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Isotope Effects on Chemical Shifts in the Study of Hydrogen Bonded Biological Systems

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ABSTRACT

This review deals with biological systems and with deuterium isotope effects on chemical shifts caused by the replacement of OH, NH or SH protons by deuterons. Hydrogen bonding is clearly of central importance. Isotope effects on chemical shifts seems very suitable for use in studies of structures and reactions in the interior of proteins, as exchange of the label can be expected to be slow. One-bond deuterium isotope effects on ^{15}N chemical shifts, and two-bond effects on ^{1}H chemical shifts for N(D)H_x systems can be used to gauge hydrogen bond strength in proteins as well as in salt bridges. Solvent isotope effects on ^{19}F chemical shifts show promise in monitoring solvent access. Equilibrium isotope effects need in some cases to be taken into account. Schemes for calculation of deuterium isotope effects on chemical shifts are discussed and it is demonstrated how calculations may be used in the study of complex biological systems.

Contents

1. **Introduction**
2. **Deuterium isotope effects on ^{15}N chemical shifts**
3. **Deuterium isotope effects on ^{19}F chemical shifts**
4. **Deuterium isotope effects on ^{13}C chemical shifts**
5. **Deuterium isotope effects on ^{1}H chemical shifts**
6. **Solvent isotope effects (SIIS)**
7. **Theoretical calculations**
8. **Equilibrium Isotope Effects**
9. **Summary**

References

1. Introduction

The present review covers primarily findings published since the year 2000.

Isotope effects on chemical shifts, especially those caused by deuterium, have become an important tool in the investigation of hydrogen bonding. As hydrogen bonds form a vital part of the structures of biological molecules, valuable information can be gained. Furthermore, as it is OH, NH or SH functional groups that are usually involved in hydrogen bonding, deuterium exchange is easy to accomplish. Preparation of biosynthetically deuteriated molecules also leads to a large number of deuteriated molecules. However, recent reviews containing information about this topic are available [1,2,3], as is an overview of isotope effects of ionizable functional groups of amino acids [4]. Accordingly, the present review will concentrate on deuteriation other than at carbon. Enzymatic actions often involve a number of amino acid residues, co-factors etc. acting together, and investigating this using liquid state NMR can often be difficult. To add extra information, such as that available from interpreting isotope effects sensitive to hydrogen bonding and charges, can be of great help. The nature of hydrogen bonds in biological molecules is not special. Therefore, when new types of isotope effects are found in biological systems, it may often be useful to study model systems. In the present review isotope effects are defined as: $^{n}\Delta X = \delta X(H) - \delta X(D)$, where n is the number of bonds between the label and the nucleus, X, in question. Note that in some other papers the opposite sign convention is used; in this review, such data are converted in accordance with the present definition and marked with an $^{\#}$. It is important to note that in non-equilibrium systems, the isotope effects are intrinsic, whereas in equilibrium systems the observed effect is comprised of both an averaged intrinsic effect and an equilibrium effect (see Sect. 8). Intrinsic isotope effects are due to the fact that the vibration for a deuterated bond occurs lower in the potential well than for a hydrogen one. If the potential is asymmetric this will lead to a different average bond length for XH and XD, which in turn causes different chemical shifts leading to the observed isotope effect. In the equilibrium case, deuteriation also leads to a change in the chemical equilibrium, causing a change in the chemical shifts. [2]

To observe deuterium isotope effects as two separate signals clearly requires slow exchange of the deuterium. This can impose a limitation, especially for the XH(D) type of system investigated in the present review. A number of pulse schemes have been constructed to overcome this problem. The HISQC sequence [5] was specially designed to deal with NH_3 groups to avoid the problems of exchange. Mackenzie and Hansen [6] used a ^{13}C-detected ^{15}N double-quantum experiment to detect ^{15}Nη in arginines. This limitation can also be overcome by measuring at a series of H_2O/D_2O ratios (see also SIIS). One example is the study of isotope effects in genistein [7]. This procedure is of course more time-consuming, but can extend the study of isotope effects to systems with fast exchange.

The natural nuclei for observation will be ^{13}C and ^{15}N and, less frequently, ^{1}H. However, ^{19}F has in a number of cases also been studied in molecules in which ^{19}F is present. The isotope pair for which the isotope effect is measured will in most cases be ^{1}H, ^{2}H.

In biological compounds the hydrogen bonds relevant to isotope effect measurements are typically intramolecular. Most of the hydrogen bond donors are of NH, OH or, less frequently, SH type. The majority of NH donors are in backbone CONH groups involved in helixes and β-sheets, but side chain CONH groups can also be involved in hydrogen bonding. A few of the hydrogen bonds are of the Resonance Assisted Hydrogen Bond (RAHB) type, typically in DNA and RNA. Resonance assistance is characterized by a situation in which the hydrogen bond donor (OH,NH,SH) and the hydrogen bond acceptor are connected by a “double” bond path. COO- … NH^+ (salt bridges) may either be buried or, more frequently, exposed. As biological molecules are hydrated, isotope solvent effects clearly occur. All these situations may be studied using deuterium isotope effects. Tautomeric systems also occur. In this case isotope effects are particularly useful in detecting and describing the tautomerism. Calculation of isotope effects on chemical shifts will also be treated briefly (see Sect. 7).

