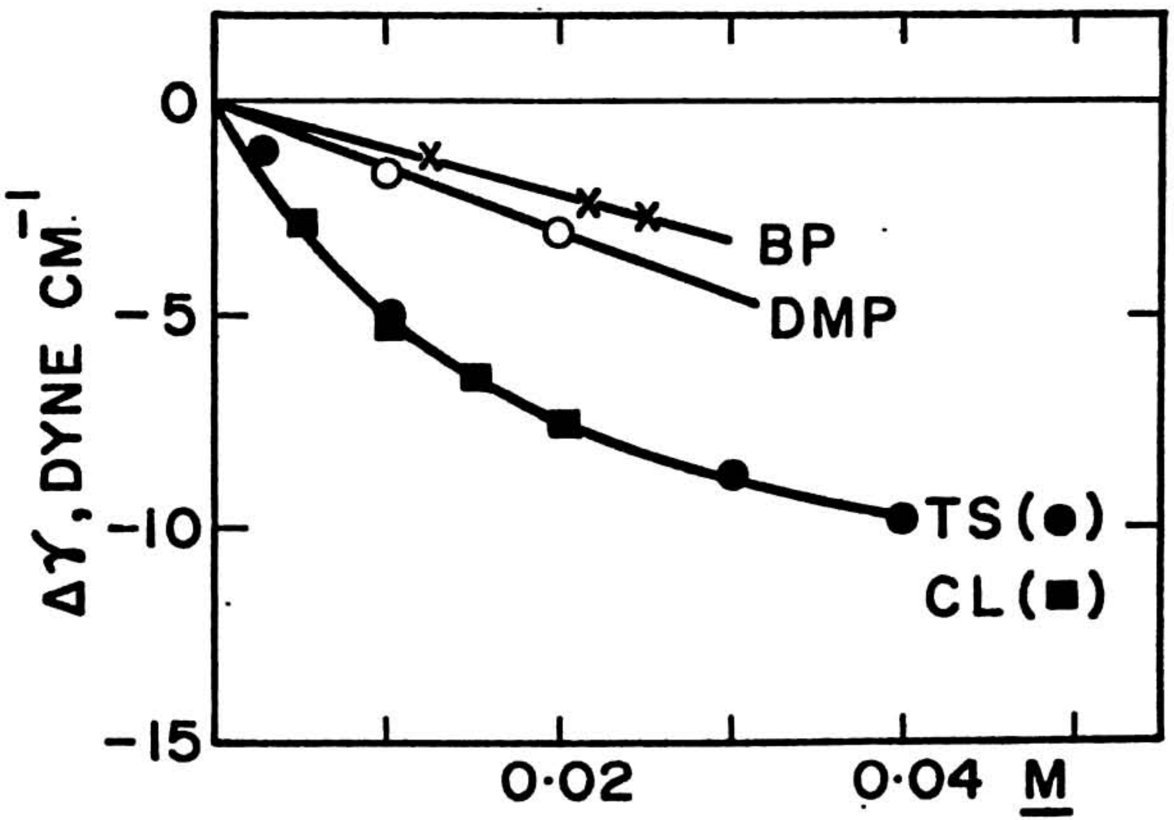

Figure 3.3 The interfacial tension lowering effects of benzophenone (BP), o-dimethyl phthalate (DMP), testosterone (TS) and cholesterol (CL) at the CCl_4:H_2O interface.

earlier (vide supra)[24]. McBain and McHan[26] had concluded several years ago that dimethyl phthalate is located similarly.

3.4.3 Polarity sensed by the keto moiety:

The results of the previous section suggest that when water is added to a solution of a compound like testosterone in a water-immiscible solvent such as CCl_4 or hexane, a measurable fraction of the solute should accumulate at the water:oil interface. In an effort to test this prediction, we have used ^{13}C NMR spectroscopy; use is made here of the polarity-dependent variation of the chemical shift of the carbonyl carbon nucleus[22,23]. First, the keto carbon chemical shifts of the probe were measured in solvents of varying polarities (E_T values), and the variation was as anticipated. Then to a solution of the probe in an organic solvent pair (CCl_4:hexane) of specific gravity unity, water was added and the mixture agitated in a vortex mixer to produce isodense buoyant microdroplets (polydisperse with diameters in the millimeter to submillimeter range), and the chemical shift alteration measured. This procedure was adopted in order that the oil:water interfacial area be large and the interfacial population of the probe molecules be enhanced, if possible[10]. The difference in the chemical shift of the keto carbon of the probe before and after the dispersion of water would then be a measure of the polarity that the probe experiences at the oil:water interface. Such an experiment on testosterone produced a downfield shift of 0.45 ppm in the keto carbon chemical shift in the microdroplets medium, compared to the bulk solvent value.

