Chemical basis of ordinary aqueous mixtures.
Chapter 5, Volume 2 of the Protein Primer Lumry 2-10-2
There are two versions of this chapter and lacking time and energy to edit them together I am sending them together. Never get done otherwise. 9-21-2006

This chapter has several immediate objectives all depending on enhanced understanding of "hydrophobic hydration". The first is to explain the properties of water mixtures such as those with ethylene-glycol. A second is to explain those properties in terms of structure and thermodynamics of aqueous mixtures, in general, as a consequence of the two species of liquid water. These considerations lead to a better description of hydrophobic hydration and the similarly so-called hydrophobic effect both descriptions both descriptions lacking veracity despite recent contributions from Koga and associates on aqueous mixtures. With the results such as those of Lüscher and Teeter and their respective coworkers to supplement hydration data for proteins it becomes possible to extract the details of protein structure that have evolved to support enzymic catalysis. That evolution is described in chapter 9 of this volume..

Thermodynamic changes in chemical reactions of small molecules are usually stoichiometric because they include complete exchange of atoms. Exceptions are common only in the associated rate processes because the thermal activation on which they depend varies to greater or lesser degree with efficiency of neighboring groups as thermal reservoirs. The processes of biology are quite different because thermal activation has proved in evolution to be an inadequate device for accelerating chemical reactions and an insensitive determinant of the delicate specificity exemplified in enzymic catalysis. The only promising device from familiar small-

molecule chemistry is "transition-state stabilization" suggested by Pauling but that has not proved to be adequate to provide the well-established rates and specificities of enzymes probably because it does not include conformation change as an essential feature.. Discovery of electronic tricks special to proteins has been the other popular goal for enzyme research but thus far has also proved to be more a source of desperation than success. By exclusion these failures leave only provision of mobile potential energy as the basis of the successful evolution of life. Conformation change is necessary to enable rate activation by potential-energy transfer rather than heat That alternative examined in detail with many families of enzymes reveals a common mechanical mechanism once called a "rack mechanism" but now after Carloni's suggestion more appropriately called "the nutcracker". The rate and specificity of an enzyme-catalyzed process depends on the extent to which the nutcracker closes when triggered by a substrate. Closure is no more than coordinated contraction of the two large matrix substructures of enzyme proteins during which potential energy of the matrices is transferred reversibly to the reaction assembly of substrate with its chemically participating contact groups from the protein. The catalyzed process may or may not involve chemical change as defined in small-molecule processes but the ubiquity of enzyme construction requires a single mechanism with speed and specificity dependent on the degree of matrix contraction along the matrix contraction-expansion coordinate in each enzyme. Exact stoichiometry is not a feature of these rate processes nor is it an exact feature of the overall change in the process catalyzed. The protein conformation as a non-obligatory participant in the process provides potential energy, entropy and thus free energy by linkage among the participating subprocesses. That flexibility makes it possible for a single enzyme to provide a wide range of nutcracker specificity for substrates and inhibitors by small conformational adjustments. Biology has little in common with the rate processes of small-molecule chemistry so the latter are a poor guide to understanding the former.

Protein chemistry will begin to become scientific only when it is realized that evolution has found better ways to effect chemical change than have so far been found by the most brilliant chemists. Its Darwinian nature has made biology possible and in so doing has completely changed the game.

The variability of free-energy management in linkage systems is sufficiently small to allow treatment by extrathermodynamic model to provide a non-stoichiometric framework usually called a "compensation" mechanism. That is, the variability and range of change in protein linkage systems is usually adequately approximated by a linear relationship between enthalpy and entropy changes. Conventional mass-action relationships would impose constraints that evolution has had to find ways to avoid. Compensation behavior is simple to illustrate with protein machines but even more simply so with the interactions of water and small solutes underlying the Hofmeister series of relative solubilities in water, one end of which lists *structure breakers* and the other the *structure makers* including: amphiphiles, hydrophobes, ions and polymers. It is shown in this discussion that with the use of such data the Hofmeister series can be made quantitative. One of the most familiar and most accurately linear examples of enthalpy-entropy compensation behavior is that of the non-branched alkanols. The plot of standard enthalpy changes against standard entropy changes, called "a compensation plot" with slope called the "compensation temperature", has its antecedents in the "Linear free energy plots" of Hammett and Brönsted. A typical example is the transfer of an alkanol as a representative amphiphile from a suitable reference state such as pure liquid alkanol or alkanol vapor to liquid water. Using liquid alkanol as standard state the slope of the compensation plot for the linear alkanols is 354K. Branched alknanols fall only slightly off the line. The plot was explained by Lumry and Rajender (R. Lumry and S. Rajender, Biopolymers 9, 174 1970)) as a consequence of the existence of two chemical species of pure water, a popular guess at that time although that unique characteristic had not

been established nor had the clathrate description of amphiphile-water complexes

proposed by Glew. As detailed in chapter 4, volume 2, structure-making solutes all at

the sulfate-ion end of the Hofmeister have strong (low formation free energy) cages

such as the pentagonal dodecahedra for methanol, ethanol, methane, probably argon

and. as well the recently established twenty-water molecule cages for sulfate ion and

the protonated water molecule.. According to the two-state model water has two

chemical species of which the lower-density structure L is itself a small highly

cooperative cluster from which the clathrate cages are formed rather than from the

higher-density H form predominate at room and higher temperatures. Formation of a

cage depletes the amount of L water immediately rebalanced by conversion of H to L

water as required by the equal chemical potentials of these two pure-water species.

The slope of the line is then the temperature at which the standard chemical

potentials of the two are equal. The exceptionally high constant-pressure heat capacity

due to the pure water equilibrium requires small corrections in the equivalence

temperature but the common appearance of the compensation temperature as 285K

seems to be fully explained by the equilibrium between the two (and only two)

chemical species of water. Complications can appear with additional hydration

equilibria of one or more solutes .

    This "chemical hydration" of argon, ethanol, ethylene glycol and by analogy

molecules of similar chemical character and small size have on average 10-mer cages

reduced by hydroxyl or amino groups of the solute that displace water molecules in

the cage. There are rapid fluctuations from 0 to 20 since the lifetime of the L species

itself is only slightly longer than 0.5 ps at 285K and the clathrate at least an order of

magnitude slower. Assuming argon requires a single pentagonal dodecahedron cage

the changes for the cage alone based on the further assumption that argon has no

cage in pure hydrazine are -4.9 kcal and -15.3 cal/MK per mole of cage, that is 10

water molecules but at shown below the bound water molecules are in equilibrium

with free so the total number of participating water molecules is closer to 20, neither

very large nor very small. It is not surprising larger amphiphile solutions such as 30%

glycerol, 1% PEG and concentrated sulfuric acid have such unusual physical

properties entirely unlike pure water.. Sulfuric acid mixtures contain sulfate, bisulfate

and protons all of which are major structure makers. Similarly amino alkanes have the

amino groups and the aliphatic groups both structure makers so primary amines are

more effective structure makers than the corresponding alkanols. Ionization of amino

compounds and carboxylic acids produce major changes in many water molecules

contributing to the peculiar thermodynamic changes (cf. supra)

Structure makers even in low mole fraction use up most normal water and

become dominated by the extent of clathrate formation and the clathrate relaxation

behavior. Solutes of the clathrate variety compete for water and are coupled to each

other as a consequence. The latter well-known "hydrophobic effect" is a

manifestation of this competition and cooperativity. It knits all solutes including

proteins in a given aqueous mixture into a single linkage system probably sufficiently

strongly to be of major importance and certainly a hazard in trying to interpret

thermodynamic data until correct models become established.

Means sizes for many solutes can be determined from simple solution data

using compensation analysis and the empirical compensation temperatures provide

detailed information not available from conventional mass-action-law analysis

(described by Lumry, Biophysical Chemistry 2002)..A classic tool for this purpose is

the paper by Wen and Hung (JPC, 74, 170(1970) reporting solubility data for two

amphiphiles or hydrophobes as cosolutes in each mixture holding the member of one

series constant while varying concentrations of a member of the second series and

vice versa for both congener series. The reference state for all is the second solute in

pure water. They used the solubility apparatus developed by Ben-Naim and Baer

which has precision and accuracy rivaled for gas-solubility studies only by the mass-

spectrometry equipment invented by Benson and Krause and modern calorimetry as perfected by Brandts et al. When the standard enthalpy and entropy changes are computed all such combinations were found to give precise compensation lines with slopes of 285K. There series were compared in this way: alkanes up to C-4, alkanols and tetraalkyammonium salts up to tetrabutylammonium bromide. These elaborations on the alkanol studies discussed above provide quantitative comparisons of the competition among solutes for water to form clathrate cages. Simple theory provides the mean clathrate cage size which in turn with reasonable assumptions can be converted to mean cage size for each member or each series. The reasoning is the same as for the simple alkanol solutions showing that the single compensation pattern is due to changes in the populations of pure-water species. Enthalpy, entropy and free energy changes per water molecule in each cage can be determined using data in their 1970 paper with an appropriate linkage mechanism from Wyman. .