2. Deuterium isotope effects on ^{15}N chemical shifts

^{15}N is a very useful nucleus, first of all because it is often present in biological molecules, and secondly because of its large chemical shift range and often very large chemical shift differences between e.g. single and double bonded nitrogen. The most frequent parameter is the one-bond deuterium isotope effect, $^{1}\Delta N(D)$. A characteristic feature of this parameter for NH groups involved in hydrogen bonding is that it decreases the stronger the hydrogen bond. This is true both for sp^3 [8] and sp^2 hybridized nitrogens [9].

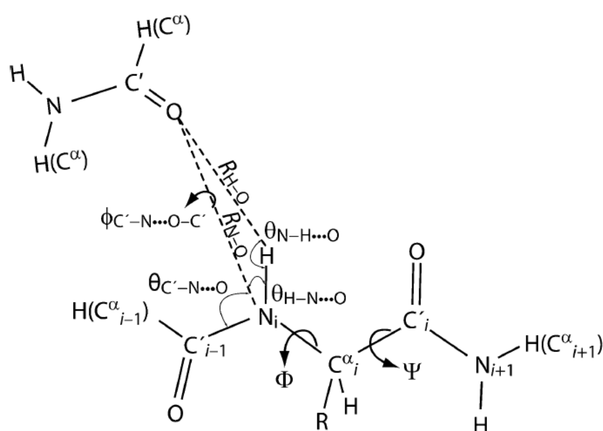

Figure 1. Angles and distances used to predict $^{1}\Delta N(D)$. Taken from Ref. [9] with permission from J. Biolmol. NMR.

Backbone CONH(D) isotope effects have been measured in ubiquitin and correlated to backbone angles, but also to a term containing both electric field effects and anharmonicity [9] Eq. 1:

$$^{1}\Delta^{15}N(D) = A\,\Phi' + B\,\Psi' + C\cos(\theta_{N\text{–}H\cdots O})/R_{H\cdots O} + D \quad \text{Eq. 1}$$

where $\Phi' = \cos(\Phi + 90°)$, $\Psi' = \cos(\Psi - 70°)$, $A = 0.058 \pm 0.008$ ppm, $B = 0.041 \pm 0.005$ ppm, $C = 0.05 \pm 0.02$ ppm·Å, and $D = 0.65 \pm 0.01$ ppm. Symbols are those of Fig. 1. An important feature of this study was that the hydrogen bond positions were recalculated using DFT calculations.

However, this equation is only valid for amino acids without charged side chains, highlighting that charges in the protein need to be taken into account. Zhang and Tugarinov [10] arrived at a slightly different equation using data from ubiquitin and GB1, Eq. 2:

$$^{1}\Delta N(D) = 689 + 13\sin(\phi+117^{o}) + 43\cos(\psi-60^{o}) + 16\cos(\Theta_{N\text{-}H\ldots O}) \quad \text{Eq. 2}$$

Here, isotope effects are specified in ppb and glycines are excluded. $\Theta_{N\text{-}H\ldots O}$ is the angle formed between N-H and the direction of a hydrogen bond in crystal structures of ubiquitin and GB1.

For perdeuteriated amino acids the effects of deuteriation at carbons also play a role. Maltsev, Ying and Bax [11] investigated α-synuclein and found that the value of the observed isotope effect at ^{15}N depended on the preceding residue. Clearly a glycine had the largest effect, roughly twice that of the other residues, due to the fact that it has two CαH protons. Strangely enough, Ile and Val had the opposite sign to the other residues. In relation to this, a more specific study of the effect of deuteriation at the C-β carbon has been conducted by Tugarinov [12]. Recently, Smith et al. [13] found similar values for perdeuteriated proteins in a solid state study of HET fibrils, but they did not study these further for reasons of variying pH.

Liu *et al.* [14] measured differential deuterium isotope effects on ^{15}N ($^{1}\Delta^{1}\Delta N(D) = {}^{1}\Delta N(D^{E}) - {}^{1}\Delta N(D^{Z})$) of amide groups of the amino acid side chains of Gln and Asn. Such measurements will in most cases detect hydrogen bonding, as one hydrogen is involved and the other not. Most values are positive, but for one particular Asn in the protein studied (Asn51 in yeast cytosine deaminase) it is negative, reflecting that it is the Z-hydrogen that is deuteriated. $^{1}\Delta^{1}\Delta N(D)$ can be expressed by a number of terms: $^{1}\Delta^{1}\Delta N(D) = \Delta c + \Delta j + \Delta h + \Delta e + \Delta o + \Delta a$. The two first terms represent a configurational effect, the second is due to differential one-bond couplings, the third term reflects hydrogen bonding, the fourth represents the changes involved

in hydrogen bonding, the fifth term is due to hydrogen bonding through the oxygen atom of the carboxamide and the last term is due to trans hydrogen bonding. Hydrogen bonding through bifurcated hydrogen bonds is also described in Ref. 15. The use of these differential effects also enables side-chain amide groups involved in hydrogen bonding to be distinguished from those not taking part in interactions.

One bond isotope effects, $^{1}\Delta N\eta(D)$, have also been measured in side-chains such as arginines. Very little variation was seen for arginines of the protein T4L99A [6]. One-bond deuterium isotope effects on ^{15}N chemical shifts, as well as $^{2}\Delta H(D)$ isotope effects, were essential in proving that some salt bridges seen in the solid state for the B1 domain of protein G do not exist in solution [16] (see also theoretical calculations, Sect. 7). Similar examples were seen in a study of barnase [17]. In a study of HoxD9 homeodomain bound to DNA, a lysine group is forming a buried salt bridge with an oxygen of a phosphate group. A $^{1}\Delta N(D)$ of 0.35 ppm can be estimated [18] (see also Sect. 7).