The observations of such a downfield shift in the microdroplets system is consistent with the interpretation that testosterone accumulates significantly at the oil:water interface and experiences the water environment. Clearly, the observed signal is an average of the contributions from the ketone dissolved in the bulk oil drops and from the fraction that is positioned at the interface. However, the alternate possibility exists of the ketone enhancing the solubility of water in the organic medium and thereby sensing a greater polarity. This possibility has been discounted on the basis of the following experiment. A small amount of CCl_4 was added to the vortexed mixture to increase the specific gravity of the organic phase slightly and the system allowed to phase separate. The amount of water present in the organic phase was determined by NMR (intensity of the water proton signal). This was found to be essentially the same as the amount of water solubilized by the same organic solvent mixture in the absence of any dissolved testosterone.

In an effort to enhance the surface area/volume ratio of the solubilizing system, we next solubilized testosterone in an oil-in-water microemulsion[27] containing potassium oleate, cyclohexanol, benzene, and water (weight percentages of 11.4, 26.1, 8.0 and 54.5 respectively). Such microemulsions are spherical assemblies of radii <u>ca</u>.10-60 nm, and should thus provide a larger surface area per unit volume than the microdroplets mentioned above. The keto carbon chemical shift of testosterone solubilized in this microemulsion system was measured to be 201.61 ppm, a downfield shift of about 5 ppm from the bulk CCl_4 value, suggesting that the probe experiences a more polar environment in this medium. Next, when testosterone was solubilized in systems of even smaller dimensions, i.e. micelles of CTAB and SDS, the keto carbon chemical shifts of the probe were measured to be 202.35 ppm and 203.45 ppm (downfield shifts of over 5.6 ppm and 6.7 ppm from bulk CCl_4 values) respectively. The sharp increase in the downfield shift of the keto signal upon going from the microdroplet system to the oil-in water microemulsion and the micellar phases is consistent with the concomitant large increase in the surface area to volume ratios (= 3/r, where r is the radius of the spherical assembly, and which, when expressed in units of cm^2 cm^{-3}, is of the order of 30-300 in the droplets, around 10^6 in microemulsions and around 10^7 in micelles), and consistent with the results and predictions of Mukerjee and Cardinal[10] that an enhancement in the surface-site population is expected when mildly polar and surface-active compounds are housed in assemblies of nanometer dimensions.

3.4.4 Solubilization site of enone probe:

We have also been able to establish the actual site of location of testosterone in micelles using variation of the NMR method used for aromatic optical probes. In the present case, since the probe molecule used is not aromatic in nature, the approach adopted was to compare the proton NMR chemical shifts of the same probe molecule when it is solubilized in micelles of surfactants with and without aromatic headgroups; e.g. the pairs CPC and CTAB, and SDBS and SDS. The assumption made here is that the environment of the solubilizate in each of the pairs of micelles is essentially the same, excepting that the probe would experience the ring-current shielding effect of the aromatic headgroup in CPC (but not in CTAB) and in SDBS (and not in SDS), if it were to be largely positioned in the headgroup region of the micelles. If the probe were to be located in the micellar interior, one would not expect the surfactant ring-current effect to be felt by the probe. When such comparative experiments were done under otherwise identical conditions, the chemical shift of the H-4 proton of the A ring of probe molecule testosterone was found to be upfield shifted by 0.07 ppm in CPC micelles when compared to the value obtained when it was solubilized in CTAB micelles. Similarly the same proton signal was found to be upfield shifted by 0.22 ppm in SDBS micelles when compared to the value in SDS micelles. (The H-4 proton was chosen because its signal could be unambiguously assigned from among the multitude of peaks due to the aliphatic protons of the probe and of the surfactant molecules in the sample, and also the alterations seen in the other relevant proton signals were far smaller). With the assumptions held valid, these results would indicate that the A ring part of the solubilized sterone is located largely in close proximity to the aromatic headgroup regions of CPC and SDBS, and hence in the same regions of CTAB and SDS as well.

Testosterone contains two polar moieties, the enone of the A ring and the hydroxyl group of the D ring. Considering this feature and also the dimensions of the whole molecule, it is possible that when the molecule locates itself in the headgroup region of the host micelle, both the keto and the hydroxyl ends are near the polar head regions of the aggregate; in other words, testosterone might be inserted transverse to the surfactant chain direction rather than longitudinally. This possibility, which would be similar to that of bile salt molecules such as deoxycholate, has not been tested as yet.