The method can be extended to measure the extent of structure breaking using structure breakers for one series and, of course, the more complex behavior at high solute concentrations can be made quantitative. The distribution of the enthalpy and heat capacity change on mixing around the magic mole fraction becomes narrower as the size of the structure makers increases as expected for increasing size of the clathrate reflected by those data. The cooperativity of the clathrate rises with the average size and falls even more rapidly when the free water is used up also very cooperatively. Both the stability of the clathrate and the degree of cooperativity in its formation are so large it is surprising that neither have attracted any general attention. Vaslow suspected that behavior for the sulfate ion but did not complete the proof. Instead of partial clathrates the full clathrate is formed at very low sulfate concentration and the increments in enthalpy and heat-capacity changes per mole of bound water are less sensitivity to the degree of coverage. One result is a more abrupt change from the concentration range in which the average clathrate size is increasing

to that in which it is decreasing. The difference is qualitative in nature as well as quantitative making possible more accurate descriptions of the qualitative changes which will considerably improve the use of such high quality data as those of Huot, Battistel et al on the mixtures of water with ethylene glycol (Fig. @). A large fraction of the studies of the thermodynamic information from aqueous mixtures will have to be repeated and the necessary studies are complicated by the two-state equilibrium of water Although the proportions of the two states can be measured using the raman method of Walrafen or the infra-red method of Worley and Klotz, few useful data have been published.

The data of Wen and Hung as replotted in Lumry and Rajender revealed that the linked process responsible for the linear enthalpy-entropy compensation behavior is simply the two-state process of pure water. Although two structure-making solutes were used in each experiment, the total concentrations were too low to display the magic mole fractions of Arnett and McKelvey. Recent Koga et all have extended the solute concentration ranges up to and beyond the magic mole fractions thus generalizing the clathrate explanation of the magic mole fractions. Koga and coworkers also measured the second moments of the distribution functions of H and S as well as the entropy-volume covariance. However, their extensive interpretations do not include the linked water process nor the relationship between the magic mole fraction and the saturation size of the clathrates about their structure-making solutes. Their distribution functions for the first time demonstrate that the corporality in clathrate formation is high even for their largest solutes. That is consistent with the deduction from the ice clathrates that all first-shell water molecules form the clathrate structures, a deduction also made by many other investigators but the critical importance of clathrates with closed shells has not been obvious. These characteristics make reliable estimates of clathrate size and often geometry possible but those parameters are largely determined by the size of the solute, the size of its non-polar

parts and the size and distribution of the polar parts of the guests from total polarity

in sulfate and protonium ions to hydroxyl and carbonyl atoms of polyhydroxy solutes.

Franks and coworkers have illustrated the last using related families of polyols with

different arrangements of hydroxyl group, axial versus equatorial. Another worker @

(1983 Trans. Faraday but not yet found) has found that the partial molar volumes of

more than one hundred structure making amphiphiles can be estimated to 1% by

adding 0.05 Å to the mean van der Waals radii. Primary amines have been found by

others to be even more efficient structure makers. Koga et al attempt to relate

"hydrophobic hydration" to magic mole fraction without taking the specific detail and

limitations of the possible clathrate cavities into account. The following figures from

their papers illustrate their wealth of essential information to be found in their papers..
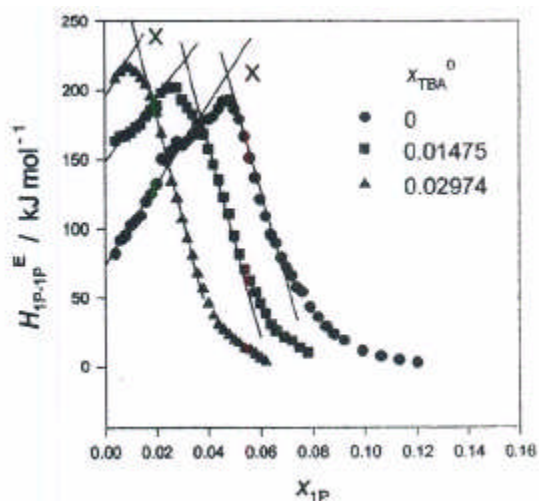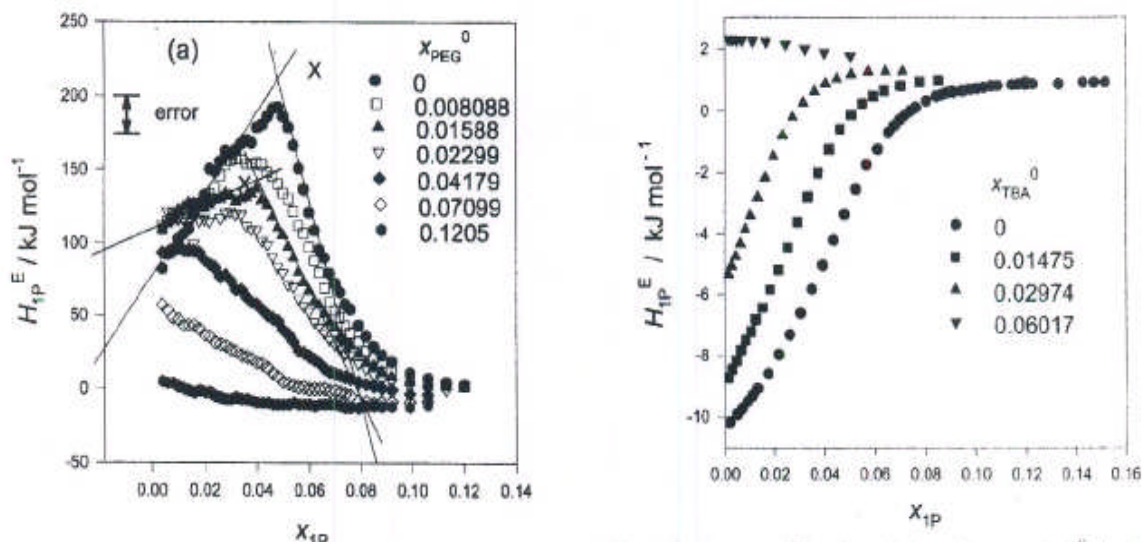
A new description of "hydrophobic hydration

Such data brings interest and research on water problems long moribund to a
new renaissance. Some results have been employed in the discussion of the
distribution functions of Huot et al given earlier in this chapter. The work of Koga
and associates has finally provided magic mole fractions for a range of "hydrophobic"
solutes sufficient to provide a new and surprising explanation for the concept of
"hydrophobic hydration" and as well the related "hydrophobic effect" both initially
popularized by Tanford and Kauzmann. As already explained in this chapter the
temperature at which the standard chemical potentials of the two water species are
equal in pure water is 285K and the enthalpy change per mole of cooperative unit, an
average of four and five water molecules, is 2.5 kcal determined with the use of
Frank's suggestion that hydrazine may be a good approximation for inhibited water,
that is, water without waters special solubility properties. What Lumry and Rajender
nicknamed a magic mole fraction is the mole fraction at which a solute in water has
maximum solution enthalpy, maximum heat capacity of solution and maximum
viscosity. Its most distinctive feature is that it is the largest value of the mean mole
fraction for a given structure-making solute in the series of its mixtures with water.
Arnett and coworkers discovered the magic mole fractions for methanol and several

other small amphiphiles in 1963 with heat and heat-capacity data and he magic mole

fraction name arises from the fact that each structure-making solute has a single

characteristic magic mole fraction but even in 1968 no obvious explanation for this

unique behavior was known. In the last few years Koga et al reported the magic

fractions of enough solutes to support a quantitative foundation with wide generality

and wide use in correcting current misunderstand of chemistry in aqueous mixtures.

They provide new insight into urea-water mixtures with unusual complications for

structure breakers but simple structure breakers like hydrazine and hydrogen peroxide

display no clathrate formation because they destroy the equivalence of hydrogen-bond

donators to acceptors unique to water thus eliminating the L water species.. At lower

mole fractions they destroy the clustering characteristic of that lower-density species

of water. At high concentration they also disrupt or at least greatly change the

"random-connectivity" description now thought to describe the higher-density

uniform behavior of H water. Water with hydrazine mole fractions above 0.25 is a

strongly associated liquid like pure hydrazine. In contrast the structure makers found

at the other end of the Hofmeister series are sometimes so effective that their full

structure-making capacity is expressed at concentrations so low as to have escaped

detection. As demonstrated above with the recast data of Wen and Hung, structure

making is detectable at very low solute mole fraction by a compensation temperature

near 285-290K That convenient feature makes separation of the thermodynamic

changes quantitatively as well as qualitatively. A complication arises when the linked

process such as the pH dependence on a protein process is passive which term means

it is poised at its own equilibrium point and thus does not contribute to the total free

energy change. Then its compensation temperature extracted by a properly designed

family of experiments is equal to or near the mean experimental temperature. These

uses and complications are discussed by: Lumry in Biophysical Chemistry (ref. here

@) and in Chapter 2. Vol. 2 of this monograph. The review in 1970 on enthalpy-

entropy compensation phenomena by Lumry and Rajender made a strong case of the two-state model of water as the explanation for many examples but that model was not established as correct until Walrafen's Raman studies in 1983 and the infra-red studies of Worley and Klotz a few years earlier. Then by repotting the data of Wen and Hung on solubility of pairs of amphiphiles the clathrate basis of such phenomena and thus the importance of L water in all "hydrophobic hydration" phenomena became established.

