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Spectroscopy of aqueous solutions: protein and DNA interactions with water

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Abstract. This review emphasises the need to use spectroscopy in order to understand the behaviour of water, and summarises the background of the subject. The various forms of spectroscopy that are especially informative are described, with particular reference to near-infrared (NIR) spectrophotometry. The key results are outlined, first those obtained with small molecules and ions, and second those involving proteins, DNA and cell membranes. Finally, some interpretations are offered which include the novel but possibly controversial concept of free OH and free lone-pair groups.

Key words. Infrared (IR) and near-infrared (NIR) spectroscopy; electron paramagnetic resonance (EPR) spectroscopy; water; aqueous solutions; biomolecules.

Introduction

My study of aqueous solutions began many years ago with an underutilised ultraviolet (UV) spectrophotometer. As iodide ions have an intense absorbance band in the 220 nm region, I thought that this spectral feature might prove sensitive to the environment of these ions. The absorbance indeed proved to be very sensitive, and so a new area of solvation studies was born [1–4]. I decided that rather than trying to generate complex theories to fit the facts, it was better just to 'look and see'. This endeavour started with studies of carbocations and radicals, but was later extended to aqueous solutions, where the method worked equally well.

My next instrument was an infrared spectrometer. I subsequently realised that EPR spectroscopy was a very powerful weapon for studying ion pairs [5, 6], following the pioneering work of Weissman and his co-workers [7]. Thereafter, NMR spectroscopy arrived, and finally I returned to spectroscopy, but in the near-infrared (NIR) region, one that had been missed by conventional UV-visible and normal IR spectrometers.

The key results

The following are the key results in these areas which seem to be of particular significance. Although our own work is that usually cited, I must stress that others have also contributed extensively to these fields, as I try to show below.

Pure water

A major contribution to our understanding of the structure and behaviour of pure water comes from IR vibrational spectroscopy. When the spectra for isolated water molecules (monomers) are compared with those for liquid water or for ice, there are major differences, quite apart from the expected effects due to rotational fine structure. In particular, in the regions sensitive to O–H bond stretching known as the ν_1 and ν_3 bands, there are very large shifts. These can best be studied using deuterated water, HOD in D_2O, since in this case the combination bands are absent from the O–H stretch regions.

These spectroscopic differences indicate a shift towards lower energies. This clearly shows that the O–H bond strengths for O–H···O and soon hydrogen-bonded structures are much less than those for the monomers. This in turn means that some degree of proton transfer must have occurred in the H-bonded units. This is of great importance, both theoretically and for our understanding of biological systems. Hydrogen bonds are often treated as purely dipolar in nature, an interpretation which can be very misleading. This arises in part because, in ice, each water molecule is tetrahedrally co ordinated via its two OH groups and its two 'lone-pair' groups. Such an elementary description of the non bonding electrons can be justified because it is descriptively useful, provided that there are two H bonds to the water oxygen. If there is only one other group linked to the oxygen atom, then this simple concept may no longer apply.

The shifts in the peak positions of the IR bands are a function of the hydrogen bond strengths in the aqueous systems under consideration. In addition, as the shifts increase, the bands also become increasingly intense, as well as broader. It is important, however, to note that these are not linear shifts (cf. fig. 1). Initially, for very weak H bonds, the shifts are relatively small, but they become nearly linear for strong H bonds. Eventually, when both bonds are equal, as in $(F\cdots H\cdots F)^-$, the shifts are extremely large. This method thus not only establishes the nature of the H bonds present but also provides an easy method for measuring their relative strengths.

For pure water, in the 'fundamental' IR region, there is a clear shift to weaker H bonds upon going from ice to water, and upon heating the water. At the same time, a new band appears in the NIR region close to the gas-phase band for HOD molecules. This region contains the first overtone of the O–H bands, $2\nu_3$. Fortuitously, the selection rules are such that in the fundamental IR region, the oscillator strengths for H-bonded O–H oscillators completely dominate, whilst in the overtone ($2\nu_3$) region, the reverse is true. Fortuitously, the selection rules are such that in the fundamental IR region, the oscillator strengths for H-bonded O–H oscillators completely dominate whilst in the overtone ($2\nu_3$) region, the reverse is true. Hence, for experimental studies of H bonds, both regions should be examined.