3.4.5 Reactions of the enones in the micellar phase:

Menger and Bonicamp[6d] had found the rate of the chemical reduction of CTAB-micelle-solubilized steroid enones by aqueous phase $NaBH_4$ to be comparable to the rate of the same reaction in homogeneous aqueous phase. This was used by them as an evidence for the porous cluster model of the micelle, with the suggestion that the enone is housed in the 'pores and fatty patches' of the cluster. Our finding that testosterone is located in the headgroup region of micelles provides an alternate interpretation of their results. We have reinvestigated this reaction with the enones solubilized in several types of micelles and also in aqueous solution, and typical results on testosterone are given in Table 3.2. The second order rate constants of the borohydride reduction of testosterone were found to be 0.010 $M^{-1}s^{-1}$ in water, about the same in octyl glucoside micelles, and significantly higher in CTAB micelles. The reduction did not occur perceptibly at all when the enone was solubilized in SDS micelles. These results are basically similar to those obtained on the reaction of hydroxide with triphenylmethane dyes[28] in that: (i) inhibition of the reaction occurs in anionic micelles, while enhancement is seen in cationic micelles; this is consistent with the interpretation that the approach of the attacking anion is inhibited by the headgroups of SDS and aided by those of the cationic CTAB micelles, an electrostatic catalytic feature; (ii) no significant enhancement of the reaction occurs in nonionic micelles in comparison to the reaction in water or in methanol; and catalysis in the nonionic micelle depends on the anion concentration suggesting that the anion may be distributed differently in the bulk and micellar phases, and (iii) the triphenylmethane dye, being aromatic, would be located in the headgroup region of the micelles (vide supra)[28], just as testosterone is shown to be.

Table 3.2 Borohydride reduction of enones in micelles and in homogeneous solutions

Enone	Medium	$NaBH_4$	k_2, $M^{-1}s^{-1}$
Testosterone	Methanol	60 mM	0.009
	Water	60 mM	0.010
	OG micelles	60 mM	0.014
	CTAB micelles	20 mM	0.253
	SDS micelles	60 mM	-*
Benzophenone	Water	60 mM	0.105
	CTAB micelles	20 mM	0.562
	SDS micelles	60 mM	-*

<u>Note</u>: Second order rate constants k_2 were calculated from pseudo first-order runs and rate constants (k_1) as $k_2 = k_1/[NaBH_4]$. k_2 values are averages of several experiments and are ± 5%. Temperature 24 ± 1°C.

*In SDS micelles, no perceptible reduction was observed even at higher $NaBH_4$ concentrations.

These results are consistent with the observation that the enone is largely located in the headgroup region of the host micelle, accessible to the borohydride ions of the bulk phase and that the differences seen in the reaction rates in different micelles are explainable in terms of electrostatic enhancement or inhibition in ionic micelles, and the concentration of the borohydride ion in the nonionic micellar phase.

3.5 CONCLUSIONS

Based on the results described, we conclude that the 'water penetration probes' acridine[18] and 1-methyl indole[19] are preferentially positioned in the headgroup region of the various types of micelles in which they are solubilized; in Brij 58 micelles their location is at the palisade region. Hence these probes sense and monitor not the micellar interior but the headgroup: water interface. In light of these, and earlier results on benzenoid aromatic solubilizates, it would appear that all such probes listed in Table 3.1 are similarly located and report on the polarity and water content of the headgroup region of the host micelles.

The preferential location of such solubilizates is consistent with the idea that even a mild surface activity of a solubilizate gets highly amplified in hosts of micellar (or membrane) dimensions. We have also shown that ketones such as benzophenone, dimethylphthalate, testosterone and 4-cholestene-3-one are inherently surface active. Further the effect of this mild surface activity is enhanced when the interfacial area is increased upon reducing the size of the solvent phase from bulk to micro-droplet and to micellar dimensions.

Since the predominant site of location of these ketones in micelles is the headgroup region, the polarity felt by the probes and their ready reduction in the micelle solubilized state, by the bulk phase borohydride, are understandable. These results therefore do not reflect the extent of water penetration inside micelles.

The fact that only a shift of the existing NMR signal is observed, and not multiple signals, indicates that the probe exchanges its location between the head-group region and the interior at a rate faster than the NMR time scale, yet with the head group region weighted proportionately larger, or that the surfactant monomers in the micelles exhibit differential temporal protrusions into the bulk phase[17]; the present experiments cannot distinguish between the two possibilities.

Also, it is not clear from the present results whether water penetrates upto the γ-CH_2 of micelles such as CTAB or DTAB or whether the topography of the ring-current effected shielding domains of such bicyclic and tricyclic aromatic compounds is such that maximal effects are observed for the γ- and β-CH_2 groups even if the probe is positioned at the Stern layer.

In summary, the results presented in this chapter underscore the necessity of establishing the actual site of location of a probe molecule before the results obtained using it can be interpreted in favour of one model of a micelle or the other. Certainly, the support for the 'porous cluster model' for micelles, using the aromatic and ketonic probes described in this chapter, is not unequivocal.

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VITAE

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