Lumry, Battistel and Jolicoeur proposed a cooperative cluster model for L water satisfying Benzinger's rule that chemical species to be different must have different electronic Hamiltonians.(@ L-H equilibrium species) That fact, a consequence of the revision in thermodynamics necessary to treat processes occurring at constant temperature, is not well known and as a result it is responsible for the many inadequate models of L water which postulate only rearrangement of hydrogen bonds without redistribution of valence electrons.

The many high-precision studies of aqueous mixtures suggested that the distribution functions for enthalpy and volume become possible with Picker's heat-capacity machine and Jolicoeur densimeter would add necessary detail to models for the L species, as also discussed by Jolicoeur. Despite their importance such studies are very rare. In fact that of Jolicoeur' students Huot and Battistel with contributions from several other coworkers measured the constant-pressure heat capacity, compressibility and thermal expansion coefficient for ethylene glycol mixtures has been unique until the work of Koga et al in the last few years although abundant data for ethanol-water mixtures have been accumulated item by item over many years.. Some results from Huot et al are shown in Fig. @. Thermal fluctuations at temperature equilibrium generate a distribution function of enthalpy or internal energy of which the second temperature derivative of the mean H or U measures the variance of H or U. In the same way compressibility provides an estimate of the

volume variance and there are covariance terms of the same order relating, for example, volume and enthalpy correlation. Jolicoeur with other coworkers studied in this way several other cosolvents arriving at a general description of the concentration behavior in terms of three qualitatively different concentration regions. Koga and coworkers have added many new amphiphilic solutes to the collection and often improved both precision and accuracy when their solutes form stronger though not larger clathrates. Ethylene glycol and ethanol have the same magic mole fractions but the former has much weaker clathrate formation because it is poorly accommodated in available clathrate holes. That is a consequence of the fact that ethylene glycol in one face is a structure maker and in the other a structure breaker. Glycerol has nearly the same magic mole fraction but forms a stronger clathrate because its hydroxyl groups fit into available holes to stimulate their formation. An extreme example of the latter is ammonium sulfate ion which depends entirely on favorable electrostatic interactions of sulfate ion in the pentagonal dodecahedron cavity to be the most efficient structure maker. The protonated water molecule also has twenty water molecules in its clathrate cage so acid and base chemistry has major thermodynamic complexity. Thus the minimum proton species water in normally dilute solution is the pentagonal dodecahedron with 20 water molecules rather than the popular Eigen-DeMaeyer proposal having only three water molecules.

Trehalose with two glucose rings in C-2 orientation appears to be a champion clathrate former in both size and stability. Because it sequesters so many water molecules, it has been sometimes suspected of having unique solute properties but the size of its clathrate cage rather than any special clathrate chemistry is probably its special feature. Even at lower concentrations is sequesters all the normal water molecules so like other even smaller clathrate formers it provides an efficiency way to reduce freezing temperatures and thus maintain non-thermostated organisms a sub-zero temperatures. That is not just the classical reduction of melting point by solute

but rather a consequence of chemical change. As already discussed, PEG because of

the chain structure produces a tubular clathrate shell with very unusual properties..

At low mole fractions the solutes sequester water in large clathrate structures

such as a full pentagonal dodecahedron for small solutes, argon, methane up to but

not quite including propanol requiring twenty water molecules per solute molecule.

Hvidt and coworkers showed that propanol requires a larger clathrate cage (cf Koga et

al , Fig. @). Large solutes sequester more, often many more water molecules and it is

the large numbers which quickly use up free water countering classical assumptions of

uniform, homogenous mixtures. The magic mole fraction for ethanol (Fig..@)

equivalent to only 11 water molecules is the average over 0 and 20 from no clathrate

to a complete pentagonal dodecahedron clathrate. At still higher mole fractions this

average value decreases as the full clathrate structures become less and less probable.

Temperature and guest size are important but especially guest size since the magic

mole fraction varies rapidly with guest size but little with temperature until the later

reaches 354K at which the entire picture reverses and the clathrates become

destabilized. It is not surprising that viscosity and enthalpy of mixing have maxima at

or very near the magic mole fraction but the heat capacity may not peak quite so

sharply because it arises from two equilibria. The first between the two chemically

different macro states of water a feature thus far not known to be demonstrated by

other pure liquids, and thought to be the random-connectivity higher-density species

and a small family of distorted tetrahedral clusters each form having some shorter and

some longer hydrogen bonds Fig. @ is illustrates that possibility. The second species

is held between guests in clathrate cages and guests in free-water cages. Frank and

Evans first proposed that the lower-density clusters are the building blocks for the

clathrates that Glew later suggested to be structurally the same as the ice clathrates.

The species to be chemically different must have different electronic Hamiltonians

(vide supra) and that requirement is satisfied by the electron rearrangements on the

oxygen atoms in the lower-density clusters. That condition for water clusters was

proposed much earlier by Frank and Wen under the rubric "Flickering clusters".

Lumry, Battistel and Jolicoeur called the lower to higher density, L↔H, process

"geometric relaxation" and the formation of clathrates for hydration of structure-

maker solutes "chemical hydration" because of the change in electronic Hamiltonian.

Angell et al showed association of the lower-density clusters on cooling into the

supercooled region toward a limit of -45C but apparently toward critical behavior

rather than that of a homogeneous solid.
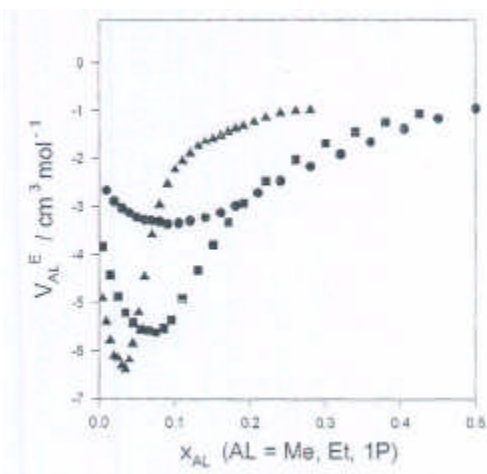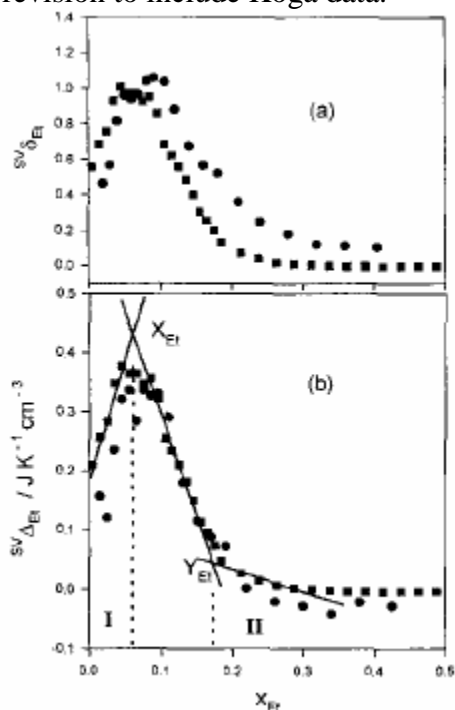
Fig. 8. Partial molar cross (entropy–volume) fluctuations of ethanol at 25°C. (a) $^{SV}\delta_{Et}$. (b) $^{SV}\Delta_{Et}$. Circle, $^{SV}\delta_{Et}$ or $^{SV}\Delta_{Et}$; square, H^E_{Et-Et}. X_{Et}, the onset of the transition from mixing scheme I to II; Y_{Et}, the end point of the transition for ethanol.
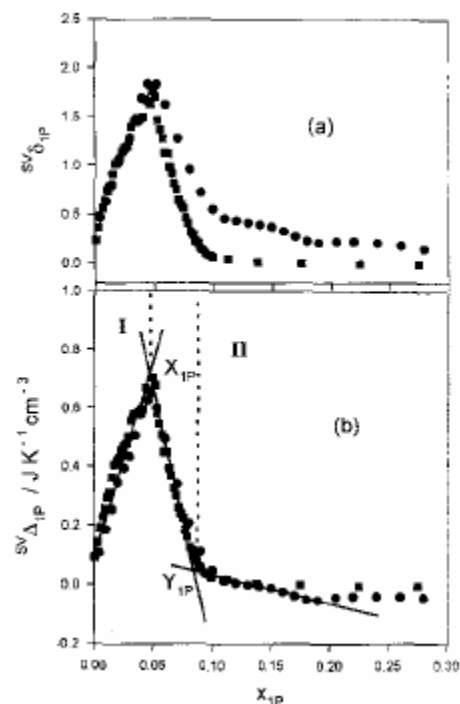
Fig. 9. Partial molar cross (entropy–volume) fluctuations of propan-1-ol at 25°C. (a) $^{SV}\delta_{1P}$. (b) $^{SV}\Delta_{1P}$. Circle, $^{SV}\delta_{1P}$ or $^{SV}\Delta_{1P}$; square, H^E_{1P-1P}. X_1, the onset of the transition from mixing scheme I to II; Y_{1P}, the end point of the transition for propan-1-ol.