Isotope effects are particularly useful in the study of DNA and RNA, as ring current effects, which would dominate structural interpretations of chemical shifts, play no role. LiWang et al. [19] have measured isotope effects across hydrogen bonds, $^{2}\Delta C2(ND)$, in A-U and A-T base pairs (See Fig. 2). They found that the isotope effects correlated with the corresponding ^{1}H chemical shifts of imino protons. This supported the view that isotope effects can be useful in gauging hydrogen bond strength [20]. As $^{2}\Delta C2(ND)$ values are more negative for RNA than for DNA it was concluded that the RNA hydrogen bonds are stronger than those of DNA. In a similar study it was concluded that hydrogen bonding and π-π base stacking interactions are coupled in DNA [21]. This conclusion is also supported by theoretical calculations (see Section 7).

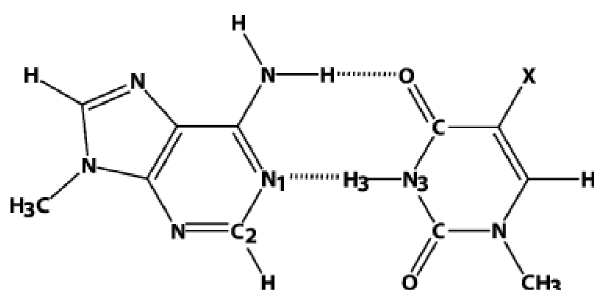

Figure 2 . A-U and A-T base pairs. X= H or CH_3, respectively.

Studies of Schiff bases are described under theoretical calculations (see Section 7).

3. Deuterium isotope effects on ^{19}F chemical shifts

^{19}F nuclei, because of their large chemical shift range, are very suitable for detection of long-range deuterium isotope effects. A model system is shown in Fig. 3 in which isotope effects over nine formal bonds can be detected [22].

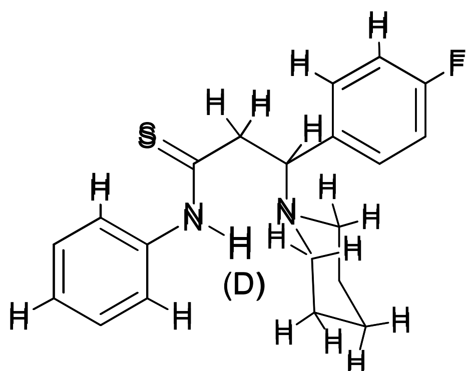

Figure 3. The long-range deuterium isotope effect on ^{19}F from exchanging the NH for ND is 35 ppb.

In biological systems, deuterium isotope effects on ^{19}F can occur in fluorine-substituted proteins (see also Section 6 on solvent isotope effects) or in fluorine-substituted DNA´s or RNA's. ^{19}F is also very frequently present in drugs. An effect over four formal bonds has been reported in 5-fluorocytidine deuteriated at the NH_2 group due to exchange with the solvent and built into an oligonucleotide [23]. The authors suggest the intramolecular hydrogen bond between C-5 and H-ND-4 important,but also the effect due to the non-hydrogne bonded NH(D) is clearly seen.

4. Deuterium isotope effects on ^{13}C chemical shifts

Deuteriation at the NH position in a protein will lead to isotope effects at the adjacent carbons. For Cα Zhang and Tugarinov [10] have extended the original study of ubiquitin [24]. For $^3\Delta C\alpha(N_{i+1}D)$ it turns out that residues preceding glycine deviate from the Karplus-like curve. Also, for $^2\Delta C\alpha(N_iD)$ a number of outliers are found. For $^3\Delta CO(N_iD)$ the early prediction that the OHN distance was important [25] is limited to distances less than 3.5Å, and the interaction determining the magnitude of the isotope effects is not purely electrostatic. For $^2\Delta CO(N_iD)$, by extending the data from BPTI [25] with data from ubiquitin and GBI Zhang and Tugarinov [10] found an average value of 0.082 +- 0.006 ppm. The variation is unfortunately rather low. $^3\Delta C\beta N_iD)$ values have also been measured [10].

The isotope effects of exchanging the OH proton of tyrosines buried in the interior of the protein enables an estimation of exchange rates. In peptidyl-prolyl *cis-trans* isomerase b, two tyrosines showed slow exchange whereas a third showed fast exchange [26]. In a similar way the exchange of the SH proton of cysteines has been studied in an environment with low water access and slow exchange of the label [27].

5. Deuterium isotope effects on ^1H chemical shifts

Although the ^1H chemical shift range is small and the observed isotope effects are usually also small, such effects may be useful. For nitrogens with two hydrogens attached, it is possible to observe a two-bond deuterium isotope effect. Two-bond deuterium isotope effects in ammonium ions [8] and lysine amine groups change sign from positive to negative as the heavy atom distance between the nitrogen and the hydrogen bond acceptor is reduced [16].

