Fluorine NMR

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SUMMARY

Current synthetic methods make possible the production of fluorine-containing analogs of amino acids, nucleosides, lipids and sugars, as well as a wide variety of molecules that are important drugs and agricultural chemicals. Fluorine-19, at 100% natural abundance, is a spin ½ nucleus. The technology for obtaining fluorine NMR spectra is virtually the same as that involved in getting proton NMR data. However, because a fluorine nucleus in molecules is on average surrounded by 9 electrons, rather than a single electron as is the case with hydrogen, the range of fluorine chemical shifts and the sensitivity of fluorine chemical shifts to the details of the local environment are much higher for fluorine than hydrogen. For example, a protein in which all tryptophan residues have been replaced by a fluorinated tryptophan analog will typically exhibit a resolved signal in its fluorine NMR spectrum for each tryptophan position of the sequence, while the proton spectrum of the same tryptophan residues in the native protein will be appreciably overlapped. Similarly, formation of a complex between a fluorinated small molecule and a receptor is often accompanied by an appreciable change in the chemical shift of the fluorine.

Fluorine spins relax by dipole-dipole interactions with the proton spins that surround them and because of the anisotropy of the fluorine chemical shift. The dipolar interactions can lead to $^{19}F\{^1H\}$ and $^1H\{^{19}F\}$ nuclear Overhauser effects that provide information about internuclear distances in the same ways that $^1H\{^1H\}$ NOEs lead to such data. Analysis of fluorine relaxation can also produce quantitative estimates of mobility. Like other NMR experiments, fluorine NMR experiments are sensitive to the rates of processes which interchange the environments of the observed spins and can produce quantitative data about rates of processes such as conformational change and ligand exchange.

Some examples chosen from the recent literature demonstrate the abilities of fluorine NMR experiments to provide data about ligand binding, unfolding, mobility, and other aspects of biological systems.

1. INTRODUCTION

1-1 FLUORINE IN CHEMISTRY AND BIOCHEMISTRY

Fluorine chemistry is a large field. It is represented by its own section of the American Chemical Society and several specialized journals. Methods for the synthesis of fluorine-containing molecules of potential biological significance have expanded in their scope and effectiveness since the early 1960's and fluorinated analogs of virtually any molecule are now available (Banks and Tatlow, 1986; Mann, 1987; Welch, 1987, 1990; Hudlicky and Pavlath, 1995; Hudlicky, 2000). The biomedical applications of organofluorine compounds range from pharmaceuticals to medical diagnosis (Filler, Kobayashi, and Yagupolskii, 1993). Reiss and Krafft (1998) discuss potential uses of fluorocarbons, fluorinated surfactants and fluorinated colloidal systems in biomedical applications.

Examples of fluorinated materials with impressive biological activity include **I** (diflucan, fluconazole), once heavily advertised in popular magazines as a one-dose treatment for vaginal yeast infections; compounds **II** and **III**, which are antibacterials that have been developed in response to the increasing resistance of bacterial infections to currently used drugs; Lefenuron (**IV**), a growth regulator used to control fleas on household pets; and pyrrole **V**, which finds application as an insecticide. Insertion of fluorine into a potential drug or agricultural chemical can produce an incredible range of biological effects, from complete metabolic inertness to very highly enhanced specificity for binding at a particular receptor site. Thus, it is not surprising that the preparation and study of fluorinated inhibitors, substrate analogs, anti-metabolites, "transition state analogs", suicide substrates and inhibitors, and other organofluorine compounds is a highly active research area (Resnati and Soloshonok, 1996; Ojima, McCarthy, and Welch, 1996; Seebach, 1990; Ramachandran, 1999; Banks, 2000).
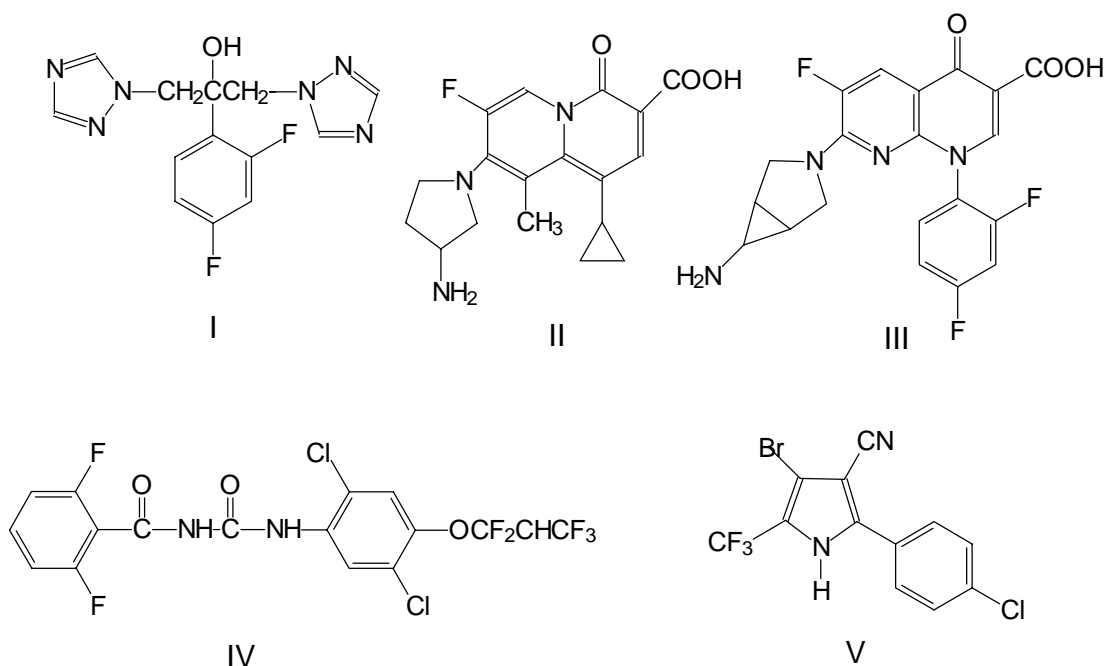
1-2 NMR IN BIOCHEMISTRY