These figures from Koga et al illustrate the use of the variances and covariances to determine the width of the distributions of volume, enthalpy and entropy and their covariances. What is plotted here are the square roots of those quantities. The abundance and excellence of the data published by Koga and coworkers makes possible detailed descriptions of the phenomena responsible for the magic mole fractions. Those workers distinguish three regions in which the dependence on composition is quite different. The same regions were found in the ethylene glycol-water data of Huot et al and in subsequent studies with different amphiphilic solutes by Jolicoeur and coworkers (insert ref to that fig here). Region I is that in which the clathrates are completed; region II in which they decompose toward progressively smaller average numbers of waters per molecule of amphiphile and region II in which the special features attributable to the solute disappear. Clathrate formation is seen to be very cooperative and very stable demonstrating the importance of the complete

clathrate shell. Judging from the ice clathrates only water molecules in the inner shell are involved, a deduction that has been made many times from other kinds of data but without full realization that the complete clathrate shells are required for maximum interaction with water.

Linear enthalpy-entropy compensation behavior in region I is due entirely to the two-state equilibrium of water(as demonstrated by Lumry and Rajender using the data of Wen and Hung). Compensation behavior in Region II is not sharp thus consistent with the variety of partial clathrates that describe this region but it does show some similarity with the knot palindromes found in region I. At the upper end of region III compensation behavior once again becomes sharp but the compensation temperature is well below 100K and Jolicoeur et al find the final state of water in this region is pairwise association.

The two-state equilibrium of water is the major coupled reaction for most processes studied in water and as Klotz showed in 1999, it is responsible for most of the high heat capacity of pure water, The deduction was based on older evidence from Worley and Klotz who used infra-red spectra to confirm Roentgen's proposal in 1892 that the unusual behavior of water is due to its two chemical species. Using depolarized Raleigh scattering Montrose et al (C.J. Montrose, J. Buscaro, J. Marshall-Coakley and T. Litovitz, J. *Chem. Phys.* 1974, **60**,5025; see also H. Endo, *Ibid*, 1982, **76,** 4578 for acoustic confirmation) found 0.6 ps at 293K in a Lorenzian peak accurately fitted by the two-state Arrhenius model with 2.4 kcal and 6 cal/K per mole of cooperative cluster. Recently similar times have been reported by Zewail et al mostly from their femto second relaxation-time data from protein solutions. The formation equilibrium in which building blocks associate to form the clathrates enclosing a guest relaxes at 20ps as noted above for the small clathrates of PEG and extend to at least 48ps for clathrates at protein surfaces (Zewail et al and Teeter et al) depending on temperature and guest size. These characteristic times are all short with respect to the

matrix relaxation times of enzymes which are about 1ns at 298K estimated from acoustic relaxation and fluorescence quenching. That is presumably the breathing time governing the rare of proton exchange at matrix sites. The maximum contraction at 298K is about 4%.

Argon forms clathrates in water but not in hydrazine so its enhanced solubility in water is due to the clathrates but the enthalpy of formation of its clathrate hydration is only 4.5 kcal per mole of argon (Fig, @) consistent with just two moles of the L clusters. That is not large when distributed over approximately ten water molecules but it is nevertheless the basis of hydrophobic hydration above 273K and enhanced solubility of argon and other structure-making solutes below. What appears to have been confusing is the instability of clathrate structures in the absence of a structure-making solute. Thus the L clusters associate to form the stable pentagonal dodecahedron only under the stabilizing influence of argon. There remains also a misunderstanding as thermodynamic basis of the enhancement of the solubility of permanent gases and amphiphiles originally arising from early suggestions such as that of Kauzmann in 1959 that that characteristic is due to the negative entropy change in hydration. Shinoda and Fujita@ and Lumry, Battistel and Jolicoeur showed that instead of entropy loss on insertion of a structure making guest such as argon the poor solubility is the result of enthalpy increase (Fig.@). It is the positive reaction-field enthalpy developed between non-polar guest and shell of water for non-polar groups at one extreme and at the other the favorable electrostatic interaction of clathrated water and charged species such as sulfate ion overcoming the otherwise poor reaction field. Thus the clathrates are stabilized for different reasons depending on the reaction fields. With argon, a typical example of poor solubility due to poor reaction field, the number of water molecules actually participating in its hydrophobic hydration is closer to 20 than 10 so the clathrate size fluctuates between zero and 20 with an average of 10. Just as the high heat capacity of pure water arises from its two-

species equilibrium, that of structure makers in water also very large, arises from the added clathrate-formation equilibrium. In the latter at mole fractions up to the magic mole fraction roughly half of the total water is free H or L explaining why the viscosities at the magic mole fractions though large are not larger. Although the physical characteristics the aqueous mixtures are very different, because the enthalpy and entropy changes in clathrate formation near 290K cancel in the free energy, the underlying differences are not easily detected from the free-energy changes alone. The differences become even more extreme above the magic mole fractions.

Hydrophobic hydration of ethanol is quantitatively similar to that of argon, methanol influences fewer water molecules. N-propanol requires a bit larger clathrate structure but still one on average half melted at the magic mole fraction (near 285K). Newer data for small alkanols and still larger amphiphiles demonstrate more dramatic behavior around the magic mole fraction making the high degree of cooperativity in clathrate formation more obvious. Highly charged proteins like the serum albumens at its isoionic point and hemoglobins at physiological pH values produce even more dramatic effects sufficient to make them useful stabilizers Above the magic mole fractions the average clathrate structures diminish in size as solute concentration increases and with it the structure making since there is little if any water to structure in that concentration range.

There is another way to rationalize the effects of the clathrates and thus to judge the importance of the clathrate relaxation. The reciprocal of the magic mole fraction is the number of water molecules per mole of solute and the latter can be computed from the concentration at which the magic mole fraction appears. The product of these two numbers is the number of moles of water per mole of solution associated with clathrate formation but the argument given just above shows that the number must be multiplied by two or the appropriate factor for the experimental

temperature to take account of the clathrate melting equilibrium. Except for

methanol, methane, helium and neon the total number is very close to the total

possible which per mole of solution is slightly less than 55 depending on the size of

the solute. This means clathrating uses up all the water and is a considerably more

stable formation process than has been thought. Most mixtures of water with

structure-forming solutes contain half free water and half clathrate-bound water. The

fractions changes only with temperature and pressure and as demonstrated in Chapter

4 using ethanol-water mixtures (Fig. repeated here), the clathrates become unstable at

345K. Recall also the vanishing Young's modulus at that temperature found with

proteins by Morozov and Morozov. The fully clathrate of ethanol was found to form

athermally at 354K (Fig. @) with ideal mixing entropy. The entropy may be difficult

to estimate in other clathrate forming processes and particularly in that occurring at

protein surfaces where the full cages are not possible. The cage cooperativity and

stability is much higher than has been appreciated but significantly less so in partial

cages and thus difficult to estimate. That limitation also applies to the enthalpy of

mixing so both will have to be put on a quantitative basis using such data as those of

Koga et al. The most useful aspect of the mixing studies is their athermal character

since that means that the enthalpy of conversion of H to L water to form the

clathrates is just canceled by the enthalpy of clathrate formation from L water at

354K. Spider dragline silks are stable at least to 500K and thus stable in the absence of

hydrophobic interaction. Many exotic organisms grow in the deep-sea vents but the

highest temperature at those vents is not much more than 350K.

A better explanation of hydrophobicity

The high clathrate stability is usually said to be due to the hydrophobicity of the

solutes implying the electrostatic mismatch with water observable when trying to

obtain stable mixtures of bulk water with bulk oil but the basis of the oil-water

incompatibility is somewhat more complicated. It depends on the fact that bulk water has 50% free volume and much fully exposed surface of its O-H-O bands neither of which can be reduced except by solutes such as hydrazine destroying the several lattice forms, structure breakers, or solutes allowing electrostatic relaxation. The last is probably correctly described by Frank as the drawing back of the first water shell into bulk water to take advantage of its high dielectric constant. The distance is estimated as about 0.05Å and this separation actually reduces the unfavorable reaction field between water and solute sufficient to overcome the enthalpy increase in formation of the reaction field. Note, however that ions like sulfate and protonium stabilized clathrates by favorable electrostatics. Such guests must match the requirements of the clathrate structures so most ions fail in such fitting. The reduction in unfavorable reaction field would be larger were the cavity empty but arrangements of the L species toward significant clathrate size are very improbable both because of the larger free space and the large unfavorable reaction field. Formation of empty clathrate cavities are very improbable at higher temperatures and must have very short lifetimes even at subzero temperatures where they are assembled into polymers of the L form to give critical behavior as the temperature drops toward -45°C. With amphiphilic guests the major improvements then are the van der Waals interaction between the side-on O-H-O bonds of water and the increased local density of water. Whether or not the latter is correctly called 'hydrophobicity" is to some extent more a historical accident than a useful definition. It is not adequate for the most efficient structure-maker, sulfate ion nor for PEG when examined in detail but such special cases still ultimately rest on the equivalence of donor and acceptor sites on the water molecule.The latter is probably the most correct basis of "hydrophobicity".

