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Infrared investigations of biologically important hydrogen bonds in halogen containing solvents

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Abstract - It has been known for many years that halogenated solvents hinder the formation of hydrogen bonds. Infrared spectroscopic studies indicate that in certain cases this might have a bearing on biologically important H-bonds. Our original low temperature work led to the suggestion that the anaesthetic potency of halogen containing molecules is connected with the shifting of the free/associated ratio in favour of free or less associated species. More recently we were able to provide evidence for this on model gramicidin ion-channels. (R. Buchet, C. Sandorfy, T.L. Trapane and D.W. Urry). The self-association of alcohols by H-bonds has been shown to be independent of the length of their hydrocarbon chain in saturated paraffin or CCl_4 solutions. In more polar media, like AOT (aerosol of sodium di-2-ethylhexyl sulfosuccinate) which forms inverted micelles this is no more the case, however. In these experiments cholesterol behaved like a short chain alcohol. The degree of protonation of Schiff bases (modelling the chromophore of visual pigments) is not influenced by solvents like CH_2Cl_2 or $CHCl_3$.

INTRODUCTION

It has been known for many years that halogenated solvents hinder the formation of hydrogen bonds. (H-bonds) (refs. 1 to 4); in other terms, perturbation by halogenated molecules tilts the free/association equilibrium in favour of the free (or less associated) species. This can provoke spectacular changes in the infrared spectrum at low temperatures. Examples were given in some of our previous publications on N-H...:N, N-H...:O=C, O-H...:O-H...:O-H type H-bonds. (refs. 4 to 8) Contrary to expectation, as temperature is lowered, the intensity of the free band increases and that of the association bands decreases. The solvent in these experiments was an 1:1 mixture of $CFCl_3$ and CF_2BrCF_2Br but the effect is quite general for halogen containing molecules.

Now, such molecules possess anaesthetic potency. Thus several years ago we started investigations to ascertain if there exists a relationship between H-bond breaking ability and anaesthetic potency. Later, we directed our attention towards the association of alcohols (which also have anaesthetic potency), first in halogen containing solvents and then, in micellar media. This then developed into a more general project of spectroscopic and theoretical investigations on biologically important H-bonds. The most recent facet of this series of works relates to H-bonds in rhodopsins. In subsequent sections some of our new results will be presented and discussed.

HALOCARBONS AS ANAESTHETICS AND SOLVENTS

Most anaesthetics are soluble in lipids but only very slightly in water. It is their solubility in lipids which takes them to the site of action in the lipid membranes of neurons. It is therefore believed that a study in solvents like carbontetrachloride or chloroform can have relevance as a model study on the mechanisms of anaesthesia. Our previous results have shown that there is a smooth relationship between H-bond breaking ability as inferred from infrared intensities and anaesthetic potency. (ref. 7) This, we believe, can be considered as a counterpart to the well-known correlation between anaesthetic potency and lipid solubility. (The Meyer-Overton rule) (For a review see ref. 9, for example). In order to obtain an improved relationship which can be extrapolated to the low anaesthetic concentrations used in clinical practice, H-bond association constants have been determined (ref. 10). As a model system N-ethylacetamide was chosen which forms an N-H...:O=C H-bond, typical of peptides and proteins, with a number of halocarbon anaesthetics. The concentraton of the latter was varied and the H-bond equilibrium constants determined for equilibria of the type:

$$N\text{-}H\ldots O{=}C + CHCl_3 \rightleftharpoons Cl_3\,C\text{-}H\ldots O{=}C + NH$$

It is a well established fact that halocarbons containing a so-called acidic hydrogen, like chloroform, halothane, methoxyflurane have high anaesthetic potency (refs 11 to 13). They also have a strong influence on H-bond equilibria. Halocarbons containing no acidic hydrogen have a much weaker anaesthetic potency and also a much weaker effect on H-bond equilibria (refs. 7,8). However, even the latter dissociate an appreciable proportion of the H-bonds at low temperatures. The association constants for a number of halogenated compounds could be determined at room temperature in CCl_4 solutions by varying the concentration, using a modified version of Drago's procedure (refs. 14, 15). The self association of the amide was taken into account. We only show a few representative results.

TABLE 1. Hydrogen bond association constants and anaesthetic potency of some halocarbons.