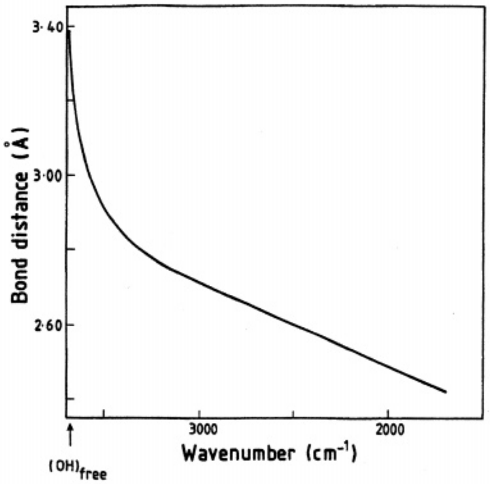


Figure 1. Shifin the stretching frequency of hydroxyl groups. Stretching frequencies are indicated by the IR absorbance band positions in a range of OH···O hydrogen-bonded units as a function of the total O···O separation.

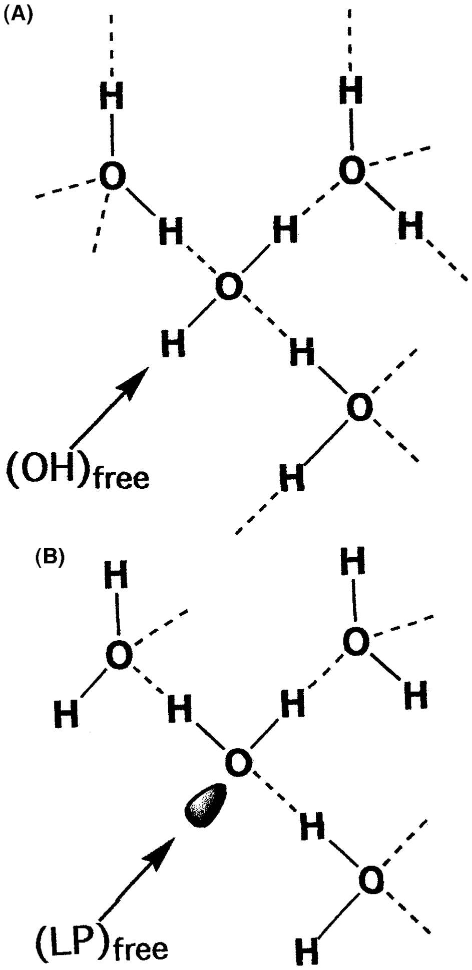


Figure 2. Structures for OH_{free} and LP_{free} groups. (*A*) A typical cluster of water molecules showing H-bonded and free OH groups. (*B*) A typical cluster of water molecules showing bonded and free lone pairs on oxygen atoms.

The NIR results show clearly that there are many free O–H oscillators in liquid water. These may be designated $(O\text{–}H)_{free}$ groups (fig. 2a). At 20 °C, there are about 8% such free groups, and this percentage increases considerably on heating. Unfortunately, in the case of water, there is no corresponding clear band for the lone pairs (fig. 2b), although an IR band between those for the free and those for the strongly bound units was clearly resolved for methanol (MeOH) in deuterated methanol (MeOD). This was shown to derive from MeOH molecules forming only one O–H···O bond to another oxygen atom [8]. However, it may be pointed out that since $(O–H)_{free}$ groups in water are formed by breaking H bonds, an equal number of free lone-pair $(LP)_{free}$ groups must be formed concurrently.

Because NIR bands for very weakly bound OH groups fall very close to that predicted for an $(O–H)_{free}$ group, it has been argued that the observed bands are not due to $(O–H)_{free}$ groups but to such weakly bound groups [9]. There are two arguments against this interpretation. The first and most compelling is that it is surely impossible to have very weak H bonds but no broken bonds. The second is that it is difficult to explain the fluidity of liquid water if all H_2O molecules are tetrahedrally bound as in ice. For ice, there is no detectable band in the $(O–H)_{free}$ region. This set of bands is shown for HOD in D_2O, as a function of temperature, in figure 3 [10].

Furthermore, for methanol there is no resolved band in the $(O–H)_{free}$ region, but simply a shoulder. This is as expected because whenever an H bond breaks in liquid methanol, the resulting $(O–H)_{free}$ groups are surrounded by a sea of $(LP)_{free}$ groups. They will therefore form new H bonds at once (fig. 4). Hence, these spectra show what is to be expected when only weak hydrogen-bonded OH groups contribute.

At present, studies involving the UV spectrum for pure water, which lies in the 'vacuum UV' region, are not readily experimentally available, because standard spectrophotometers do not go below the 190 nm wavelength region. However, there is a considerable absorbance 'tail' in this region, which increases upon heating. This may represent a shift of the main UV absorbance band, but broadening is the probable main explanation. ^{1}H NMR spectroscopy shows a steady shift of the NMR features towards their gas-phase values on heating; of course, these latter bands are very narrow because of rapid averaging. This shift strongly suggests that an increasing number of H bonds are broken upon heating. The NMR changes, including the estimated concentrations of $(O–H)_{free}$ and $(LP)_{free}$ groups, agree well with the NIR data. However, in my view, the latter remain the most reliable indicators. In summary, heating breaks H bonds, but the value at 99 °C shows that there are still less than about 40% broken bonds prior to the boiling transition.