The term "hydrophobic effect" is used for cases in which one hydrophobic solute enhances the solubility of another and appears to be a consequence of size and shape but must also depend on the availability of water for clathrate formation. As

just described, there is competition among structure-makers for water (see discussion

of the work of Wen and Hung in chapter 4). One way simplify the competition is to

package solute molecules together and other possibilities can be suggested. From the

point of view of the biology the competition binds all the competitors into single large

linkage systems such as in single cells of organisms. Near 285K H, S and V can

experience large fluctuations throughout such systems but the free-energy fluctuations

though important are small as result of compensation. At physiological temperatures

near 37.5C even the latter can be important. The importance for function depends on

the characteristic temperatures of the physiological processes. Thus the matrix

expansion-contraction process is governed by its high compensation temperature of

470K making it susceptible for large free-energy fluctuations.

It has become apparent in the work of Koga et al and even more in these

volumes that the terms "hydrophobe" and "hydrophobic hydration" need revision.

Any solute favoring structure defined as association and stabilization of clusters of L

water must be classified as belonging to a single group and that group is found at one

end of the Hofmeister series. It includes ionic and neutral species but sometimes the

electrostatic behavior of ions requires some modification. Sodium chloride is at the

middle being neither a structure maker nor a structure breaker. On the other hand

sulfate ion and a protonated water molecule are among the most effective structure

makers each with clathrate shells of 20 water molecules. The shells are pentagonal

dodecahedra and are favored by their intrinsic possibilities for association of the L

building-block units. The large cluster associations stimulated by the tetrahedral

symmetries of sulfate ion and H_3O^+ are among the most effective. Perchlorate ion on

the other hand is too large for the minimum cavity despite its symmetry. Symmetry,

size and charge distribution are important but with solutes such as sugars the

positioning of the hydroxyl groups is also important. At the latter extreme

polyethyleneglycols have a unique influence usually attributed to the fact that the

oxygen-oxygen distance between monomeric units fits precisely into the pentagonal

dodecahedron.

The hydration of the proton is particularly important as an illustration of the

magnitude of the errors that have been made and the degree to which chemistry must

backtrack to make corrections. It has always been apparent that ionization of acids

such as acetic acid required much smaller enthalpy and entropy changes than those

estimated by conventional means and the deficiencies often have been attributed to

neglect of the contributions from changes in hydration. The bare proton became the

hydronium ion and then the $H_9O_4^+$ of Eigen and DeMaeyer and finally the recent

mass-spectroscopy estimates of twenty water molecule. That may be the final

improvement the clathrate is probably no larger than the pentagonal dodecahedron.

Formation of the clathrate resulting from transfer of the proton from free acid to

water produces the large negative enthalpy and entropy contributions required. Similar

confusion in ammonium ionization are explained in the same way and both

complicate ordinary chemistry by making rigorous thermodynamics possible only be

taking into account all species in any given solution. Note however that Benzinger's

errors in application of thermodynamics to processes carried out at constant

temperature already eliminate any experimental possibility of rigorous resolution of

those problems.

Ions can be placed in the Hofmeister series using their Setchenow constants

but charge changes render such positioning complicated in interpretation. Ionization

of weak acids and basis illustrates the additional problems with electrolytes.

The compensation temperature for amphiphiles in general and most

hydrophobes on transfer from pure liquid reference to water is 354 K in

agreement with many previous studies. Detail provided by Kessel'man and

Omifrier, Russian J. of Physical Chemistry 50, 1714 (1976) on ethanol into water

confirms Arnett-McKelvey pattern (reference list) over wide range of

temperature and composition. The Russian workers found that the heat of

mixing goes to zero at 354K at 0.5 mole fraction of each and thus at ideal mixing

entropy. The magic mole fraction is 0.089 only slightly varying with temperature.

The data of Wen and Hung in 1970 for mixtures of structure-making solutes

shows precise linear enthalpy and entropy compensation with a single

compensation temperature for transfer of their several families of solutes from

water to water mixtures with fixed mole fractions of similar solutes from several

congener families to be 285K. As discussed in chapter 4 of volume 2 that

temperature is shown to be the condition of equal standard chemical potentials

for the two water species and also since neither species has its maximum average

clathrate structure, the behavior measures the relative clathrate formation ability.

The method is an improvement over comparison of the magic mole fractions of

the paired solutes but not much. More useful specific chemical information can

be extracted from the thermodynamic changes that occur in transfer of a single

solute from light to heavy water though this method is also complicated unless

the solute concentration is not well below its magic mole fraction. The data

collected by Arnett and McKelvey and also described in chapter 4, volume 2 lie

within considerable errors on two nearly parallel compensation lines with slopes

of 285K. This means that the temperature at which the standard chemical

potentials of the two species of heavy water is the same within error as that of

the two species of light water in which case the standard enthalpy change in the

transfer process is a multiple of the single water change. Then so long as the

actual experimental mole fractions are small, that multiple or its equivalent

standard-entropy multiple is an important measure of the relative affinity for

water, The method can be calibrated with the argon results in Table @. In 1992

Scharlin and Battino reported the changes in H and S for transfer of 10 small,

non-polar gases from light water to heavy water (Fig. @, table @@). These

accurate data show very precise linear compensation behavior as previously

suggested by the data compilation for a wide variety of solutes by Arnett and

McKelvey and by Ben-Naim and Baer but now revealing some surprises. *A priori*

it might have been suspected that chemical changes for any of the solutes would

nearly cancel in the transfer process so that the relative number of water

molecules in the hydration could be estimated for each solute from the relative

change in enthalpy or entropy. The table shows that some of the chemical

differences are not cancelled in the transfer nor is there a simple correlation with

size or polarizability. The linearity of the plot implies a single correlation which is

not apparent. Arnett and McKelvey compilation reveals the same compensation

behavior within considerable error for a wide variety of solutes including

electrolytes and the two compilations suggest more complexity than is revealed

in the data from Wen and Hung. Their experiments have been repeated under

wider range of solutes and experimental conditions by Koga and coworkers and

in the following section we attempt to expand with those data on the

conclusions suggested in chapter 3 of volume 2.
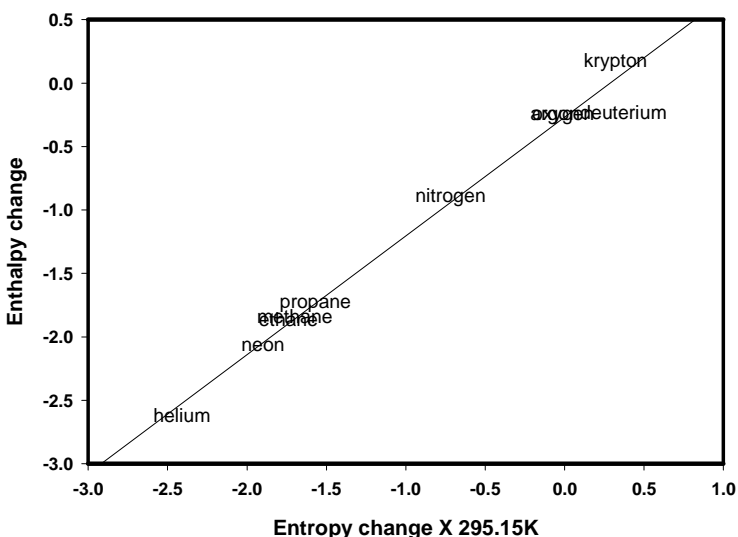
Transfer from H_2O to D_2O of low pressure gases

	$\Delta\Delta H$	$\Delta\Delta Sx295K$	Hydration Difference
	kcal/mole	kcal/mole	
Helium	-2.62	-2.41	2.28
Neon	-2.06	-1.90	1.79
Argon	-0.24	-0.06	0.21
Krypton	0.18	0.32	-0.16
Deuterium	-0.23	0.37	0.20
Nitrogen	-0.88	-0.72	-.76
Oxygen	-.024	-.0.01	0.21
Methane	-1.84	-1.70	1.60
Ethane	-1.86	-1.74	1.62
Propane	-1.72	-1.57	1.49

Data from Scharlin and Battino (J. Solution Chem.(21(1992) 67)

The compensation temperature from the plot of the tabulated values is 285K

which is also the temperature at which the standard chemical potentials of the

two normal water species are equal. Recall that there are 4 or 5 water molecules

in the mole unit but 285K is still surprisingly large compared with 277K so the

density drop in forming the L species is quite small. The two species of heavy

water give a maximum density temperature of 284K so the density of the L

species is considerably larger. The temperature at which the standard chemical

potentials of the two heavy-water species are equal can be computed using the

excellent density data available. The numbers of water molecules directly

involved can be determined from the table above using a reference substance if

chemical differences cancel but that is neither likely nor obvious.