	K (M^{-1})	log (1/P)
$CFCl_3$	0	
CH_2Cl_2	0.27±0.07	1.52
$CHCl_3$	0.74±0.09	2.08
$CF_3CHClBr$ (halothane)	1.08±0.20	2.11

K stands for the H-bond association constant. Log (1/P) is a measure for anaesthetic potency where P is the pressure in atmospheres at which the righting reflex of half of the experimental animals is suppressed. The parallelism of K and log (1/P) is to be noted. Extrapolation of the data to low concentrations shows that the H-bond breaking effect of these strong general anaesthetics is weak but still significant at clinical concentrations. Which are the H-bonds that could be affected by these halogen containing molecules? The functioning of the nervous system hinges on the permeability of the neuron membrane to ions, mainly K^+ and Na^+. The ions have to pass through ion-channels which are formed in proteins lying across the lipid membrane. The permeability is determined by the structure and dimensions of these ion-channels in which H-bonds play a decisive role. The peptide H-bond, N-H...:O=C is expected to be the most important of them. Actually, while the hydrophobic or lipid theories of anaesthesia still carry much weigth among anaesthesiologists, evidence is accummulating pointing to the protein as the site of anaesthetic action. Here we refer particularly to the works by Haydon and his coworkers. (refs. 16 to 18). It is then logical to expect that anaesthetics perturb the ion-channel. This could be done indirectly by disorganizing the surrounding lipid or directly by acting upon the protein containing the ion-channel. Another possibility is to perturb neurotransmitter release at the synapse. (refs. 19, 20) In both cases the anaesthetic would provoke a change in the permeability of the neuronal membrane to ions. In several papers of our laboratory a "pluralistic" theory of anaesthesia has been advocated (refs. 8, 21, 22, 23); a weak anaesthetic action would be possible through perturbing only the lipid, (what almost every molecule can do), but for strong action the protein itself, (which contains the ion channel), must be affected.

Recent experiments on model gramicidin-A ion-channels (ref. 24) strongly support this view. Gramicidin A is a channel forming peptide containing fifteen aminoacids whose sequence has been determined. (refs. 25,26) Hladky and Haydon (refs. 27,28) were the first to charactarize this system and to recognize its potentiality for research on the functioning of the nervous system. Haydon and his coworkers (refs. 16 to 18) and Urry and his coworkers (refs. 29 to 32) applied it extensively in their investigations on the mechanisms of anaesthesia. When incorporated into planar lipid bilayers, gramicidin A exhibits conductance properties similar to those of ion-channels in living organisms. (ref. 26). As lipids Urry et al. used 1-alkyl (C16:0, C18:0)-2-acetoylphosphatidyl-choline (AAPC) or L-α-lysophosphatidylcholine among others. They were able to show (ref. 30) by measuring the current across the gramicidin A ion-channels that halocarbon anaesthetics like halothane strongly decrease the rate of opening and closing of the ion-channel at physiological concentrations. (About 1m M or less) (See also ref. 33).

The Fourier-transform infrared investigations of Buchet et al. (ref. 24) have given strong indication for the direct interference of halocarbons containing an acidic hydrogen with the peptide type H-bonds of the ion-channel. Urry et al. (ref. 29) have demonstrated that the model gramicidin A ion-channel is formed by two gramicidin molecules, "...each in a β helical conformation, associating amino end to amino end by six H-bonds to form a continuous lipid spanning transmembrane channel about 26A in length". The infrared spectra of films of gramicidin/lipid/water samples were recorded on a Nicolet 6 DXB FTIR instrument. The amide NH stretching band could be distinguished at 3285 cm^{-1}, the ester carbonyl of the lipid at 1741 cm^{-1}; two amide I (mainly carbonyl stretching) bands were observed at 1670 and 1639 cm^{-1} and an amide II (mainly NH in-plane deformation) band was found at 1552 cm^{-1}. Upon addition of methoxyflurane ($CH_3OCF_2CHCl_2$) the NH stretching band shifted to 3307 cm^{-1} showing that some of the H-bonds were broken. The ester carbonyl band was unaffected and could therefore be used as an internal standard for the significant changes which occurred in the relative intensities of the amide carbonyl bands. The 1639 cm^{-1} bend greatly

increased in intensity while the 1670 cm^{-1} band was reduced to a shoulder; instead a weaker band appeared at 1680 cm^{-1}. Water does make a contribution in this part of the spectrum but this does not change these conditions. The 1670 and 1639 cm^{-1} bands can only belong to amide groups. Now, there are three types of H-bonded carbonyls in the model gramicidin A channel (ref. 29). Those forming the strongest H-bonds (2.95A) are within the monomers; the band at 1639 cm^{-1} is related to these. Six weaker H-bonds (3.20A) hold together the two monomers at the head-to-head junction; the 1670 cm^{-1} band is naturally assigned to these. This band might receive a contribution from the three C=O groups at the entrance of the channel which are probably weakly H-bonded to water. The spectacular decrease in the intensity of the 1670 cm^{-1} band clearly demonstrates the fact many of the H-bonds which provide for dimer formation at the head-to-head junction have been broken. Furthermore, proof has been found in the spectra that instead of the N-H...:O=C bonds, CCl_3H...:O=C bonds are formed. Using $CDCl_3$ as the perturber an associated C-D band is found at 2222 cm^{-1} besides the free C-D stretching hand. Both spectroscopic (refs 4 to 8) and quantum chemical (refs. 22, 23) evidence has been given to the effect that in such cases a