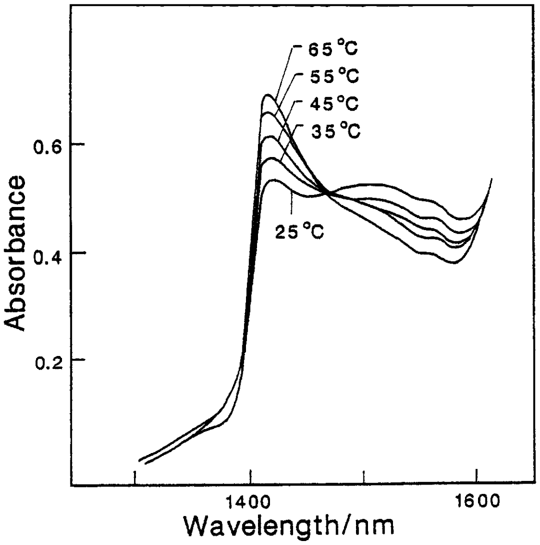


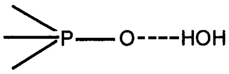
Figure 3. NIR spectra for water. HOD solutions in D_2O, showing the intensity and shape of the $(O–H)_{free}$ band as a function of temperature.

Solutions of aprotic polar solutes in water

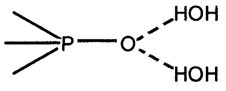
Vibrational spectroscopy is also very helpful in studies of the behaviour of neutral hydrophilic solutes in aqueous solution. In the NIR overtone region for pure water, there are linear decreases in the intensities of the $(O–H)_{free}$ band upon addition of strongly basic cosolvents such as dimethyl sulphoxide (DMSO) and hexamethyl phosphoramide (HMPA) [11, 12]. The results obtained with triethyl phosphine oxide (Et3PO) as a cosolvent as well as those with other solutes are shown in figure 5. I try to show what these results imply below. Such solutes can also be studied using their characteristic spectra in the fundamental IR region [13, 14]. Conclusions as to H-bonding patterns based on some of these results are shown in figure 6.

We used molecules such as Et_3PO at very low concentrations not as cosolvents but as a way of probing binary solvent systems throughout their whole range. The advantage of using vibrational spectroscopy is that in most cases, signal averaging is very slow, and hence features grow in and are lost, as shown in figure 7. In contrast, NMR time averaging is very fast, so it is not possible to gauge thereby how many different structures are involved in systems being examined, and it has previously been necessary to estimate the ground states involved.

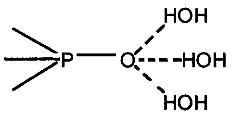
Now, however, such guesswork is no longer needed. For Et_3PO, for example, going from the aprotic solvent through to water, the non-H-bonded >P–O stretch band fell, and an intermediate band grew in. This then gave way to a second intermediate band before the final band characteristic of pure water, appeared. Hence the first band was assigned to the singly H-bonded species A, the second to the doubly H-bonded species B and the third to the triply H-bonded species C (fig. 6).



Species A



Species B



Species C

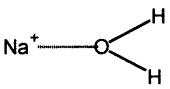
It is clear that this is the final state for this solute in pure water, and from these and similar results we have suggested that water brings out the maximum solvation number for such solutes as well as for anions. On the other hand, the solvation number for alcohols is less. Using such results, we were then able to reinterpret the NMR results [15, 16].

Inert solutes

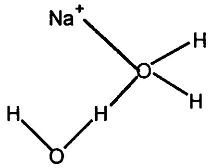
Water loves water, and so most inert ('hydrophobic' or weakly 'hydrophilic') solutes do not dissolve beyond a very low limit. Even oxygen at its atmospheric pressure only produces a low equilibrium concentration in water. Sometimes, instead of totally rejecting such solutes, water forms cages around each molecule to give clathrate compounds [17]. In the liquid phase, at least at low temperatures, it seems probable that such cages form incipiently around all inert solutes, but only extensively when the solutes are held in solution by solvated molecular segments or by solvated cosolutes. One such example is *t*-butyl alcohol, and another is the tetramethylammonium salt $(CH_3)_4N^+Cl^-$. For the latter, it is the strong solvation of the chloride ions that pulls the $(CH_3)_4N^+$ cations into solution. In the case of *t*-butanol, water is thought to form a partial cage around the *t*-butyl group. As the concentration increases, these groups tend to share their individual water cages. The making and breaking of such cages gave rise to the ultrasonic absorption characteristic of these systems [18]. This cage sharing is a feature of 'hydrophobic bonding'. It is important to stress that there are no chemical bonds involved in such structures.