**Another version of chapter 5 of Volume 2 of the Protein Primer. The
two need to be edited together and uploaded to my web site but in a long
time that has not happened because I am tired and still have much to do.
So as of Sept 21, 2006 I am resigned to sending the two. R. Lumry**

Chapter 5 verion 2

Introduction. The more complicated features of protein hydration can be

put on a quantitative foundation using the linkage to the two-state process of

water to measure competition with reference cosolvents. In this chapter the

objective is to provide a quantitative description of the construction and

hydration of the protein interface required to tune enzymes for isoergonic

nutcracker operation. The path to this objective is to explain so-called

"hydrophobic hydration", the major device found by nature as a useful device

for that tuning. Much of the discussion presented in this section has been already

included in chapters of volume 1 of the Protein Primer as for example the basis

of hydrophobic hydration in competition for water among all varieties of

hydrophobic solutes including also such ions as sulfate and the protonated water

molecule. In time logical assembly of a wide array of information on water and

aqueous solutions will be possible; for the present we continue to try to simplify

that jigsaw puzzle starting with a systematic review of established or suspected

lines of logic. For Lumry, Battistel and Jolicoeur that process began with the

proposal by Henry Frank that hydrazine could be used as a model for "inhibited

water" by which he mean water free of the unusual, probably unique

solubilization properties of normal water. Professor Frank grounded this

proposal in the similarity between water and hydrazine illustrated by his Table,

Table @ below and further supported by the heat-capacity measurements on

water-hydrazine and water-hydrogen peroxide mixtures of Oguni and Angell.

With data for the latter supplied by Professor Angell, Lumry et all were able to

identify as basic building block for the lower-density species of water a

"flickering cluster" formed by electron redistribution with an average of five and

four water molecules and with a lifetime of about 0.5 ps. The clustering requires

equal numbers of hydrogen-bond donor and acceptor sites and is not otherwise

formed so additions of hydrazine progressively reduce the probability of

formation of the clusters. At 0.24 mole fraction, cluster probability is very small;

further additions of hydrazine or hydrogen peroxide further modify the

extensive connectivity of the higher-density water species eventually destroying

percolation. Above 0.24 mole fraction water-hydrazine mixtures increasingly

resemble hydrazine, a strongly associated liquid but without the cooperation on

which L and H water depend. Most studies of that and other structure-breaking

solutes reveal two distinct concentration regions but they also appear in studies

of mixtures of water and structure-makers.

First consider the solubility properties of water with structure breakers

illustrated by studies with argon and later with studies of surfactants by Ramadan

and Evans. The transfer per mole of argon from hydrazine to water produces an

enhanced stability for the mixtures as it does for all permanent gases. The

standard-free energy change is zero at 322K and thus near the 328K value at which the partial molar activity coefficients of the noble gases in water at infinite dilution is a maximum (Benson and Krause) as are those for methane and ethane (Rettich, Battino and Wilhelmy). Note for future reference that 328K is the temperature at which protein matrices become suddenly labile for normal denaturation fluctuations. (Gregory using proton-exchange data). At of near that temperature the two steps of hydration make equal and opposite contributions to hydration stability. Specifically the cost of converting H water to L water is just equal to the gain in stability arising from clathrate or equivalent incorporation of solutes in clusters formed from L water. That is, the free energy changes are equal and opposite in sign. Above that temperature it becomes more difficult to form the unstable species of water, L, and at 354K as shown in Chapter 4 of this volume the total free energy change in hydration of the structure-making solutes is zero. The two processes involved are the conversion of H to L water and the incorporation of the solute in clusters formed from L water. The former has a compensation temperature of 285K for H_2O and the latter 328K or slightly lower.

Wen and Hung used pairs of structure-making solutes but each at such low concentration as to clearly reveal only the single coupled process, the conversion of H water to L water to accommodate increasing solute concentrations. Koga et al in more recent papers used higher concentrations so that the magic mole fractions became clearly distinguished (Figs #@@) generalized the phenomenon and revealed several more of its general characteristics. However before that discussion several of the previously discovered characteristics must be discussed.

The compensation behavior exhibited by transfer from H_2O to D_2O reported by Arnett and McKelvey in 1967 received little attention until Ben-

Naim developed it as a general tool some fifteen years later. However accurate

data on solubilities in heavy water illustrated in Table are still in short supply.

Koga and coworkers were familiar with the seminal papers on "icebergs"

published by Frank and Evens of 1945 may have appreciated Glew's proposal

that the clathrates formed in liquid water are similar to those found in the ice

clathrates. However Koga et al have not used the characteristics temperatures of

water: 285K H-L equilibrium, 328K maximum activity coefficient for structure

makers; 354K hydrophobic hydration contributions change sign becoming

destabilizing above that temperature. With ethanol, other than ethylene glycol

the most completely studied amphiphile; the magic mole fraction disappears at

354K.(Fig.@, chapter @) The enthalpy distribution is clearly outlined up to that

temperature and the maximum though not so sharp as with larger amphiphiles

changes very slowly in mole-fraction value with increasing temperature. The

proton and sulfate ion may not have the same temperature dependence (it can be

extracted for proton and hydroxyl ion from tabulated acid and base ionization

data) and such special examples as PEG may have different temperature

dependence. However since all structure makers are likely to depend on the

same properties of water, differences in qualitative behavior are probably rare.

        The thermodynamic stability of clathrated structure makers is large as

indicated by narrow magic-mole-fraction peaks, by 328 K transition temperature

and probably by 354K disappearance temperature but that remain to be

examined (infra). The number of water molecules for several of the most

effective structure makers have been established as 20 (the hydrated proton and

hydrated sulfate ion) consistent with a variety of other information suggesting

the pentagonal dodecahedron and the high degree of cooperativity in clathrate

formation that make it the dominant clathrate for small solutes can be

determined from the data of Koga et al. as discussed below.

Note: Fennel Evans and coworkers found stable micelles in water up to their highest

*experimental temperature,***169°C??***. Evans and @s found micelle formation in liquids in*

which the cohesive factor is short rang such as hydrazine and simple structure breakers because

their arrangements of free volume depend on local H bonding. Micelles also form in ethyl

ammonium nitrate, a fused salt in which the local balancing of positive and negative charges

produces strong local cohesion. Thus micelle formation does not distinguish water from these

other solvents and does not support a special strength of the water clarthrates. Ramadan, Evans

and Lumry found hydrazine-water mixtures acted increasingly like hydrazine in the same way

found with argon solubility. It is the L species clustering that is destroyed but not the solvations

of the surfactants. Normal micelles place polar groups including charges at their outer surface in

contrast to the most hydrophobic molecules of interest and ions such as sulfate and protonium

generally without charges. Ions like sultate and H_3O^+ are small complete or nearly complete

tetreahedra and apparently fit preciscly into clathrates cages such as the pentagonal

dodecahedron. The have tight shells of twenty water molecules so it is charge and geometrey

which governs their hydration. Larger ions such as perchlorate are not so well accommodated.

*   The Hofmeister series organizes ions according to solution properties but the details of*

may be different. For example calcium ion, lithium ion and sodium ion, all chlorides have some

qualitatively different properties in aqueous solution. It is particularly interesting that the

sodium and chloride ions are both entirely inactive in determining properties of other solutes

except when salt is high where its effect can be attributed to reduction in water. Other

interesting solutes and guanadinium sulfate the anion being a very effective structure maker and

the cation a very strong structure breaker so the net effect on aqueous solutions is small.

Ethylene glycol is somewhat similar though it does not ionize in water rather it combines in a

single molecule both structure-making and structure breaking characteristics. As Huot et al

found, mixture with water have qualitative properties the same qualitatively as those of larger

amphiphiles but quantitatively much smaller. Although water displays great versatility in cavity production, shape is generally more important than chemical character as illustrated by the difference between sugars with the same number of hydroxyl groups but different placement. Trehalose has attracted an unusual amount of attention because of its unusual effectiveness in stabilizing proteins against heat denaturation but it is not anomalous in terms of the number of water molecules in its clathrate (@). Like sulfate ion its shape and symmetry stabilize a large cage with at least 50% more water molecules that its glucose parent glucose. It prevents freezing of some beetles in coldest winter temperatures. Even more dramatic is the finding from Engberts and (Thesis of student names R something-in my office) that of the pair 1-4 dioxane and 1-3 dioxane one is a structure maker and the second a structure breaker. Clathrates as exemplified by the inclusion associations in water ice do not depend on bonding interactions with water shells but rather on the ability of water to arrange to make a minimum cavity for the gases. However the deductions from the magic mole fractions show the latter do interact chemically with their water cages. t Only the first water shell has major importance as Frank recognized and explained in terms of the contraction possible in familiar clathrate structures such as the pentagonal dodecahedron. First-shell water is drawn out into bulk water because of the higher dielectric constant. Only about 0.05Å increase in cavity size results but the net change in reaction fields between guest and water and water and water in contrast to the water-water interactions in pure water is sufficient. This is not familiar chemistry but the rearrangements produce a change in electronic Hamiltonian that simple rearrangement of the water-water hydrogen bonds does not. What counts is redistribution of charge that makes the L species of water possible. Benzinger showed that only the changes in potential energy appear in the Hamiltonian and thus in the partition function changes so in constant-temperature processes; the changes in heat enthalpy and entropy do not. A pertinent result for the present discussion is that in water charge distribution on oxygen atoms is required to change the Hamiltonian and water is able to support such changes.