High resolution NMR is an indispensable tool for the synthetic chemist as she attempts to characterize the structures of newly formed molecules. Rudimentary instruction in interpreting NMR spectra is part of every introductory organic chemistry course and most of our ideas about structure and the conformational mobility of organic and inorganic molecules are based on results obtained by NMR spectroscopy. The striking abilities of NMR experiments to provide detailed structural information and insights into molecular dynamics have not been lost on those working with proteins, nucleic acids, and polysaccharides. Indeed, the first published proton NMR spectrum of a protein (taken with an 11 mM solution of ribonuclease A at 40 MHz) appeared contemporaneously with the first commercial instruments capable of providing routine proton NMR spectra of smaller molecules.

As other chapters in this text demonstrate, the evolution of several technologies has now made it possible to obtain multi-dimensional proton NMR spectra at radio-frequencies in excess of 900 MHz. Computers are indispensable for collection, management and interpretation of this spectral data. All of these advances in hardware and software, as well as the intellectual advances represented by the design of the experiments performed, are necessary when using NMR to study biological systems. This is because the complexity of the spectra of even a small protein or nucleic acid fragment is such that a simple one-dimensional spectrum is rarely able to provide much information that is directly indicative of structure or dynamics. However, given the ability to prepare materials with specific carbon-13 and nitrogen-15 isotopic labels, it is possible with current procedures to obtain essentially complete assignments of the proton spectrum of a protein of up to about 30 kDa (Cavanagh, *et al*., 1996; Reid, 1997) or a fragment of RNA or DNA containing about 14 base pairs (Varani, Aboul-ela, and Allain, 1996). These limits are very approximate, since what is possible with a specific system will depend greatly on the sub-unit composition of the system, the degree to which a single conformation is present and is stable to conformational interchange, the particular effects of tertiary structure on chemical shifts, and the intrinsic rate of transverse spin relaxation.

1-3 FLUORINE NMR IN BIOCHEMISTRY

NMR methods for determination of structure and dynamics require that specific resonances in a spectrum be associated with specific nuclei of the molecules under study. That is, it must be possible to *resolve* resonances of interest from other signals in the spectrum and, having achieved this, *assign* those resonances to a specific chemical group within the system. Once enough signals have been resolved and assigned, NMR experiments such as the NOE provide constraints on what can be the tertiary structure of the system and perhaps provide indications of the rate(s) at which processes such as conformational change or ligand binding take place.

Other chapters in this volume describe isotope-enrichment and other strategies that are used to provide NMR data that can then define the structure and motions of proteins or other biological macromolecules through proton NMR spectroscopy. Although the details and reasons will vary, it is clear that at some point the standard multi-dimensional, multi-isotope enrichment proton NMR approaches to examination of structure or dynamics of a biopolymer will not be productive simply because proton and other resonances will overlap to such an extent that the resolution needed to specifically assign a proton signal cannot be achieved. This could happen when molecules are so large or the samples so viscous that molecular tumbling is slow, when interchange between two or more significantly populated conformations takes place on a time scale that leads to exchange

broadening, when paramagnetic groups are present in the structure, or when there is high degeneracy in the types of subunits that are present in the biopolymer.

The abilities of NMR experiments to provide information about proteins and other biological molecules can be extended by application of the same paradigm that guides the use of nitroxide "spin labels" in studies of biological systems. In the classical spin-labeling experiment, a stable paramagnetic group is introduced into the system and then examined by ESR spectroscopy. Usually there is only one paramagnetic center present, so the spectroscopic experiment does not suffer from a "resolution" problem. And because the experimenter often knows the details of the chemistry that was used to place the paramagnetic center in the molecule, the "assignment" problem is also minimized. Thus, two factors that limit the application of proton NMR in studies of biological systems are largely overcome. However, the price for doing so is high: the information that is obtainable from such a spin labeling experiment is limited, and is relevant to structure and dynamics only in the immediate vicinity of the paramagnetic species. It may or may not be the case that the conclusions reached on the basis of such experimental observations are relevant to the native (not spin labeled) system.

A nuclear "spin label" for NMR spectroscopy can, of course, be any nucleus compatible with the system of interest that produces a resolvable and assignable resonance. When such signals cannot be adduced from the spectrum of the native system then exogenous spins can be introduced. Similar to the use of stable paramagnetic species, utilization of the signals from such spins will be easiest when the "labeling" spins are a different type than those that are found naturally in a biopolymer and when they offer sensitivities to detection that are competitive with the ease of detecting proton signals. As indicated in Table 1, two nuclei that fit these specifications for an NMR "spin label" are tritium and fluorine. Tritium (hydrogen-3) has a detection sensitivity that exceeds that of hydrogen-1 by about 20% and has the additional advantage that, in terms of structure, it is essentially identical to the proton. Arrayed against the advantages of tritium NMR experiments with biological systems are the complications involved in dealing with the significant amount of radioactivity that would be present in such samples and the relatively small chemical shift sensitivity of hydrogen to structural changes.