Yuan *et al.* [28] observed two four-bond deuterium isotope effects at N-Hε (0.15 ppm) and N-Hδ (0.05 ppm) of His48 of phospholipase A2 hydrogen bonded to a transition state analogue inhibitor. The N-Hδ was observed at 18 ppm, showing a very strong hydrogen bond (See Fig. 4). The finding that this leads to a larger isotope effect at Hε (12.67 ppm) than at Hδ is in line with observations in smaller molecules such as substituted 2,6-dihydroxyacetophenones [29], and may have a much wider application as histidines often are part of the catalytic sites of enzymes.

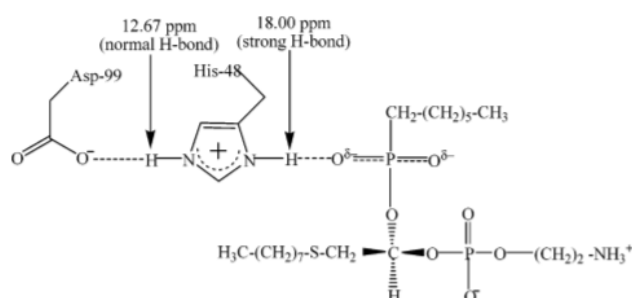

Figure 4. Phospholipase A2 and a phosphonate transition state analogue. From Ref. 28 with permission from the American Chemical Society.

6. Solvent isotope effects (SIIS)

Solvation is clearly also an interaction that very frequently involves hydrogen bonding. Using mixtures of H_2O:D_2O one can, for sensitive nuclei like ^{19}F, measure a solvent deuterium isotope effect by making a series of measurements at different ratios and extrapolating to 0 and 100% D_2O. The lack of such an effect could indicate a lack of water access. This was first established by Hull and Sykes in alkaline phosphatase [30] and later confirmed by model studies in cyclodextrins [31]. Model studies showed also that the

solvent isotope effects were largest for high electron density at the fluorine atom. A typical probe used in proteins is fluorine-substituted phenylalanine, tyrosine or tryptophan. This technique has been used in a number of contexts. Evancis et al. [32] used this technique together with paramagnetic effects to probe protein-protein interactions, specifically peptide binding to a SH3 domain. Bai, Luo and Peng [33] studied deuterium SIIS in α-lactalbumin with ^{19}F labelled amino acids and found that most of the fluoroalanines and tryptophans were buried in the native protein. They also noted small SIIS values of opposite sign for these sites. In the unfolded state all SIIS had the same magnitude, corresponding to full water access, whereas in the molten globule state W104 and W118 remained significantly buried, whereas the other amino acids were partly exposed. Solvent exposure in glucagon [34] and high-potential iron protein [35] was likewise studied. Cho and Zhou [36] interpreted the lack of a SIIS as a proof that the fluorine labelled 7-fluoro-N-acetyl-2-aminoofluorene (FAAF) was intercalated into a DNA helix. Likewise, a *Nar*I-Induced -2 frameshift mutagenesis was studied [37]. Jin et al. [38] studied cPK-ADP complexes with MgF_3^-,AlF_4^- and BeF_3^- and concluded that the larger SIIS found for fluorine A pointing towards S53 in the magnesium complex was due to a high local proton density. SIIS can easily be measured accurately. The finding that for buried residues isotope effects of the opposite sign can be found could probably be due to long-range effects from NH protons being deuteriated.

it is also relevant to mention briefly an isotope effect due to ^{18}O. In a model study [31] no effect was observed by using $H_2{}^{18}O$. However, Arnold and Fischer [39] report an effect on 5-fluorouridine, but not on 5-fluorouracil. The effects can be of either sign, depending on the degree of ^{18}O enrichment. The effect on fluoride ions was also measured as a function of the degree of enrichment and the concentration, but no change of sign was found.

7. Theoretical calculations

Theoretical calculations of isotope effects on nuclear shieldings can be done as described by Jameson, [40] leading to the following expression:

$$\langle\sigma\rangle - \langle\sigma^*\rangle = \sum_i \left(\frac{\delta\sigma}{\delta r_i}\right)_e \left[\langle\Delta r_i\rangle - \langle\Delta r_i\rangle^*\right] + \sum_{ij}\left(\frac{\delta^2\sigma}{\delta r_i \delta r_i}\right)_e \left[\langle\Delta r_i \Delta r_j\rangle - \langle\Delta r_i \Delta r_j\rangle^*\right] + \sum_{ij}\left(\frac{\delta\sigma}{\delta\alpha_{ij}}\right)\left[\langle\Delta\alpha_{ij}\rangle - \langle\Delta\alpha_{ij}\rangle^*\right] + \ldots \qquad \text{Eq. 3}$$

* refers to the heavier isotope. r refers to bond length, α to angles.

This equation can often be simplified so only the first term is included. This then requires calculation of the change in the nuclear shielding and an estimate of the change in the XH bond length upon deuteriation, which is obtained by calculating energies as a function of the XH bond length, leading to an asymmetric potential energy curve of the type shown in Fig. 5.

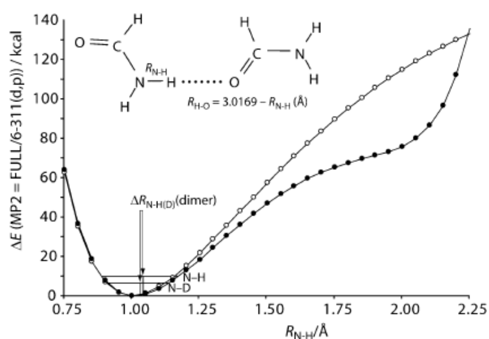

Figure 5. Example of theoretical hydrogen bond potential, in this case calculated for formamide. Open symbols are for the monomer, closed symbols for the dimer. Taken from Ref. 9 with permission from J.Biomol NMR.