Another source of the uniqueness of water is the cooperativity in the first-shell made possible by charge distribution and rearrangements on assemblies of L water. Thus, for example, it is the cooperativity among the 20 water molecules completing the first shell that makes clathrate formation in the pentagonal dodecahedron shell highly cooperative and that cooperation amplifies the small potential energy change per water molecule so as to make the gross effect so important. That has become obvious only in the new studies of supercooled water since the L species becomes dominant only below 285K in pure water but hydration of structure-makers depends on conversion of H to L water up to much higher temperatures.

Several attempts have been made to extract a chemical correlates for variations in the solubility of amphiphiles. The history of these attempts many stimulated by physiology, medicine and food preparation is extensive (see for example "The kinetic basis of molecular biology", Johnson, Eyring and Polissar @), Ben-Naim, Tanford and Hansch in recent years have added some insight for small solutes in water but the correlations sought to explain protein folded stability have only limited utility because the latter is highly dependent on free-volume management in native conformations and not on chemical variation as in polarizability.. Even with small amphiphiles correlation is limited. Koga et al have shown some correlation with the number of oxygen atoms but any detailed success must depend on simple systematizing of the clathrate possibilities in water, neither likely nor likely to be very useful. Consider polyethyleneglycol; it has extraordinary probably unique physical properties because of its clathrates which form a cylindrical tube from oxygen atom to oxygen atom that organizes water in parallel tubes with very low flow viscosity enabling firemen to increase flow rates though small hoses up to those though 300% increase in hose diameters. Chelation about this solute uses the same basic features but differs in detail from that found with small structure makers. Urea clathrates were discovered many years ago and establishes urea as a structure maker as well as a major structure breaker but urea clathrates use special association for many urea molecules instead of water so the former characteristic though important in the history of protein chemistry does not fit it into the class of water clathrates.

The extensive broadening of the magic-mole-fraction concept by Koga and his associates now makes possible attempts to elucidate important characteristics of the clathrates of water in liquid mixtures of the latter primarily with amphiphiles as structure makers. The immediate objectives of this search is to explain why water and oil do not mix but it has become clear that the isoergonic characteristic of the nutcracker mechanism used by all enzymes is obtained by adjustment of composition at and between protein surface regions. Apparently evolution of extremophile depends on such adjustments as much as on the residue composition of the protein. The first objective has been met in preliminary conceptual way in the discussions given in the preceding pages but fundamental features of the clathrates themselves require attention in the following pages.

Properties of water clathrates

Already several reasons have been given to suggest that water clathrates can be stable consistent with tetrahedral structure proposed for the L clusters which in the pentagonal dodecahedron forms a complete first shell the clusters oriented to minimize the reaction field with most guests. That orientation appears to improve the favorable interaction with the second hydration shell but that improvement is probably due to the expansion of first shell into second shell proposed by Frank It has not been possible to explain the partial molar volumes of many amphiphiles without that expansion of 0.05Å added to the van der Waals radii. Note however that the precision of x-ray diffraction does not provide accuracy in detecting such small distances in the clathrate ices (Jeffries) so there is little hope for liquid clathrates. The H species of water is preserved in normal ice , Ih, but in supercooling becomes unstable with respect to such other frozen solids that are available but the L species apparently does not form a stable crystalline solid bur must be the more stable supercooled form as

temperatures drop toward -45C Proteins and presumably other large amphiphiles can be cooled to two different solid state but rapid cooling is required for the lower stability form in which the unstable form is unstable because of its stressed conformation in the expanded matrix state. Klibanov et al and Gregory have utilized the resulting conformation trap to stabilize the less stable ice form and there is now a long history of non-freezing water in cold proteins starting with Kuntz. The distinction between Kuntz water and cold denaturation is still not well established and the inconsistencies among different experimental results suggest that they have often been incorrectly identified. There is a phaselike transition near 200K which appears to be the matrix contraction process driving the nutcracker at higher temperatures. However it has been difficult to separate normal thermal denaturation of proteins from native forms with frozen hydration. Rifkind finds that the distal imidazoles of the four-chain hemoglobins fall onto the iron ions at that temperature. Kuntz' estimate of the relationship between numbers of residues and the amounts of non-freezing water suggests cold denaturation.

A comparison of the thermodynamic data for solubility of argon in water and hydrazine has been used to measure the advantage of water as solvent for pure hydrophobic solutes. The difference in standard enthalpy at and standard entropy are given in Table @ both being attributed to the changes in water on clathrate formation. The number of water molecules involve can be estimated from the changes for conversion of 1 mole of H water to L water, the cooperative unit indicated to be 4 or 5 water molecules. The numbers suggest that 10 water molecules assemble into hydration cluster per argon atom. However, there are 20 water molecules in a pentagonal dodecahedron and the experiments were carried out at temperatures near the 285K temperature at which the standard free energy change in the L↔H conversion is 0 so the

number must be adjusted for the additional ten molecules that change to

maintain a constant ratio of L and H mole fractions. The estimate is close but

not exact since the large heat-capacity change in the water two-state process has

not been accurately included. In any event errors due to neglect of changes in

second and higher hydration shells have been shown to be small. According to

this description the magic mole fraction will be temperature dependent due to

the shift of the L-H equilibrium toward the H species with increasing

temperature. The dependence can be determined using the data for ethanol-

water mixtures (Fig. @) and is not large indicating that the ethanol clathrate is

little altered with temperature increase consistent with its geometry and

indicating that the clathrates are favored forms up to high temperatures.

Improvements in estimates of the characteristic relaxation times for the

two-state process and the clathrate-formation process have been improved by

Zewail and coworkers. The time of 0.5ps suggested by Lumry, Battistel and

Jolicoeur from the findings of Montrose and Litovitz remains a reliable estimate

for 298K increasing at lower temperatures. The relaxation time for clathrate

formation estimated on assumptions of the measured processes using laser

studies of relaxation in protein-water interfaces is a less certain extrapolation but

lies between 28 and 48ps at 100 K (@ check Zewail PNAS reference.). Shikata,

Takahashi and Sakamoto (JPC B116(2006) 8945) found a relaxation time for the

clathrate formed about the oxygen atoms of polyethyleneglycol of 22 ps and a

hydration number limit to be 3.7 water molecules per PEG unit. These numbers

are consistent with the proposed description of the L clusters in pure water. For

comparison the times for relaxation of the matrix substructures of enzymes

estimated by dielectric relaxation is 1ns at 298K and thus much slower than

relaxation in the protein-water interface.

There is still the question of the degree of cooperation in formation of the clathrates. There are two limits to consider. The first is such a low degree that the formation process is essentially one water molecule at a time. The second is complete cooperativity for which micelle formation is a good model. The water clathrates formed about single or small collections of small amphiphiles have first shells which are formed with high cooperativity. It can be suspected that the clathrating is poor with guests that do not stabilizing complete shells. Micelles may be just a special branch of structure makers so large that the shape of the aggregate of surfactants establishes the shape of the water shells. The qualitative differences are probably due to the additional feature of charge in the micelles. So long as micelle formation does not involve incomplete formation their theory is straight forward and can be developed in several familiar ways. Among the first such developments is that of Roux and Desnoyers. Before recapitulation of their work, the all or none feature of micelles can be checked for water clathrates in a simple way using the magic mole fractions.

The magic mole fraction, a term introduced by Lumry and Rajender, is the mole fraction at which the average number of water molecules per clathrate is a maximum. In the all-or-none model of complete cooperativity the reciprocal of the magic mole fraction multiplied by the concentration in moles per liter gives the total number of water molecules in the clathrates. If that is a large fraction of the total numbers of moles of water, cooperativity is very high so simple mass-action law uncomplicated by intermediate steps in ligation is suitable. The times estimated in the previously paragraph are more likely to be due to the latter process but in any event clathrate relaxation is rapid and modulates the cooperativity.

Given the previous statements it is easy to show that clathrate formation supplemented by the L↔H equilibrium of free water uses up all the water in the

mixture. Thus the reciprocal of the magic mole fraction for ethanol is 11 and the

concentration in 1 liter of binary solutions is given by

$$x_L = \frac{([L])}{([L]+[W])} \quad [W] = 55.5 - [L]$$

$$[L] = x_L([L]+55.5-[L]) = x_L 55.5$$

But $1/x_L$ is the average number of water molecules per solute so $\frac{[L]}{x_L} = 55.5$, the

total number of moles of water per liter. Thus the concentration at the magic mole

fraction is the number of mole of water bound by the total concentration of solute.