A second approach to nuclear "spin-labeling" involves the use of fluorine. With only a few exceptions there are no natural occurrences of fluorine in biological systems (O'Hagan and Harper, 1999). Fluorine-19 has a natural abundance of 100% and has a sensitivity to NMR detection that is 83% that of the sensitivity of ^{1}H. Thus, introduction of fluorine produces a strong NMR signal that appears against a background devoid of signals from endogenous nuclei. Using a fluorine-labeling strategy also takes advantage of the large body of research that has been done over the years in basic fluorine chemistry alluded to above.

The utility of the fluorine spin labeling approach for NMR studies of biological systems has been recognized for more than 35 years. A number of reviews of such efforts have appeared (Gerig, 1989, 1994; Danielson and Falke, 1996; Gakh, Gakh and Gronenborn, 2000). Broader reviews of fluorine NMR spectroscopy are available in *Annual Reports* (Chemical Society) and *Annual Reviews of NMR*, and a short introduction to the uses of fluorine NMR in studies of organic compounds is useful (Everett, 1988).

Table 1. Properties of Spin ½ Nuclei

Nucleus	Gyromagnetic ratio, rad s^{-1} T^{-1}, x 10^{-7}	NMR Frequency (MHZ) at 11.75 T	Natural Abundance, %	Relative Sensitivity (constant number of nuclei)	Shift parameter range, ppm
^{3}H	28.535	533.	0	1.2	13
^{1}H	26.7519	500.	99.98	1.0	13
^{19}F	25.181	470.4	100.	0.83	400
^{31}P	10.841	200.2	100	0.066	530
^{13}C	6.7283	125.7	1.108	0.0159	250
^{15}N	-2.712	50.7	0.37	0.00134	1700

Data taken from Harris, R. K., "NMR Spectroscopy: A Physicochemical View", Pitman, London, 1983 and the URL http://rmn3.uab.es/standard.html. Relative sensitivity to detection in NMR experiments at constant number of nuclei varies roughly as the cube of the ratio of gyromagnetic ratios (Canet , 1991).

1-4 FLUORINE-CONTAINING BIOLOGICAL MATERIALS

Proteins. Several fluorinated analogs of the aromatic amino acids can be obtained commercially, including 4-fluorophenylalanine (**VI**), 6- and 5-fluorotryptophan (**VII**, **VIII**), and 3-fluorotyrosine (**IX**). (See *http://synthetech.clipper.net/specialty.html, http://www.fluorochemusa.com* and *http://www.sigma-aldrich.com.*) Synthetic methods are available for preparation of fluorinated derivatives of most of the common amino acids, as well as a variety of fluorinated nucleotides and sugars. Placement of these fluorinated materials into biopolymers has been accomplished by a number of different strategies, including chemical synthesis and biosynthetic incorporation by organisms.

VI   VII   VIII   IX
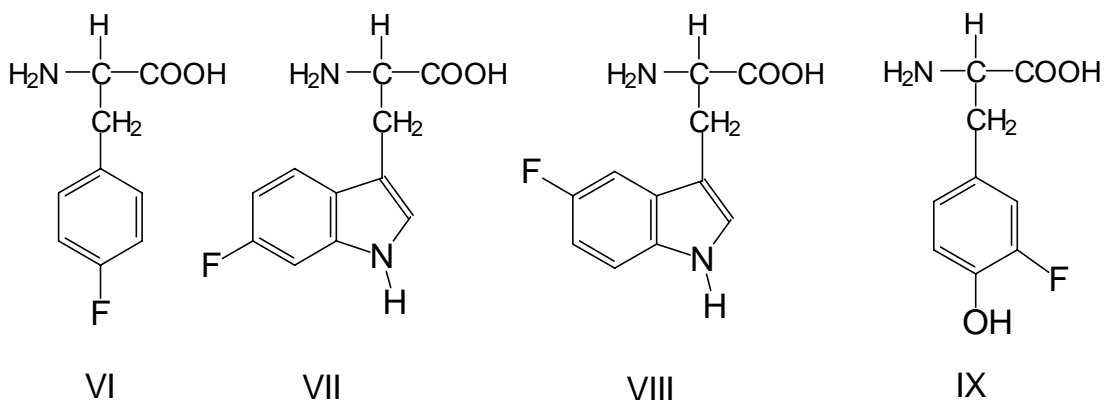
By far the most widely used method for getting fluorinated amino acids into the primary sequence of proteins is biosynthesis of the protein by a living organism. Methods for biosynthesis of fluorine-labeled proteins by bacteria and yeast have taken on increased importance because of the abilities of modern biochemical technology to express proteins of nearly any origin in these organisms. Fluorinated tryptophans are typically introduced into bacterial proteins of strains that are auxotrophic for tryptophan by inclusion of the fluorinated amino acid in the growth medium. An alternative approach, demonstrated by Kim, *et al*., (1990) involves the inclusion of glyphosphate, an inhibitor of aromatic amino acid synthesis, in cultures of *E. Coli*. The three aromatic amino acids must be included in the medium for growth to take place under these conditions but growth can be sustained when the majority of the tryptophan present is the 5-fluoro analog. 5-Fluorotryptophan (5FTrp) appeared to be incorporated into an enzyme, F1-ATPase, isolated from cells grown in this way, at levels consistent with there being essentially no discrimination between the fluoroanalog and normal tryptophan during protein synthesis.