This strategy was demonstrated for intramolecular hydrogen bonded systems [41]. The approach was further used by Piana *et al.* [42], who calculated deuterium isotope effects on ^{13}C chemical shifts in the Asp dyad of HIV-1 PR for different protonated states. They compared calculated ^{13}C chemical shifts with the experimental values and conclude that the dyad as shown in B1 (Fig. 6) was the preferred arrangement.

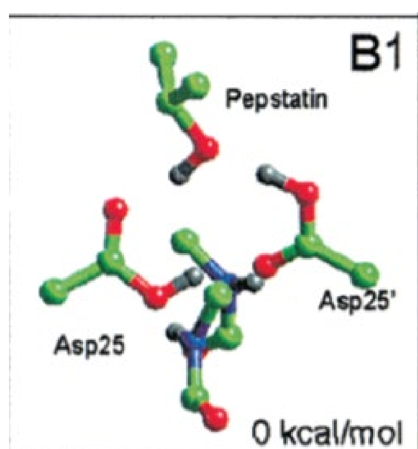

Figure 6. Preferred dyad for HIV-1 PR. From Ref. 42 with permission from the American Chemical Society.

In cases where isotope effects of many amino acid residues have to be calculated, this method is less useful, as the hydrogen bond potential has to be calculated independently for each hydrogen bond [**Fejl!**

Bogmærke er ikke defineret.]. Deuterium isotope effects on ^{15}N and ^{1}H chemical shifts, both experimental and theoretical, have played a major role in determining the presence or absence of salt bridges in protein G [16]. Although the two-bond deuterium isotope effect is small, it turned out to be more sensitive to hydrogen bonding.

LiWang *et al.* [19] has calculated isotope effects across hydrogen bonds, $^{2}\Delta$C2(ND), in A-U and A-T base pairs (See Fig. 2) and find that the isotope effects depend on the N…N distance.

A recent approach to calculating such potentials is to use multicomponent hybrid density functional theory combined with, in the first instance, the polarizable continuum model. This is a rather fast method. [43] It was tested using calculations for picolinic acid N-oxide and a very convincing result was obtained even for the primary isotope effect [44]. Subsequently, the same method has been applied to salt bridges, where it was demonstrated that $^{1}\Delta$N(D) decreases as the counter-ions move closer. This enables an estimation of the presence of hydrogen bonding, counter ions and water (see below). Calculations based on X-ray data for protein G did not lead to a good correspondence with experimental values, most likely because the salt bridges in solutions are very different from those in the solid state [45]. An extension including the ONIUM scheme has been used to investigate the hydrogen-bonding center of photoactive yellow protein [46].

The use of theoretical calculations enables one to test one of the parameters that is often unknown, namely the influence of water. The effect of hydration (see Fig. 7) has been calculated (Table 1) and, as in the case of hydrogen bonding, it is a reduction of $^{1}\Delta$N(D) [45]. However, for the case of an NH(D) bond that does not point towards water, the counter ion or the salt bridge partner (see Fig. 8), hydration leads to a small positive contribution, as can be seen from Table 1. In Fig. 8 the arrangement shown is that of a salt bridge in HoxD9 homeodomain, as mentioned in Section 2.

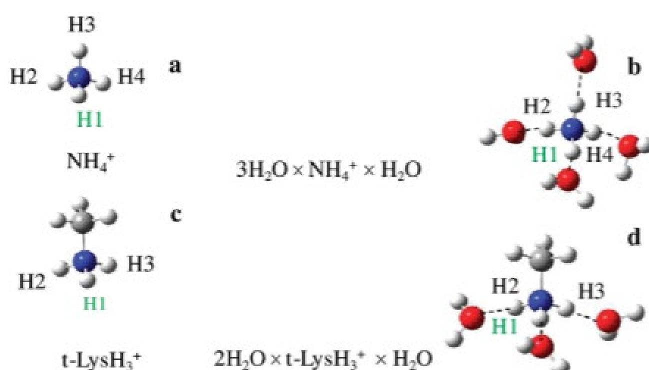

Figure 7. Atomic configurations used during MC-MO-HF calculations of the effect of water on an ammonium ion (a and c) and on lysine (c and d; lysine is truncated to methylammonium in these

calculations). The site of deuteration is marked in green. Taken from Ref. 45. Reproduced with permission from the American Chemical Society.

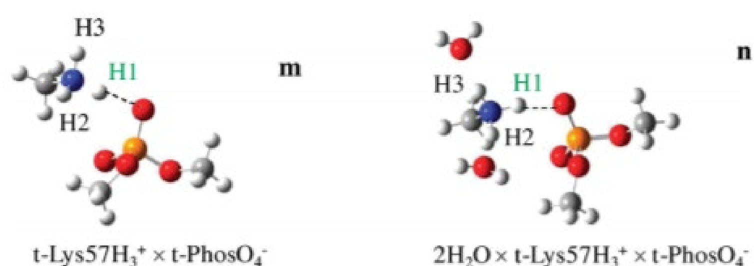

Figure 8. Atomic configurations used during MC-MO-HF calculations for a salt bridge. **m** is a model for a salt bridge between HoxD9 homeodomain and DNA, while **n** is a hydrated form of the same salt bridge. The site of deuteration is marked in green. Taken from Ref. 45 Reproduced with permission from the American Chemical Society.