Ionic solutions

The situation for anions in aqueous solution is the same as that for aprotic polar solvents. For cations such as Na^+ and K^+, coordination is via lone pairs of electrons on water oxygen atoms, just as for transition metal ions, though the bonding in the latter case is usually much stronger. For a cation, the interaction with the water oxygen can be treated as dipolar, and one structure is therefore a symmetrical one. However, this structure (species D, fig. 6) requires the breaking of two water H bonds, so it could well be that only one of the lone pairs is used, leaving three intact H bonds, as in species E (fig. 6).



Species D



Species E

The NIR results suggest that the latter structure is preferred by alkali metal cations. The extensive decrease in the intensity of the $(O–H)_{free}$ band on adding tetraalkylammonium halide ($R_4N^+hal^-$) salts to water is caused by H bonding to the halide anions. The R_4N^+ cations are not solvated. In contrast, the alkali metal halides show relatively small shifts. We have argued that this follows if the cations use up to six LP groups whilst the anions use up to six OH groups. This was supported by the fact that sodium tetraphenylboron (Na^+BPh_4) induces a strong increase in the $(O–H)_{free}$ NIR band (fig. 5) [11]. In all cases, secondary solvation is of considerable importance in determining the strengths of these H bonds.

Proteins

Although there are no major theoretical differences between the interactions of proteins and those of smaller organic molecules with water, with proteins there is a very wide range in the types of water molecules involved. Only $(O–H)_{free}$ groups are readily detectable in protein-associated water. A full interpretation of the ^1H NMR characteristics of their H-bonded groups is virtually impossible. The protonated amino groups (N-terminal and lysine ε-amino) are not easy to study because they form strong H bonds, and the resulting broad IR bands lie in the region also occupied

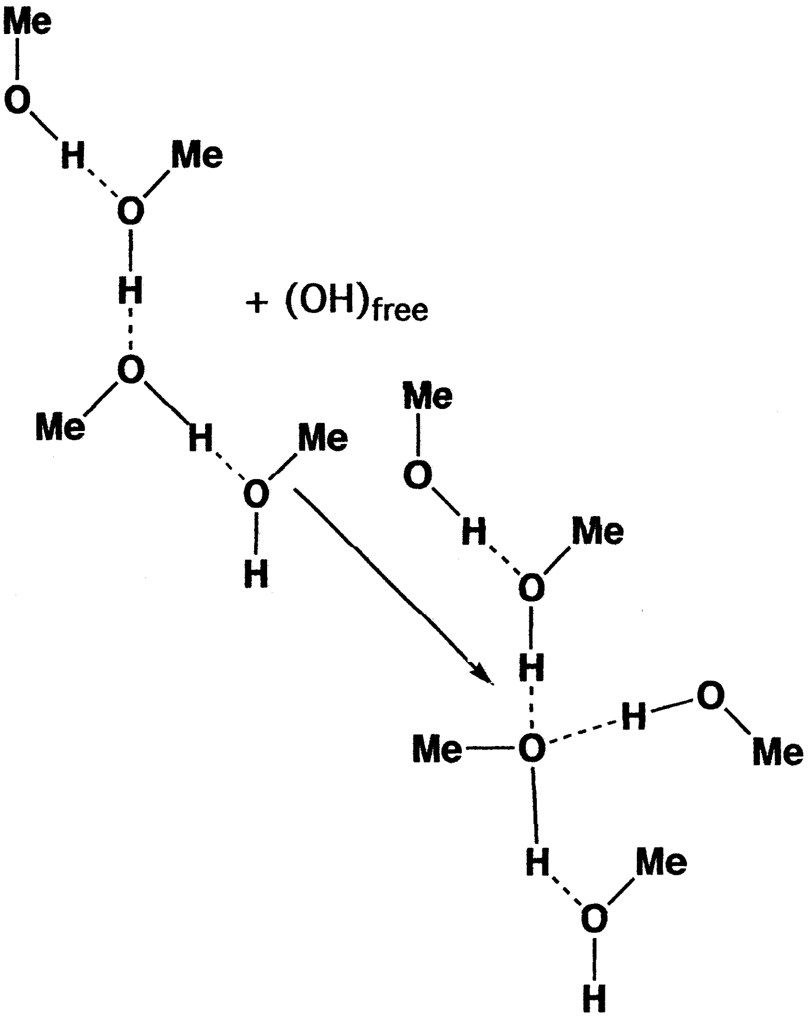


Figure 4. Why there are few OH_{free} groups in methanol. (*A*) typical H-bonded chain of methanol molecules. (*B*) How $(OH)_{free}$ groups can add to any $(LP)_{free}$ groups.

by H-bonded –O–H bands. However, the carbonyl (> C=O) groups of the peptide backbones do give resolved spectra, which show that their oxygen atoms are usually strongly H-bonded. At least some of these are bonded normally not only to NH groups on adjacent β-sheet strands or on the same α-helix, but also to one water –OH group. This is not unexpected, since two water molecules can normally bind to $R_2C=O$ groups [18], and the N–H groups of amides form H bonds of comparable strengths. Specific protein side-chain groups, such as those of cysteine (RS–H) or cystine (RS–SR), can also be studied using vibrational spectroscopy. Although this is of great use in protein structural analysis, it will not be discussed further here because such data provide little information about protein solvation.