If one can be satisfied with lower levels of incorporation, it is often possible to achieve incorporation of fluorinated analogs of some amino acids into proteins simply by having the amino acid present in the diet of an organism. 4-Fluorophenylalanine is incorporated into ribosomal proteins of growing *Tetrahymena* cells when the amino acid is added to the culture medium. Similarly, 4-fluorophenylalanine replaces a detectable fraction of phenylalanine in proteins of the rabbit and the chimpanzee when the animals are maintained on a diet containing this fluorinated amino acid. Fluorinated derivatives of phenylalanine, tyrosine, and tryptophan in the diet are incorporated into lysozyme produced by avians, at levels that afford excellent fluorine spectra in a few minutes.
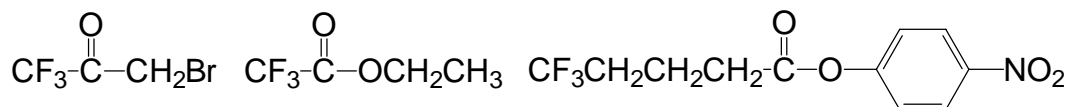
A disadvantage of preparation of fluorinated proteins by the biosynthetic methods indicated above is that all locations in the sequence occupied by residues of a given kind of amino acid become fluorine-labeled. When it is desired to have a protein in which only a single position in the sequence is taken up by a fluorinated amino acid, the only recourse is to chemical methods for synthesis. Schultz and his co-workers have devised an *in vitro* method for incorporation of fluorinated amino acids at specific locations in a protein (Noren, *et al*., 1989). In their work 4-fluorophenylalanine (4FPhe) was incorporated into the enzyme β-lactamase by use of an appropriately acylated suppressor tRNA that inserted the fluorinated amino acid in response to a stop codon. A more recent example is the work of Zhong, *et al*. (1998) exploring cation-π electron interactions in the nicotinic receptor by incorporation of a number of unnatural amino acids into the protein, including a number of fluorinated tryptophans. A current limitation of these approaches is the small amount of protein made available, although proton NMR studies of proteins prepared in this way have been reported. However, Furter (1998) has reported a method for incorporation of 4-fluorophenylalanine into specific positions of any protein expressible in *E. Coli*. that is able to supply tens of milligrams of protein.

Nucleic acids. Fluorinated analogs of nucleic acids in which particular bases or sugar rings are fluorine-substituted have been prepared and have been examined by fluorine NMR. However, such studies have been less common than is the case of fluorine-containing proteins.

Carbohydrates. The status of research with fluorinated carbohydrates, including fluorine NMR spectroscopic studies, has been considered in a series of reviews appearing in *Carbohydrate Research* (Vol. **327**, 2000).

Covalent modifications. Another way to introduce a fluorine "spin label" into a biological molecule is by means of a chemical reaction in which a covalent bond is formed between a functional group of the molecule and some exogenous, fluorine-containing reagent. A favorite target in such experiments has been the sulfhydryl group of cysteine residues because of the high nucleophilicity

of the side chain sulfhydryl group. Since there are relatively few cysteines in proteins an element of specificity is present in such modification reactions. However, the amino groups of lysine and of the peptide N-terminus, or the hydroxyl group of a serine or threonine are also possible sites of reaction with an appropriately constituted fluorinated reactant. The structures below show reagents that have been used, respectively, to link a trifluoromethyl group to -SH, -NH2, and -OH groups.

$$CF_3\text{-}\underset{\displaystyle \overset{\displaystyle O}{\|}}{C}\text{-}CH_2Br \qquad CF_3\text{-}\underset{\displaystyle \overset{\displaystyle O}{\|}}{C}\text{-}OCH_2CH_3 \qquad CF_3CH_2CH_2CH_2\text{-}\underset{\displaystyle \overset{\displaystyle O}{\|}}{C}\text{-}O\!-\!\!\!\bigcirc\!\!\!-NO_2$$

Receptor-ligand interactions. NMR spectroscopy has been an important tool for the study of protein-ligand interactions since before the advent of instruments based on superconducting magnets. Let R represent a receptor molecule, L a ligand, and RL a complex formed by binding the ligand to the receptor. Formation of the RL complex is accompanied by formation of interactions between

$$RL \rightleftharpoons R + L$$

the nuclei of the receptor and those of the ligand that replace all or some of the interactions between R and L and solvent molecules when these components of the complex were separated. The strengths of the new interactions relative to the strengths of the interactions with solvent define the equilibrium constant for complex formation by defining the rates of formation and dissociation of the complex. They may produce chemical shifts at the spins of the ligand and the receptor within the complex that are different from the shifts of the separated partners. Typically, there is also an appreciable change in the T_1 and T_2 relaxation times for spins of the ligand in the receptor-bound state. If the rate of exchange between the free and complexed forms of L is rapid enough, changes in chemical shift and relaxation that accompany binding can be experimentally detectable even when the concentration of ligand present greatly exceeds the concentration of protein.

What is observed, and observable, in the NMR spectrum of a system containing receptor, ligand, and the corresponding receptor-ligand complex depends on the concentration of RL relative to L and the rate of dissociation of the complex. Because of the high sensitivity of fluorine chemical shifts to environment and the potentially large shift difference between signals for the free and bound species, it is often possible to obtain evidence for the formation of receptor-ligand complexes by fluorine NMR when observations of other spins such as ^{1}H or ^{13}C provide no indications of complex formation.