Table 1. MC-MO-HF +GIAO calculations of $^1\Delta N(D)$ in ppm. For explanation, see text and Figs. 7 and 8.

System	$^1\Delta$N(D)	$^1\Delta$N(D)av[a]
NH_4^+	0.48	
$3H_2O$ x NH_4^+ x H_2O	0.41	
Trunc-LysH_3^+ [b]	0.63	
$2H_2O$ x Trunc-Lys x H_2O	0.48	
Trunc-Lys57H1+ x trunc-PhosO_4^- [c]	0.44	
Trunc-Lys57H2+ x trunc-PhosO_4^-	0.74	0.63
Trunc-Lys57H1+ x trunc-PhosO_4^-	0.72	
$2H_2O$ + Trunc-Lys57H1+ x trunc-PhosO_4^-	0.48	
$2H_2O$ + Trunc-Lys57H2+ x trunc-PhosO_4^-	0.43	0.49
$2H_2O$ + Trunc-Lys57H3+ x trunc-PhosO_4^-	0.57	

a. Average value
b. Methylammmonium is used instead of lysine
c. H1 is pointing towards oxygen of the phosphate

8. Equilibrium isotope effects

Deuteriation may cause a shift in a chemical equilibrium that in turn causes changes in the chemical shifts of the nuclei; such a contribution is called an equilibrium isotope effect. One particular case where such effects are important is in tautomeric equilibria (Fig. 9), and Fig. 10 illustrates the difference in the energy levels for the two tautomers. Equation 4 illustrates for a specific case how the total observed isotope effect is comprised of contributions for both intrinsic and equilibrium isotope effects, with the change in the

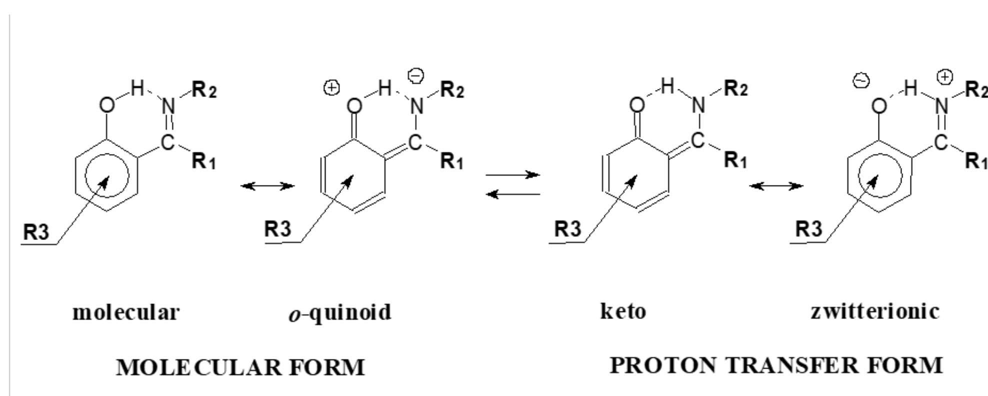

Fig. 9. Tautomeric equilibrium of an o-hydroxy Schiff base.

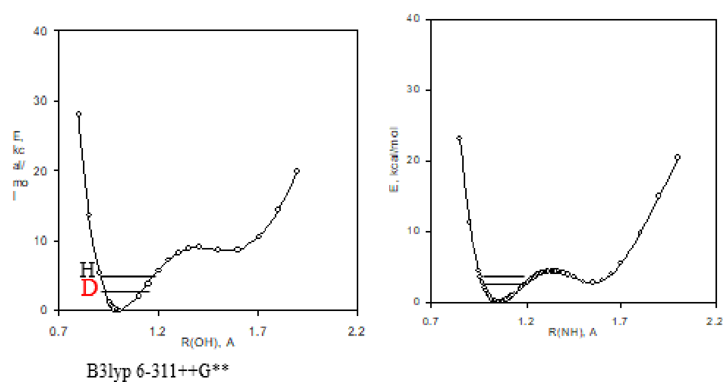

Figure 10. Potentials for OH and NH tautomers.

equilibrium described by $\Delta X_{H(D)}$, which is the change in the equilibrium position upon deuteriation. As intrinsic isotope effects are also at play, the total isotope effect in the case of an equilibrium is given in Eq. 4.

$$\Delta C_{obs} = X_M \times \Delta C_{int}(M) + (1-X_M)\, \Delta C_{int}(PT) + \Delta X_{H(D)} \times (\delta C_M - \delta C_{PT}) \quad \text{Eq. 4}$$

The first two contributions are the intrinsic ones and the last the equilibrium one. It is illustrated for isotope effects on ^{13}C and the two tautomers of Fig. 9, called molecular and proton transfer, abbreviated M and PT.