The smaller the magic mole fraction the larger the number of moles of bound water

but the ratio in the last expression is always constant at a given temperature, 2 at 284K

for example. However the term "bound" may not be correct if at experimental

temperatures the clathrate and the pure-water equilibria are balanced. Then because

the clathrate is in equilibrium with both L water and thus also with H water, there is

also significant concentrations of the latter. Thus near 285K where the standard

chemical potentials of the two species of unbound water are equal, the clathrate

concentration is less than the total concentration of structure maker. That is an

interesting situation since it buffers the clathrate concentration and limits the changes

in physical properties resulting from the conversion of structure maker to clathrate.

An example is the solution of argon which would involve only 10 molecules of water

without those equilibria buy near 285K with the correction 20 molecules are involved

as expected for a pentagonal dodecahedron. The general rule from equation @ is that

at ordinary temperatures roughly half of the total water is held in the clathrate in rapid

equilibrium with non-clathrated water. The reciprocal of the magic mole fraction is

the average number of water ligands and thus half of the average in fluctuations

between full clathrate and fully dissociated clathrate as determined by the temperature

and the two equilibrium constants on which hydration depends. Judging from the

rapid transient studies of Zewail et al the relaxation time lies between 30 and 50ps at

100K At 290K the relaxation time for the L↔H equilibrium is about 0.5 ps. Because

of the competition for free water all solutes are linked to a degree depending on the

amount of free water and that in general will be small. That linkage must often make

equilibrium interpretations of data for aqueous mixtures misleading.

The average number of water molecules estimated in this way can be

distributed so that each solute molecule has the average number but it is more likely

from the high degree of cooperativity in clathrate formation as suggested by the above

equation that some solute molecules have full clathrate shells and others have small

shells often none at all. The magic mole fraction is the dividing line beyond which

solutes must compete for water so that the average clathrate size progressively falls as

solute concentration increases. Properties, at least physical properties, of the solution

approach the limit of pure solute. Jolicoeur and coworkers studying the properties at

very low water found a frequent emergence of a water dimer. As can be seen in the

figures from Koga et al, the transition is sharp dividing the total concentration profile

into two distinct regions. Those workers also noticed that hydrogen-bonding in the

free-water fraction diminished in amount and importance as expected from division

of total water into two types: "oil in water and water in oil". Huot, Jolicoeur et al have

emphasized the division in explaining the thermodynamic properties of aqueous

mixtures of ethylene glycol illustrated in Fig. @. Koga and coworkers have attempted

to rationalize "hydrophobic hydration" in terms of chemical composition such as the

number of oxygen atoms but as observed above, this is limited by the requirements

for compatibility of solute structure and clathrate structure possibilities. The PEG

clathrate is a special example (vide supra).

The clathrate description of the hydration of amphiphiles and hydrophobic

molecules involves two features making it possible to extend the traditionally

excluded-volume methodology so elegantly applied by Timasheff and Winzor and

their coworkers (Timasheff, Biochemistry 41, 13473, 2002)). The interactions are

chemical since they involving changes in electronic Hamiltonians; hence the term

*chemihydration (*Lumry, Battistel and Jolicoeur, Faraday Society Symposium,@). Solutes

rarely reach protein surfaces because of their water sheaths which are extensive and

difficult to remove. Many more water molecules are constrained than suggested by

conventional ideas about water structure.

Incomplete understanding of the hydration of structure makers and the extend

of cohesion inside proteins continue to be seriously misleading and will have to be

revised with inclusion of the two-states of water and the clathrate structures in protein

interfaces. Consider acid and base ionization in water and dielectric behavior in

aqueous mixtures.. Although familiar theories of these phenomena often give fairly

accurate free energies, the enthalpy, entropy and volume information they gives are

incorrect often seriously in error. Electrochemical topics are particularly vulnerable to

such oversimplification.

The model of protein hydration given here is different from that developed in

major detail by Timasheff and coworkers as well as by Winzor et al. It does not

assume water to be a uniform essentially homogeneous fluid, a single component.

Instead it assumes most water molecules to be in chemical reaction with solutes so

that mass-action-expressions for the equilibria among the many components are

necessary to take into account two chemical species for pure water and the clathrate

species. The final definitive proof of the latter due to Walrafen in 1983 has not been

given in even the latest editions of chemistry and biochemistry textbooks (but see@

Grunwald's latest book, Chapter 3). Teeter and coworkers find clathrate structures at

interfaces that may be easily adjusted dynamically but thermodynamically must be

considered part of the protein with major implications for explanation of folding and

conformational features essential for physiological function as reversibility in enzymic

catalysis (Chapter 6, Vol.2 Protein Primer).

Most protein hydration appears to be quite mobile even in best-folded states

but nevertheless the participating water molecules tend to be fixed in partial clathrate

cages about non-polar residue ends, ionized acidic and basic groups and sulfate ions in

protein surfaces. In addition these cages have very motile bridges between fluid water

and protein. As noted above ionizable groups bearing charges are heavily hydrated but

their non-ionized counterparts have different structured interactions with water so

probably much less clathrate water.. Apparently all structured forms of protein

hydration make major contributions to the stability of folding the expansion-

contraction process of protein matrices (Chap. 8, vol.2, Protein Primer.).Wyman's

linkage theory as developed for the multiple localized binding of ligands appears to be

a better basis for quantifying protein hydration than the continuum theories that have

been used. The difference in orientation allows a more flexible development of

hypotheses. A good example of the latter is the probability that urea and other

structure breakers affect proteins more through their effect on the aqueous milieu

than effects of direct contact. This idea now frequently proposed is due to Timasheff

as part of more extensive descriptions of hydration and denaturation(vide infra).

Benzinger in 1967 discovered major errors in the applications of textbook

thermodynamics to isothermal processes. The resulting errors in H, S and V are rarely

correctable so much of chemistry investigation is probably pointless. Compensation

theory often provides ways to extract some significance from even isothermal

processes but does not provide a method to make the Benzinger corrections.. An

example from protein chemistry very similar in construction to the hydration example

discussed here is the study of the thermal denotation of chymotrypsinogen as altered

by glycerol. The data obtained by Timasheff and Gekko at several temperatures

around 315 K fall on linear enthalpy-entropy plots all with the same compensation

temperature of 302K. This plotting removes apparent differences among the effects of temperature and glycerol concentration. It reveals a single common linkage stabilizing the melting process below that temperature and destabilizing it above. As yet the source of that central linkage has not been established but the exact linearity of the compensation plots establishes that compensation is probably due to a single linked process modulated by glycerol mole fraction. Solvation and hydration of the noble gases yields significantly curved plots because they are due to two different variations along that congener series: size and thus surface and polarizability. .

Why does water at 1 atm freeze at 273.15 since it has two chemical species and thus presumably two solid phases but only one appears in freezing? How find true freezing temp for H water. The familiar thermodynamic freezing point depression treatment to determine free-energy changes must be extended to accommodate two species of water..). Much of the information necessary to understand proteins is summarized in the Summary of volume 1 also to be found at that URL. What is new in Volume 2 and relevant to this contribution are the following items: (see http://www.chem.umn.edu/groups/lumry)

Teeter and coworkers in 2002 (see reference list) showed by low-temperature x-ray diffraction that exposed non-polar tails of amino-acid residues in protein surfaces (crambin was used) have partial clathrate cages identical with those that form about small amphiphilic and hydrophobic solutes. Cage size depends on solute size, shape and competition with other solutes so that most free water is chemically held in the cages which on average decrease in size a mole fraction of solute increases. For ethanol the maximum cluster size occurs just below mole fractions of 0.1 and decreases slightly with increasing temperature nearly vanishing at 354K. The maxima for larger amphiphiles decrease with size down into concentration regions not easily studied and often complicated by competition with micelle formation by the amphiphiles. The prorogated water molecule has a 20mer cage presumably a

pentagonal dodecahedron but the effects are easily detectable only at low and high pH.

Quantitative estimates of the contributions of the clathrates on protein surfaces to folded stability are not yet more than guesswork but they appear to be as large as those from knot formation. Timasheff's estimates using an excluded-volume model may be approximately correct but only volume estimates have been extracted in detail. The multiple-equilibrium treatment is identical with that developed by Wyman for hemoglobin oxygenation and protein ligation in general (Gill and Wyman's monograph on linkage and Schellman's treatment of the example give complete details). Ben-Naim pioneered applications of the two-state model for water and gives much of the theory. As discussed in many of our older papers, most theoretical treatments of process in water medium such as dielectric dispersion are incorrect because they do not include the water equilibria. Note however that even with such corrections it will rarely be possible to estimate Benzinger's corrections for G, U, H, S and V.

Structure breakers as well as structure makers compete for normal water and thus compete with each other with the consequence that all solutes and that includes proteins form a single linkage system. This explains for example the so called hydrophobic effect.. Solutes can interact with proteins by direct contact or by altering the medium and the latter is the more likely as has also been found by Timasheff's using his excluded-volume analysis.

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