1-5 FLUORINE-CONTAINING PROTEINS

Proteins containing a particular fluorinated amino acid at several positions in the sequence generally exhibit a single fluorine resonance or a narrow band of resonances when the protein is denatured. That is, the fluorine shifts of the fluorinated amino acids in the sequence are essentially degenerate under conditions where the tertiary structure of the protein is removed. However, in the native conformation(s), there are large shift effects, both up field and down field, due to local differences in the individual environments of the fluorinated residues that are a direct result of the tertiary structure of the protein. Some possible origins of these shift effects will be considered later, but, for

the present, it is sufficient to underscore that one of the advantages of the "fluorine-labeling" approach is the large range of shift observed because of the high sensitivity of fluorine shielding to the details of protein structure. This range is sufficiently large that a resolved signal for each fluorinated amino acid in the primary sequence can be expected even though the individual fluorine resonances may be quite broad. When more than one conformation of the protein is possible, a separate set of signals for each significantly populated conformation may be detected.

An important aspect of making full use of the fluorine spectra of proteins containing fluorinated amino acids is assignment of a given fluorine signal to a specific amino acid/fluoroamino acid in the protein sequence. Among the strategies that can be used to make assignments when a naturally occurring residue is replaced by a fluorinated analog are (1) examination of a mutant of the protein in which a position in the sequence occupied by a fluorinated amino acid is taken by an amino acid that is not fluorinated, (2) site-specific chemical modifications which alter the chemical structure of a particular amino acid near a fluorinated residue such that the shift of the fluorinated residue is altered, (3) attachment of a spin label to a specific residue, to take advantage of the distant-dependent line broadening effect of the paramagnetic species on fluorinated residues, (4) determination of the effect of complexation of a paramagnetic ion such as Gd^{3+} or other paramagnetic species to the protein, and (5) examination of the effects of pH changes or the binding of small molecules to the protein.

The preferable strategy is to make use of site-specific modifications of the protein sequence by means of modern protein engineering techniques. To assign a particular fluorotyrosine resonance, for example, a mutation might be done which replaces a tyrosine in the sequence by phenylalanine. A signal will be missing from the fluorine spectrum of the mutated protein after incorporation of fluorotyrosine and comparison of that spectrum to one obtained when all tyrosine positions have been replaced by fluorotyrosine leads to assignment of the signal arising from the residue that has been mutated. Given the high sensitivity of fluorine shifts to structure, there is reasonable concern that sequence alterations could produce structural changes throughout the protein and thus influence the chemical shifts of fluorinated residues remaining in the protein. However, indications so far from the relatively few studies that have used this strategy for making assignments are that the change in fluorine shift that arises at a residue distant from the one that has been mutated is detectable but usually small.

2. FLUORINE CHEMICAL SHIFTS IN BIOLOGICAL SYSTEMS

The nature of organic molecules is such that a carbon atom backbone or framework defines the basic shape of a structure. Hydrogen atoms encase the framework and the underlying carbon atoms are protected from an approaching molecule by these hydrogens. This notion is illustrated in the drawings on the next page. These representations of the structure of fluorocyclohexane (with the fluorine in the axial position) show how exposed the hydrogens of the methylene groups are and how they would tend to protect the interior carbon atoms from short-range interactions with other molecules. When fluorine replaces hydrogen in an organic molecule it ends up positioned on the "outside" of the molecule as well. It also can experience direct interactions with atoms or molecules in its immediate vicinity.

The chemical shielding parameter for a spin of a molecule in solution that is tumbling rapidly (σ, or more accurately σ_{iso}) is defined by Equation (2-1-1). Here ν is the resonance frequency of the

spin of interest, γ is its gyromagnetic ratio, B_{app} is the laboratory magnetic field used for the experiment, and $\hbar$ is Planck's constant divided by 2π. A more complete discussion would show that
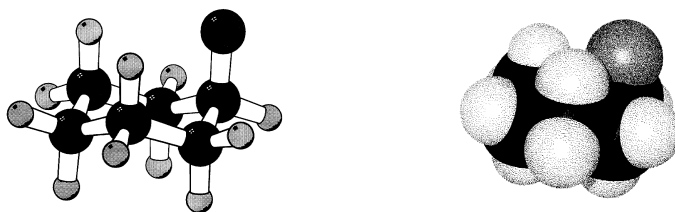
Ball-and-stick (left) and space-filling (right) representations of fluoro-cyclohexane. The darkly shaded fluorine atom is in an axial position.

the chemical shielding effect is orientation-dependent; the isotropic value of σ (σ_{iso}) observed for a molecule in solution is the result of averaging all shielding effects over all possible orientations of a molecule in the magnetic field by molecular motion.

$$\nu \ = \ \gamma \hbar B_{app} (1 - \sigma) \qquad \qquad (2\text{-}1\text{-}1)$$

The magnetic field used in the NMR experiment causes the electrons of the sample to precess around the direction of the magnetic field. These motions include those that take place locally at the nucleus being observed, as well as electron motions in other parts of the molecule. Electrons in molecules near the spin being observed are also set in motion by the magnetic field. These nearby molecules could be solvent molecules, other solute molecules, or, in the case of a biopolymer, part of the same macromolecule that has been folded in such a way that it is close to this observed spin.