Importantly, equilibrium isotope effects can be large far from the site of deuteriation. If the equilibrium contribution dominates, a simplified expression is just the last term, $\Delta X_{H(D)} \times (\delta C_M - \delta C_{PT})$. If we now make a ratio between two isotope effects, it will be as described in Eq. 5:

$$\Delta C\text{-}1/\Delta C\text{-}2 = (\delta C\text{-}1_M - \delta C\text{-}1_{PT})/(\delta C\text{-}2_M - \delta C\text{-}2_{PT}) \quad \text{Eq. 5}$$

This shows that the observed isotope effects are proportional to the chemical shift differences between nuclei in corresponding positions in the two tauomers. An example is piroxicam [47], the tautomeric equilibrium for which is shown in Fig. 11.

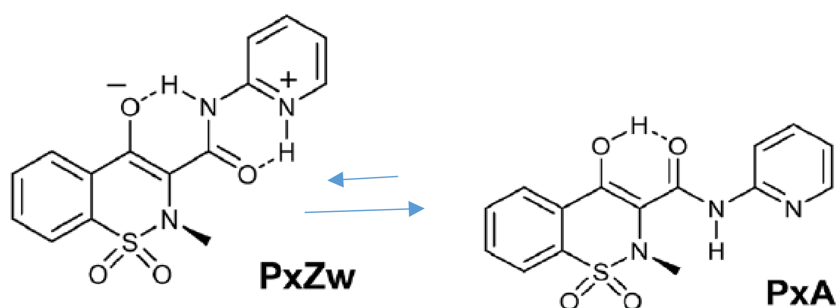

Figure 11. Tautomeric equilibrium of piroxicam

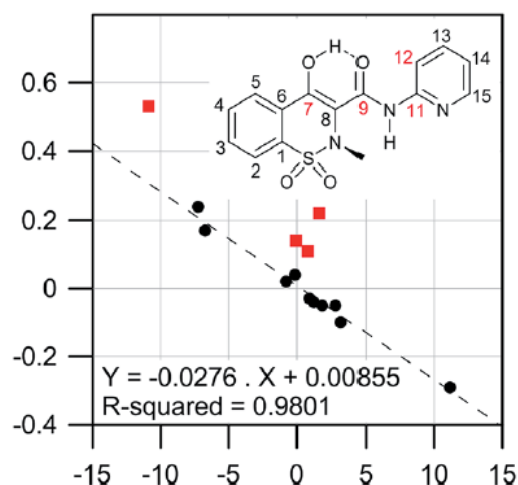

Figure 12. Deuterium isotope effects on ^{13}C chemical shifts in ppm (vertical axis) vs. ^{13}C chemical shift difference between corresponding carbon sites in the two tautomers of Piroxicam (see Fig. 11). The data points in red correspond to the carbon atoms numbered in red on the structure. Taken from Ref. 47. Reproduced with permission from the Royal Society of Chemistry.

An important example of a tautomeric equilibrium in some biological systems is that of *o*-hydroxy Schiff bases (see below). This system has been investigated and shows an “S-shaped” curve when the one-bond deuterium isotope effect on ^{15}N chemical shifts, $^{1}\Delta$N(D), is plotted against mole fraction [48]. This kind of curve is characteristic for equilibrium systems [49,50,51]. The intrinsic isotope effects of e.g. *o*-hydroxy Schiff bases can be estimated making use of the fact that derivatives of salicyladehydes (Fig. 12a, X=H) are fully in the OH-form whereas the Schiff base of gossypol (Fig. 13b) is fully in the proton transfer form (NH form), see Fig. 9. [52].

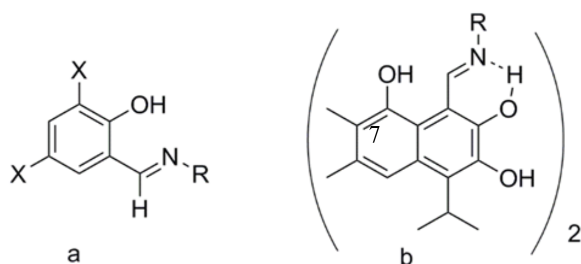

Figure 13. a. *o*-hydroxy-Schiff base. b is the Schiff’s base of gossypol. The line in position 7 is the link between the two halves

Another important biological system that has been studied is pyridoxy -5´-phosphate PLP cofactor, which the Limbach group has investigated, first using a model system like Schiff bases of 3,5-

dibromosalicylaldehyde (Fig. 13a, X=Br) and other similar compounds [53]. The hydrogen bonded part of this system can be described as : OLN in which L is either H or D.

They based their isotope effect calculations on bond valences, leading to the equation:

$$\delta(\mathrm{OL\underline{N}}) = \delta(\underline{\mathrm{N}})^{\circ} p_{\mathrm{OL}}{}^{\mathrm{L}} + \delta(\mathrm{L\underline{N}})^{\circ} p_{\mathrm{LN}}{}^{\mathrm{L}} + 4\delta(\mathrm{OL\underline{N}})^{*} p_{\mathrm{OL}}{}^{\mathrm{L}} p_{\mathrm{LN}}{}^{\mathrm{L}},\ \mathrm{L} = \mathrm{H, D} \quad (12)$$

Eq. 6

p^L are isotope sensitive bond orders. The isotope effects are then obtained by doing a subtraction when L is H and L is D, eventually to lead to an equation for what are termed “equilibrium isotope effects”.