$$\text{N-H...:O=C} + CHCl_3 \rightleftharpoons \text{NH} + \text{C = O:...}HCCl_3$$

equilibrium is indeed established and that a sizeable number of the amide/carbonyl H-bonds are replaced by amide/chloroform H-bonds. The same results were obtained with chloroform, halothane, and methoxyflurane. To the contrary, carbontetrachloride and n-pentane have only a very slight effect on the relative intensities of the 1670 and 1639 cm^{-1} bands and n-decane has none. All these observations are well in line with the weak anaesthetic potency of the latter molecules and the strong potency of the anaesthetics containing an acidic hydrogen. The dissociative effect on the peptide (and other) H-bonds is confirmed by these experiments.

ALCOHOLS IN HALOGENATED SOLVENTS

The facts mentioned in the previous section clearly show that anaesthesia is closely linked to the alteration of the pattern of associations. For this reason it can be considered as a key phenomenon for investigations of molecular interactions.

Alcohols are known to have anaesthetic potency. Both their self-and heteroassociations were the object of intensive studies for the last four decades. Their surprising behaviour with respect to their anaesthetic potency induced us to reconsider some aspects. This potency increases gradually from methanol to n-dodecanol (C_2 to C_{12}). Tridecanol (C_{13}) however, has only a lesser potency and tetradecanol (C_{14}) and the higher homologues have none at all. A great deal of effort was deployed to explain this "cutoff" (refs 34, 35, 36). On the other hand, n-alcohols with less than ten carbon atoms decrease the gel to liquid crystal transition temperature of the neuronal membrane and increase its fluidity. Alcohols with more than ten carbons have the opposite effect and C_{10} has practically no effect.

In order to find clues through infrared spectroscopy we remeasured the spectra of n-alcohols from C_1 to C_{18}. Our aim was to ascertain if the length of the hydrocarbon chain has an influence on the free/associated equilibrium of association by H-bonding. For this purpose Wilson et al. (ref. 37) recorded both infrared and near-infrared spectra of the n-alcohols from C_1 to C_{18} in the OH stretching regions for 0.2M solutions at 5,23 and 37C. The molar absorption coefficients and band width were the same for all spectra at all three temperatures except for a slight difference in the cases of methanol and ethanol; the spectra coincided from C_3 to C_{18}. While this result does not explain the anaesthetic properties of alcohols, the observed independence of the extent of association from the length of the hydrocarbon chain is intriguing. The same result was obtained when n-paraffins were used as solvents instead of CCl_4. The extent of H-bonding turned out to be independent of the length of the hydrocarbon chains in both the n-alcohol and the solvent. All this concerns self-association by H-bond formation. However, the same result was obtained for alcohol OH/ ester carbonyl association using ethyl esters of acids of different chain lengths. (The characteristic band is at 3545 cm^{-1} (ref. 37). The first overtone of the OH stretching vibration gave similar results. It appears then that the free/associated equilibrium for the self-association of n-alcohols by hydrogen bonds or their association with carbonyl groups is independent of other (Van der Waals) interactions that exist in these solutions.

It is logical to inquire if the above findings remain valid in media in which conditions approach those in true cell membranes. Ménassa et al. (ref. 38) chose sodium di(2-ethylhexyl)sulfosuccinate (commercially known as AOT) which forms inverted micelles in carbontetrachloride in the presence of traces of water. (17 molecules per micelle on the average.) (ref. 39). They determined alcohol/AOT association constants for alcohols of different chain length. First they compared a 0.2M solution of the alcohol in CCl_4 with the same with 0.02M AOT added. Both the free and multimer self-association bands (3639 and 3346 cm^{-1} for n-hexanol) decreased in intensity somewhat, indicating that AOT dissociates a part of the multimers and associates with the free OH groups. Then they increased the concentration of AOT twenty times to 0.4M. The monomer band decreased sharply and the