Moving electrons create magnetic fields. The electron motions indicated above produce magnetic fields that combine with the laboratory magnetic field (B_{app}) to define the resonance frequency of a spin according to Equation 2-1-1. The quantity $(1-\sigma)$ defines how different is this combination of magnetic fields from the applied field, B_{app}. The local electron distribution in a specific organic group tends to be similar regardless of the nature of the molecule that holds the group, so that the chemical shift parameter for this group tends to be about the same in all molecules. Thus, the chemical shifts of protons attached to an aromatic ring have approximately the same value for σ whether the structure being examined is benzene or a phenylalanine residue within a complex protein. It is the electron motions in neighboring groups or molecules that are more system-specific and they largely define the exact value for σ that is observed.

2-1 FLUORINE REFERENCING

For technical reasons it is not possible to determine directly the value of the shielding parameter (σ) for a spin. However, resonance frequencies (ν) can be determined with very high accuracy and measurement of these is used to define values for shielding parameters relative to the shielding parameter of some reference compound. The resonance frequency for the sample ν_{sample} according to Equation (2-1-1) is

$$\nu_{sample} = \gamma \hbar B_{app}(1 - \sigma_{sample})   \qquad (2\text{-}1\text{-}2)$$

while the resonance frequency for the reference signal is

$$\nu_{reference} = \gamma \hbar B_{app}(1 - \sigma_{reference})   \qquad (2\text{-}1\text{-}3)$$

Both ν_{sample} and $\nu_{reference}$ can be measured accurately, as can the difference ($\nu_{sample} - \nu_{reference}$). NMR instruments operate at different values of B_{app} so the dimensionless (and magnetic field independent) parameter δ is defined

$$\delta = \frac{\nu_{sample} - \nu_{reference}}{\nu_{reference}} = (\sigma_{reference} - \sigma_{sample})   \qquad (2\text{-}1\text{-}4)$$

This represents the *difference* between the shielding parameter for the reference signal and that of the nucleus of interest. By convention, 1D NMR spectra are displayed so that the values of δ along the horizontal axis decrease from the left side of the plot to the right side. However, as one moves across the spectrum from left to right the shielding parameters for the nuclei represented on the horizontal axis increase. Peaks that appear to the left of the signal(s) arising from a particular spin are said to be *deshielded;* the shielding parameter for the spin(s) giving rise to these peaks is smaller than the shielding parameter characteristic of the first group.

By convention the reference signals used for proton and carbon-13 NMR spectroscopy are those from tetramethylsilane (TMS). The electron densities about the hydrogen and carbon atoms of the methyl groups of this compound are high because of the electronic nature of the silicon atom and the result is that shielding parameters for both hydrogen and carbon nuclei in this compound are relatively large. The shielding parameters for hydrogen atoms in most organic compounds are smaller that the proton shielding parameter of TMS and the proton NMR signals for these appear, in a spectrum displayed in the standard way, to the left of the proton NMR signal from the TMS reference compound.[1]

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[1] By convention the δ axis is plotted in a manner that corresponds to *decreasing* values of the Larmor frequency as one moves from left to right. Under this convention algebraic signs of the numbers used to annotate the δ axis of proton and carbon-13 spectra are reversed. An aldehyde proton gives a proton NMR signal at about +10 ppm on the left edge of a standard spectrum. This means that the shielding parameter σ for this proton is 10 ppm *smaller* than the proton shielding parameter of tetramethylsilane and that the Larmor frequency for the aldehyde proton is larger than that of TMS at constant B_{app}. This same sign convention is used with the fluorine shifts (Table 2).

Fluorine chemical shifts span a very wide range and there is no single fluorine-containing compound that is experimentally convenient for use as a universal reference compound. A fluorine reference is chosen on a more *ad hoc* basis and is usually a compound that is chemically similar to the one under examination. Since fluorine shielding parameters are significantly more sensitive to sample conditions than proton shielding parameters it is important when describing fluorine NMR experiments to specify the reference compound, its concentration, and the temperature of the sample.

Table 2 gives the approximate fluorine chemical shifts for fluorine-containing part-structures typically found in fluorine NMR studies of biological systems. The shifts are given relative to fluoro-trichloromethane ($CFCl_3$), a reference compound with a small fluorine shielding parameter, trifluoroacetic acid (CF_3COOH), an often convenient, water-soluble compound, and fluorobenzene. These data are provided only as an aid in designing experiments; the exact shifts for any fluorine-containing group depend strongly on the structural details of the system under study. For example, aromatic fluorine shifts appear over a range of over 70 ppm since they are sensitive to the nature of the substituents on the fluoroaromatic ring and depend on solvent and solute concentrations.

## 2-2 FACTORS AFFECTING FLUORINE SHIELDING IN BIOLOGICAL SYSTEMS

A traditional starting point for discussion of NMR shielding has been the Buckingham assumption (Equation 2-2-1) that the observed shielding parameter (σ_{sample}) can be considered to result from additive terms representing shielding effects from the motions of electrons near the nucleus of interest (σ_{local}), contributions from motions of electrons in more distant parts of the molecule which holds this nucleus ($\sigma_{neighbor}$), and shielding effects that arise from characteristics of the environment of the nucleus. Electric fields produced by charged or polar groups present in the remainder of the molecule or in the solvent molecules have an effect on shielding ($\sigma_{electric}$) because they alter electron motions. Calculations and experimental evidence shows that short-range contacts between atoms also can produce shielding effects (σ_{SR}). These contacts could be with the atoms of solvent molecule or with atoms brought close by the tertiary fold of a macromolecule. Special chemical interactions such as hydrogen bonding could also alter electron distributions and change shielding parameters (σ_{Hbond})[2]. While it is clear that in the final analysis these various contributions to fluorine shielding cannot be completely independent of each other, the separation implied by Equation 2-2-1 is a useful one for qualitative discussions of fluorine shielding in biological systems.