One of the most interesting areas involving solvation problems is that of small channels in proteins, which often guide the relevant small molecule reactants, such as oxygen into haemoglobin, and which contain one or more water molecules. In some cases, X-ray crystallography has shown exactly where such water molecules are, and these results can be used to help understand the spectroscopic results.

One drawback, however, is that because crystals have to be used, only stationary water molecules can be seen. Some key water molecules may not stay bonded during crystallisation. This implies that general pictorial models such as those described in this overview remain useful.

With respect to ‘hydrophobic’ bonding in proteins, I should stress that globular protein molecules fold in

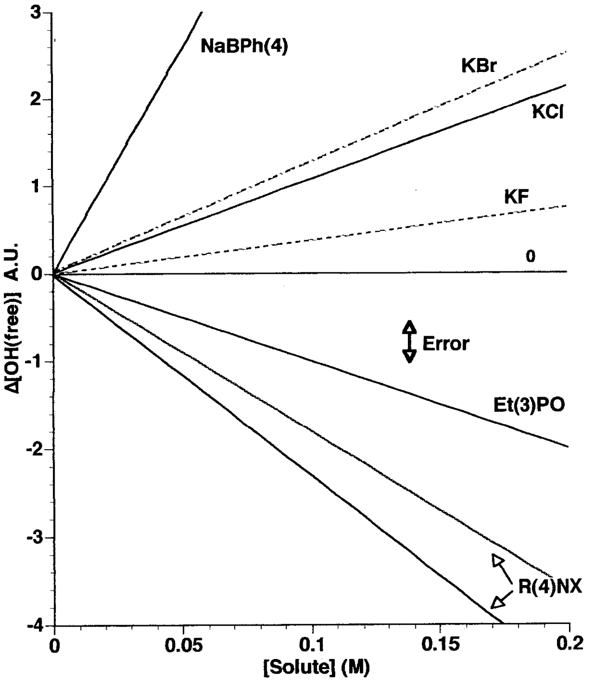


Figure 5. Intensity of the $(OH)_{free}$ band as a function of solute concentration. The band intensities (indicating the total proportion of $[(OH)_{free}]$ in solution) are plotted against varying concentrations of sodium tetraphenylboron, potassium bromide, potassium chloride, potassium fluoride, triethyl phosphine oxide and two trialkylammonium halides, all in aqueous solution.

such a way that most of the polar or ionic groups are on the outside, where they can be well solvated. The nonpolar sections come close together within the specific three-dimensional structure that the protein adopts. This is still referred to in terms of hydrophobic bonding even though water rarely plays a direct role in determining the final shape of a protein molecule.

DNA

The solvation analysis is somewhat simpler for DNA than for protein, especially for the DNA backbone phosphate groups $(RO)_2PO_2^-$ and the associated Na^+ or K^+ cations. For phosphate anions, the P–O vibrational modes proved to be very sensitive to solvation [19]. As DMSO was added to aqueous solutions of DNA, we were able to detect the loss of two water molecules from each of the phosphate groups, and the partial loss of a third water molecule. At that stage of dehydration, DNA precipitation from solution occurred. The purine and pyrimidine carbonyl (C=O) groups have also been studied. These similarly proved to be strongly solvated by water in addition to their involvement in base-base H bonding in the duplex DNA (Watson-Crick) structure.

Two other points are significant. One is that various studies have suggested that strings of water molecules lie in the major groove of the B form of DNA. However, in this location there must also be H bonding to a surface layer of water; thus in aqueous solution at room temperature, it is unlikely that there could be any precise locations or long lifetimes for particular water molecules in such strands.

Second, in studies of radiation damage to DNA, we and others have found it very useful to use frozen aqueous solutions. For these, we postulated a normal outer ice region, and then an intervening shell of glassy water that links the pure ice and the DNA strands.