$$\delta^{\mathrm{D}}_{\mathrm{obsd}} - \delta^{\mathrm{H}}_{\mathrm{obsd}} = \frac{\delta^{\mathrm{H}}_{\mathrm{a}} - \Delta_{\mathrm{a}} + \phi_{\mathrm{t}} K^{\mathrm{H}}_{\mathrm{t}}(\delta^{\mathrm{H}}_{\mathrm{b}} - \Delta_{\mathrm{b}})}{1 + \phi_{\mathrm{t}} K^{\mathrm{H}}_{\mathrm{t}}} - \frac{\delta^{\mathrm{H}}_{\mathrm{a}} + K^{\mathrm{H}}_{\mathrm{t}}\,\delta^{\mathrm{H}}_{\mathrm{b}}}{1 + K^{\mathrm{H}}_{\mathrm{t}}} \quad (19)$$

Δ is the intrinsic isotope effect for the two tautomers a and b. ϕ is the fractionation factor defined as K^D/K^H

However, as can be seen in Fig. 14, the fit to the experimental findings is not very good.

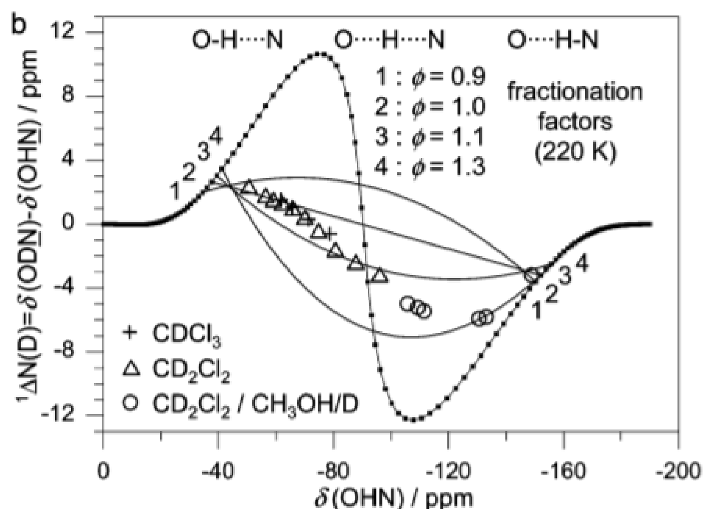

Figure 14. Plot of $^1\Delta$N(D) vs. the ^{15}N chemical shift. The symbols +, Δ and o represent experimental data points. The dotted line represents the intrinsic isotope effect. The solid lines represent the tautomerism averaged values which depend both on the intrinsic and the equilibrium effects. Reproduced with permission from the American Chemical Society.

A study of N-(pyridoxylidene) methylamine (Fig. 14) in CD_2Cl_2 at low temperature shows a $^1\Delta$N(D) of ~-3.2 # ppm. This value changes to -1.45 # ppm in methanol. In contrast, when the pyridine is protonated by

trifluoroacetic acid, an effect of 3.5 # ppm could be estimated [54]. The latter reflects a change towards a zwitter ionic form of the tautomeric equilibrium of the *o*-hydroxy Schiff base system, as seen in Fig. 15.

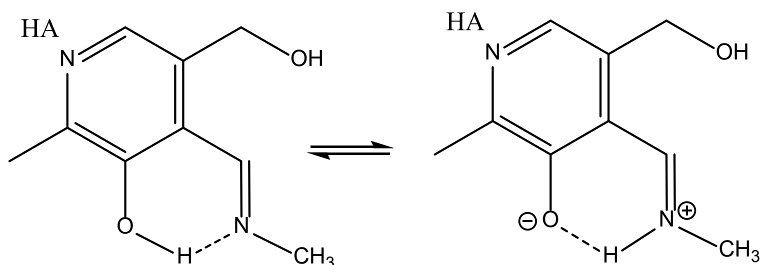

Figure 15 . N-(pyridoxylidene) methylamine

Tautomerism in an intramolecular relay system has also been investigated in 3-carboxy-5-methyl-salicylidenaniline (Fig. 16) [55].

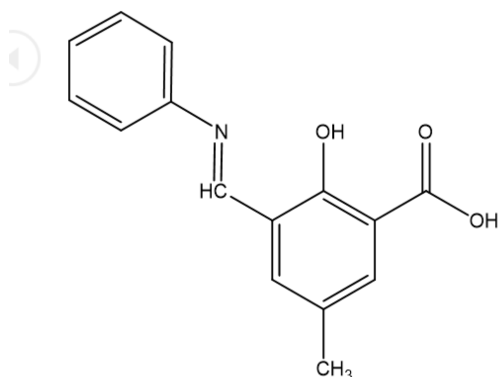

Figure 16. 3-carboxy-5-methylsalicylidenaniline

9. Summary

Deuterium isotope effects on chemical shifts (XH proton being deuteriated, X=O,N or S) can give valuable information on intermolecular hydrogen bonding, as seen in RNA and DNA. They can help in identifying salt bridges and can help describing hydrogen bond strength in catalytic sites based on e.g. histidine. The use of solvent isotope effects is clearly a very helpful tool in studying solvent access and, related to that, XH exchange. Considering that the prediction of both $^{1}\Delta N(D)$ and $^{2}\Delta CO(N_{i}D)$ for residues with charges in the side-chains met difficulties, charges probably have to be considered. Theoretical calculation of isotope effects in model systems can provide insight into often complicated biological systems. ^{19}F is very promising, both looking at isotope effects on chemical shifts and looking at solvent isotope effects.

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