$$\sigma_{obs} = \sigma_{local} + \sigma_{neighbor} + \sigma_{electric} + \sigma_{SR} + \sigma_{Hbond} \qquad (2\text{-}2\text{-}1)$$

The value for σ_{local} is the major contributor to the observed shielding parameter, σ_{obs}. Strongly dependent on chemical type, it defines in which region of the fluorine spectrum a particular resonance will appear. The shielding parameters given in Table 2 are essentially values for σ_{local}. It is the details of a specific molecular structure, including its three-dimensional shape, that define $\sigma_{neighbor}$ and can contribute in a structure-specific manner to $\sigma_{electric}$, σ_{SR}, and σ_{Hbond}. Solvent molecules that can interact with the observed nucleus may contribute to $\sigma_{electric}$, σ_{SR}, and σ_{Hbond}.

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[2] Hydrogen bonding is used here as the most likely special interaction; other interactions such as π-complexation are possible but will not be specifically indicated.

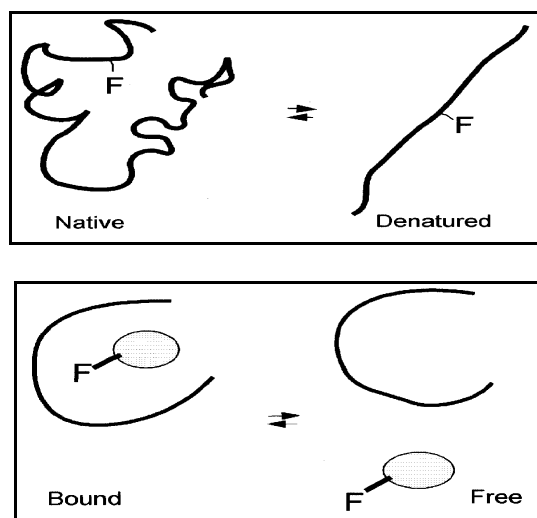**Table 2** - Approximate fluorine chemical shifts

Chemical group	σ relative to $CFCl_3$, ppm	σ relative to CF_3COOH, ppm	σ relative to C_6H_5F, ppm
⎯C(H)⎯F	-210	-131	-96
⎯C(F)⎯F	-140	-69	-26
R⎯C$_6$H$_4$⎯F	-140	-60	-26
⎯CF$_2$⎯C(=O)⎯	-125	-46	-11
CF$_3$⎯C(H)⎯	-75	4	39
CF$_3$⎯C(=O)⎯	-81	-2	33
⎯SO$_2$F	50	129	164

2-3 FLUORINE CHEMICAL SHIFT CHANGES IN BIOLOGICAL SYSTEMS

Much of the power of fluorine NMR studies of biological systems derives from the high sensitivity of the fluorine shielding parameter to changes in local environment. Two situations can be envisioned where changes in fluorine chemical shifts are expected. The drawings below portray a macromolecule containing a fluorinated group in its native (folded) state and in a denatured (un-folded) state. Those electronic factors that define σ_{local} must be the same in both states since the covalent nature of the fluorinated group is not changed by the folding process. However, the environment of the fluorine is changed significantly by folding to the native structure. In the unfolded state the fluorine experiences electric fields, short-range interactions and other chemical interactions (such as hydrogen bonding) that are predominantly due to solvent molecules and whatever interactions with the constituents of the biopolymer that persist under the denaturing conditions. When folded into the native state, the environment of the fluorine is changed. Perhaps solvent molecules

are excluded from the vicinity of the fluorine; the electric fields, short-range contacts, and hydrogen-bonding opportunities experienced by the fluorine are surely different in the folded state compared to the unfolded state. Differences in the shielding contributions $\delta_{neighbor}$, $\delta_{electric}$, δ_{vdW}, and δ_{Hbond} will contribute to a change in the observed shielding parameter.

Similarly, one can consider a fluorinated molecule binding to a receptor site. The covalent structure of the molecule is not changed by binding, but the electric fields, short-range contacts, and hydrogen-bonding possibilities experienced by the fluorine may well be different in the free and receptor-bound states, leading to a change in shielding parameter when the complex forms.

Experiments show that the fluorine shift changes that are observed upon biomolecule unfolding or when fluorinated small molecules bind to protein or nucleic acid receptors can be as much as an 8 ppm increase or decrease in the fluorine shielding parameter. This range of shift effects can be contrasted to the proton chemical shift changes observed in similar situations which rarely exceed +/- 0.3 ppm.

It should be mentioned that some fluorine-containing groups of molecules may undergo chemical reactions with components of receptor binding sites. These reactions may alter the covalent nature of the fluorinated compound and produce a large shift effect because δ_{local} has been changed. An example is the difluoroketone function ($-CF_2-CO-$). This functionality can react with nucleophilic groups such as the OH, NH_2, or SH groups of amino acid side chains. The shift effect associated with the change in δ_{local} may overwhelm any effect produced by a change in local environment.

Biological situations that could produce a fluorine chemical shift change. In the drawing at the top a fluorinated macromolecule such as a protein assumes its native conformation. Formation of a receptor-fluorinated ligand complex is portrayed in the lower illustration.