Recognition of this arrangement is key to understanding radiation damage to DNA. Using the almost forgotten technique of solid-state NMR spectroscopy, we were able to determine the temperature at which there was an onset of significant movement of bound water in DNA, because the NIR bands then suddenly narrow. This characteristic temperature proved to be very low indeed (– 30 °C) for the water molecules in the glassy regions. The number of such mobile water molecules was small, corresponding to 30 molecules per base pair. This number hardly changed until the melting point of

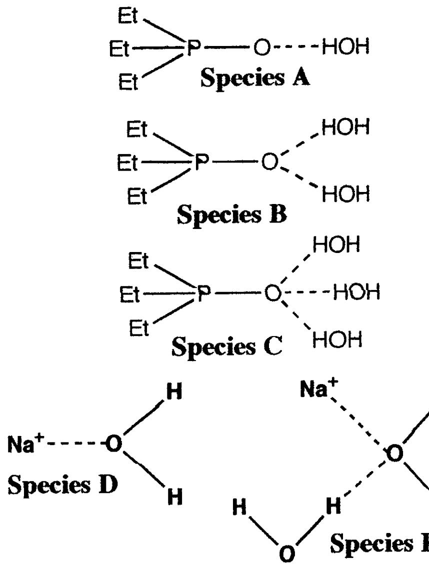


Figure 6. Structures for hydrogen-bonded Et_3PO units and O-linked Na^+ ions. A. Triethyl phosphine oxide H-bonded to one water molecule. B. Triethyl phosphine oxide H-bonded to two water molecules. C. Triethyl phosphine oxide H-bonded to three water molecules. D. Na^+ ion bonded symmetrically to a single water molecule, interacting with both oxygen lone pairs. E. Na^+ ion bonded to a pair of water molecules (direct and indirect), using a single lone pair.

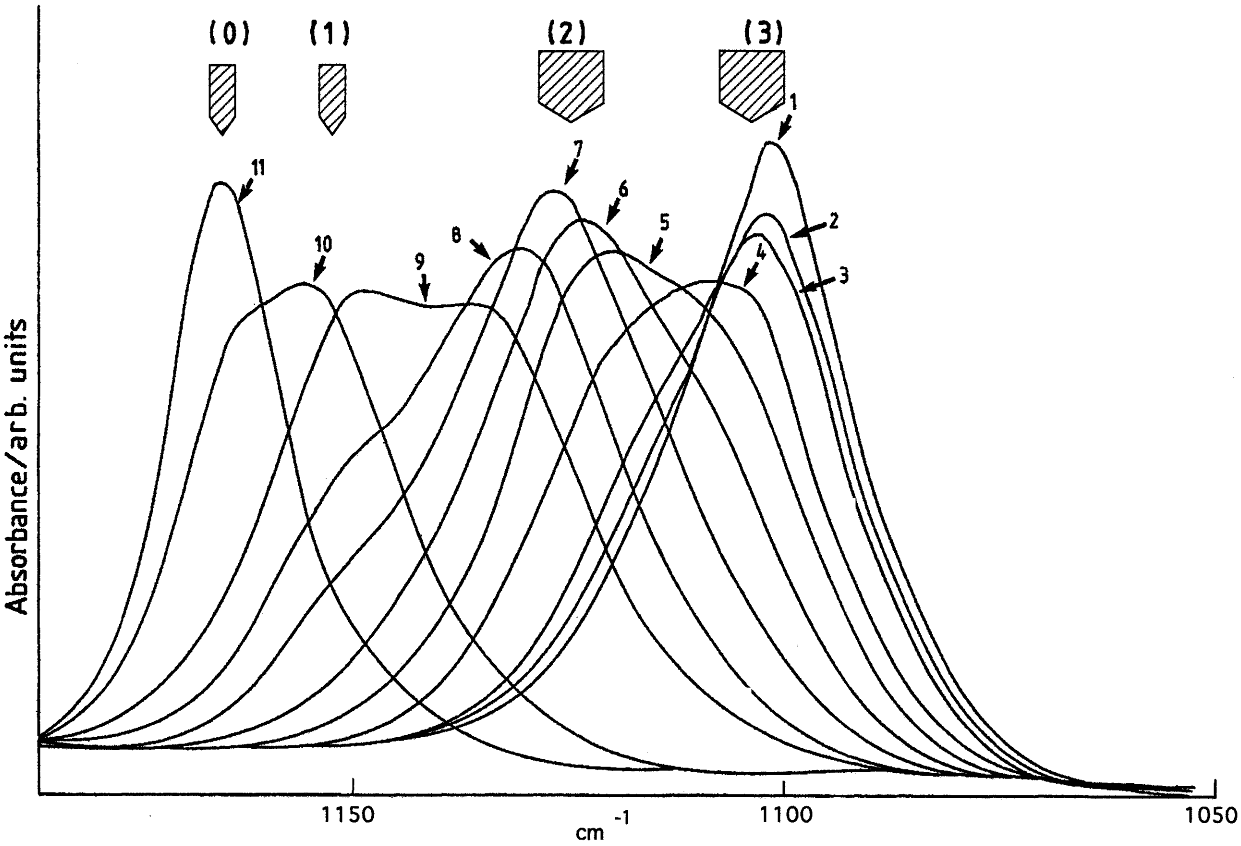


Figure 7. IR spectra for very dilute solutions of Et_3PO in H_2O/MeCN solvent systems. The arrows (0)→(3) indicate the positions of the progressive maxima (due to the proposed H-bonded species A, B and C—see text). Their widths cover the shifts observed caused by increasing secondary solvation. Spectra 1–11 are those observed as the mole fraction of H_2O increases and that of mehtyl cyanide decreases inthe solvent mixtures employed.