multimer band disappeared completely. Instead, a new band appeared at 3417 cm^{-1} due to alcohol/AOT complex formation. The association constants for this were calculated using dilute (0.02M) solutions of alcohols in CCl_4 with varying amounts of AOT in order to avoid the self-association of the alcohol. The result was clear: alcohol/AOT association decreases with the increase of the length of the hydrocarbon chain of the alcohol. AOT contains two kinds of polar groups: carbonyls and sulfonates. Now, the carbonyl band remains insensitive to the presence of increasing amounts of AOT while the SO_3^- group responded with changes in frequency (1250 to 1242 cm^{-1}) and intensity. The SO_3^- groups are located deeply inside the micelle. This indicates that the alcohols penetrate into the micelle and very likely form H-bonds with the sulfonate groups (ref. 40). Since the diameter of the micelles is about 12A it is clear that the larger alcohols will have difficulty in penetrating and a part of these molecules will remain outsides. The association constants (K, in LM^{-1}) obtained by Ménassa et al. (ref. 38) were about 18 for C_1, 11 for C_3, 7 for C_6, then they level off at about 6 for the longer chains. While AOT is a remote model for actual membranes this levelling off resembles the "cutoff" mentioned above. One might speculate that alcohols penetrate into the neuronal membrane in an attempt to reach a polar site, the penetration becoming partial as the hydrocarbon chain length increases. Short chain, branched alcohols like isopropanol seem to penetrate easily the AOT micelle (K about 13) while a long chain, branched alcohol like 4-decanol hardly penetrates. (K about 2) As expected, K is very high for water. (57!) It is most interesting that cholesterol, despite its bulky hydrocarbon radical behaves like a short chain alcohol with a K value of about 11. On the other hand the great tendency to self-association of cholesterol was noted before (refs 41, 42). It is then logical to speculate that these properties of cholesterol are connected with the role it plays in physiological events (ref. 43).

THE PROTONATION OF SCHIFF BASES IN HALOGENATED SOLVENTS

The chromophores of rhodopsins, in both visual pigments and bacteria are isomers of the Schiff base of retinal. The C=N double bond is conjugated with five C=C double bonds and is covalently bound to the surrounding apoprotein (for a review see refs 44, 45). Upon light absorption the Schiff base undergoes isomerization; at the same time proton translocation is observed (ref. 46). According to most researchers on rhodopsins the Schiff base is protonated. The identity of the proton donor has not yet been definitely established. In all likelihood it is aspartic or glutamic acid; they are known to be present in the vicinity of the chromophore (ref. 47). While the structure and functioning of these pigments is beyond the scope of this lecture, we should like to mention some of our recent results on the protonation of model Schiff bases. These studies consisted in taking FTIR spectra in chloroform solutions of mixtures of Schiff bases and carboxylic acids; our aim was to investigate if protonation of a Schiff base is possible with the relatively weak acids available in the pigments (ref. 48). For this purpose the $\nu(C=N)$ and $\nu(C=N^+)$ bands can be used as well as the carbonyl bands of the carboxyl and carboxylate groups. The $\nu(C=N)$ bands for non-conjugated imines and conjugated imines with one, two and five C=C bands (retinylidene imines) were found near: 1666, 1656, 1630 and 1619 cm^{-1}, respectively. The $\nu(C=N^+)$ are at 1712-1708, 1687-1684, 1681-1677, 1660-1650 cm^{-1} in the same order. (Lussier et al. (ref. 48)). From the intensities of these bands approximate values can be calculated for the percentage of protonation pertaining to the equilibrium:

$$-C=N\ldots H-O \rightleftharpoons -C=N^+-H\ldots O^-$$

where O^- represents a COO^- group.

The main conclusions are that, a) the weaker acids do not protonate the Schiff bases entirely but only from 50 to 80 p.c. and b) the extent of protonation increases with increasing conjugation. This means that the weak acids available in rhodopsins would not protonate them 100%; additional stabilization is required by polar groups in order to obtain this. The exact mechanism of stabilization is still an important issue in rhodopsin research.

A question of some importance is, in the present context, if the $CHCl_3$ solvent does not influence the percentage of protonation. Indeed, as we have stated, chloroform is a H-bond breaker for weaker H-bonds. To ascertain this point, the spectra were remeasured in CH_2Cl_2 which is an even weaker proton donor than $CHCl_3$. The results were the same within the limits of experimental error. Chloroform is not a major problem in protonation studies.

CONCLUDING REMARKS

Halogenated solvents hinder the formation of H-bonds. In particular those which contain an acidic hydrogen do affect H-bond equilibria. The anaesthetic potency of such molecules constitutes a convenient point of entry into investigations on biologically important H-bonds. Halocarbon anaesthetics, alcohols and the H-bond involved with the mechanism of vision are in this category. The same applies to nucleotide base pairs (refs. 49 to 51) but this topic will not be pursued here.

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