water (0 °C) was reached. However, the ^1H resonance band narrowed extensively on warming, so that reorientation times for these waters could be estimated. At 0 °C, the ice phase melts, and normal water ^1H spectra are obtained.

Cell membranes

One of the key roles of biological membranes is to keep the cell contents safe. So membrane channels allow almost free entry and exit to water molecules, but are very selective for everything else that is highly water soluble. Only non-H-bonding molecules such as O_2 and NO move freely and preferentially across the cell membranes All key molecules have special channels with gates, usually involving transmembrane proteins. Also, there are long strands or fronds of carbohydrate units attached to the cell surfaces.

Water permeates into the cell membranes themselves, but the intramembranous concentrations of isolated molecules are too low to be detected by simple spectroscopy. So far as the reviewer knows, it is not possible to learn very much under normal conditions. However, concentrations of molecules and ions are usually higher inside than outside the cells. Hence, intramembranous water molecules will be distributed unevenly, with more on the outside than on the inside of the membrane. On cooling, the outer regions will freeze at higher temperatures than the inner regions. This can be detected by NMR spectroscopy, and the results confirm the expectations.

Interpretations. Various concepts are needed to understand the behaviour of water in biological systems and to pull the results described above together. These concepts include the following: (i) the idea of primary and secondary modes of solvation; (ii) the concept of cooperativity and anticooperativity in solvation and (iii) the postulates of $(O\text{–}H)_{free}$ groups and of $(LP)_{free}$ groups.

I have already explained how secondary solvation in aqueous (and alcoholic) solutions greatly assists primary solvation through the cooperative effect. What is not always appreciated is that, if secondary H bonding

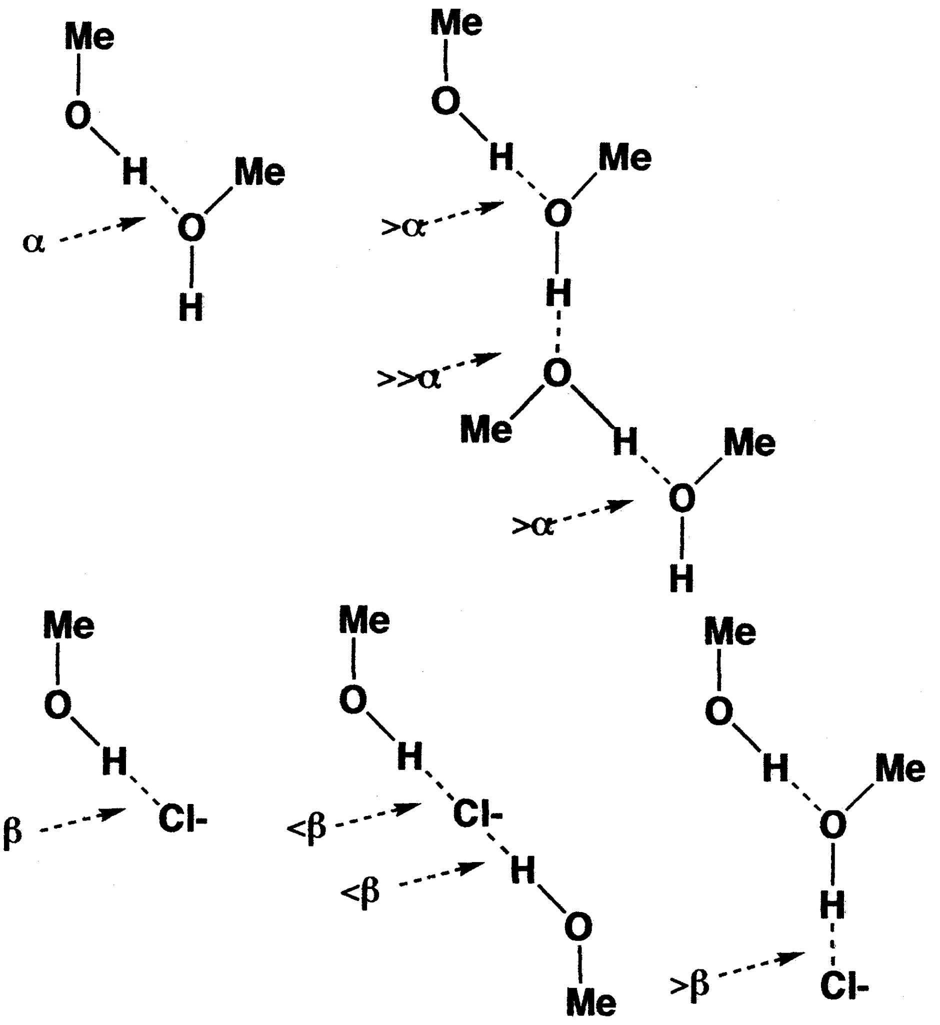


Figure 8. Examples of cooperativity and anticooperativity for hydrogen bonding in pure methanol and for chloride ions in methanolic solution. α, hydrogen bond strength for binary methanol-methanol H bonding; β, hydrogen bond strength for binary mehtanol-chloride H bonding.

occurs ‘the wrong way round’ (cf. the possible H-bonding patterns shown in fig. 5), the primary solvation can be weakened [20].

This is well illustrated with reference to our work on the solvation of chloride ions, which is of course a matter of considerable biological importance, especially as the same general principle applies to all such ions. In summary, when Cl^- forms just one H bond to water or to methanol, this is, as expected, extremely strong. Its IR spectrum is considerably shifted from the position of the pure solvent band, to lower energies [21, 22]. When a second H bond is formed, this band is lost, and a new band, shifted to higher energies, is gained. This establishes that these two Cl···H–O bonds are on average of equal strength, and both are weaker than the initial single bond. This also applies to each step in further H bonding (see fig. 8). Finally, when the full solvation number of 6 is reached, the IR band appears just on the high-energy side of the bulk solvent band. For Br^- and I^- ions, which form weaker H bonds, these bands are further considerably shifted to higher energies, showing that such H bonds are much weaker than the water-water H bonds.

I have often been told that this is wrong. It is argued that if solvent interaction with an anion were so weak, such an anion would never dissolve. There are at least two answers to this criticism. The first is to point out that $Na^+BPh_4^-$ salts dissolve. Why? As discussed above, these anions do not form H bonds, and yet they still dissolve. A stronger answer is to suggest that the negative charge 'pulls the protons in'. In fact, R–Cl compounds are very weakly solvated [23]. Perhaps it is surprising that up to six H bonds can form. The explanation lies in the application of the third postulate.

In my view this postulate, that $(OH)_{free}$ and $(LP)_{free}$ groups are of great importance, holds the key to understanding a lot of aqueous chemistry. They are not as reactive as H_3O^+ and OH^-, but the latter ions are only present at 10^{-7} M, whereas $(OH)_{free}$ and $(LP)_{free}$ groups constitute 8% of all groups at room temperature. Hence, it may be argued that the latter groups must be at least as important as the former. Indeed, they are closely related. Thus, $(OH)_{free}$ groups are predisposed to form hydroxyl (OH^-) anions, since both have an $(OH)_{free}$ group. Similarly, $(LP)_{free}$ groups are closely related to hydronium (H_3O^+) cations, since both have a $(LP)_{free}$ group (M. C. R. Symons, unpublished results). Quite apart from such properties as mobilities or viscosities, we need to consider the availability of free groups for solvation, or for reaction. Thus, for the solvation of halide ions by OH groups, it is the availability of $(OH)_{free}$ groups that favours the high solvation numbers such as those for chloride ions discussed above. The fact that such groups are not available for alcohols explains why solvation numbers for anions or polar aprotic molecules are always less for alcohols than for aqueous solutions. Even more important is the fact that the concentration of these groups is strongly temperature dependent.

This is of particular relevance for kinetic studies of reactions in solution since these are generally analysed over a range of temperatures to give ΔH* and ΔS* parameters. When the plots are not linear, this is seen as an 'anomaly'. Some of these reactions are almost independent of the concentration of 'free' groups. However, some, such as typical S_n1 reactions, depend strongly on the concentrations of $(OH)_{free}$ groups, which are required to solvate the halide (X^-) ions for R–X derivatives [24] when they dissociate. It is often stated that when R–X derivatives are in the process of giving R^+ and X^- ions, these ions are solvated 'in the transition state'. In my view, this is quite impossible, since these transition states have lifetimes comparable with those of the carbon-halide vibrations. Hence, the solvation required to pull the halide ion off has to be there before dissociation can occur.

Equally, reactions, such as deprotonations, that require the presence of $(LP)_{free}$ groups, have rates that are proportional to the concentration of these groups, and if no allowance is made for this, no real understanding is possible. I even wonder how water activities would fare if due allowance were made for these free groups. Such topics, however, would take us beyond the remit of this essay.

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