2-4 H_2O/D_2O SOLVENT SENSITIVITY

The fluorine signal from a water-soluble fluorinated material is deshielded about 0.2 ppm when the solvent is changed from water (H_2O) to deuterium oxide (D_2O). The H_2O/D_2O solvent isotope effect on the fluorine shift of 5-fluorotryptophan is linear with the mole fraction of deuterium present and this is probably also the case for other types of fluorinated molecules.

Generation of the H_2O/D_2O solvent isotope effect presumably requires "contact" between the fluorine and hydrogen atoms of the solvent. The magnitude of the effect should thus reflect the extent to which a fluorine nucleus in a macromolecular structure is exposed to solvent. This notion has been tested in studies of cyclodextran inclusion complexes of small fluorinated molecules. Those molecules thought to be encapsulated in these complexes indeed showed reduced shift changes when the isotopic composition of the solvent was altered (Hansen, Dettman, and Sykes, 1985).

Solvent isotope shift effects have been used to diagnose the accessibility of a fluorinated amino acid side chain to solvent in fluoroaminoacid-containing proteins. The assumption being made is that

the more likely a residue is to encounter solvent molecules, the larger will be the fluorine shift change when the solvent water is replaced by deuterium oxide. For example, positions of three of the fluorine signals in the spectrum of 5-fluorotryptophan-containing D-lactate dehydrogenase are invariant to changing the solvent from H_2O to D_2O, while two signals show essentially the same changes with the H_2O/D_2O ratio as does free 5-fluorotryptophan. A reasonable interpretation of these observations is that the structure of this protein places two residues near the surface where they are exposed to solvent molecules, while three residues lie within the core of the protein, "buried," and out of contact with the solvent.

2-5 THEORETICAL CONSIDERATIONS OF FLUORINE SHIELDING IN BIOLOGICAL SYSTEMS

Those factors which determine the chemical shielding tensor (σ) have been extensively investigated since the earliest days of NMR spectroscopy. Computational procedures have now developed to the point that fairly reliable estimates of the value of σ for carbon-13, nitrogen-15 and other backbone nuclei in small molecules can be made (Chesnut, 1994; de Dios and Jameson, 1994). Such methods are sufficiently sensitive that the dependence of shielding parameter on details such as changes in bond lengths and angles can be examined. Electronic structure calculations give results which basically are estimates of the contribution of local electronic motions to defining the shielding parameter (σ_{local}) and generally are applicable to the molecule in the gas phase. In any experimentally practical sample of small molecules, there are unavoidable intermolecular contributions to the observed chemical shielding and these are more difficult to calculate.[3] For macromolecules computational estimates of shielding must necessarily become more approximate because of the limitations imposed by available computing power. The effects of tertiary structure and solvent molecules on the chemical shift of a specific fluorine spin in a protein or other biological macromolecule, while large and relatively easy to measure, are difficult to interpret theoretically.

As indicated earlier, the changes in shielding parameter that accompany denaturation of a fluorine-containing protein or binding of a fluorinated molecule to a receptor arise from changes in the medium- or environment-dependent contributions to this parameter. As a means of exploring medium-induced chemical shielding effects one can consider the change in shielding parameter when a molecule is transferred to a solution from the gas phase. From such studies it appears that short-range interactions with solvent molecules are a primary source of the medium effect on σ that is observed when fluorobenzene enters solution from the vapor. In polar solvents the electric fields at the fluorine nucleus produced by the solvent molecules also appear to have an effect. While some authors ascribe the fluorine chemical shift changes associated with denaturation or ligand binding in macromolecular systems entirely to the effects of electric fields, it is likely that both short range interactions with nearby atoms and local electric fields in some combination are responsible for the observed shielding changes.

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[3] Even with a "molecular" system as simple as a monoatomic rare gas, the observed shielding parameter is dependent on gas pressure and temperature, a result of the dependence of intermolecular interactions on these variables.

3. FLUORINE RELAXATION IN BIOLOGICAL SYSTEMS

Relaxation is critical to the success of any spectroscopic experiment. In NMR, processes exist which maintain on average the correct numbers of spins in each of the allowed spin energy states. These processes, which must involve the transfer of energy to and from a molecule and its surrounding, are collectively known as spin-lattice relaxation. They are characterized phenomenologically by a spin-lattice relaxation time, T_1. Application of an RF pulse often perturbs these state populations. During and after the pulse spin-lattice relaxation processes act to return the system back to the correct (equilibrium) populations. An earlier chapter in this volume by T. C. Farrar discusses nuclear spin relaxation in detail. Only some essentials are indicated here to show how the concepts developed there apply to fluorine NMR spectroscopy with biological systems.

3-1 FLUORINE RELAXATION BY THE DIPOLE-DIPOLE MECHANISM

As is the case with other spin ½ nuclei, interactions between magnetic dipoles are an important mechanism for relaxation of fluorine in biological systems. Given its "exposed" position in organic structures (discussed above), a fluorine spin typically is nearly within van der Waals contact of hydrogen nuclei when the fluorine is present in a biological macromolecule. Equation 3-1-1 can be derived to describe the influence of a proton (hydrogen) on the spin-lattice relaxation of a fluorine using a simplistic model of a molecule containing the set of interacting F-H spins and the way it moves. In this equation γ_F and γ_H, respectively, are the gyromagnetic ratios of fluorine and hydrogen, $\hbar$ is Planck's constant divided by 2π, and ω_F and ω_H are the resonance frequencies for fluorine and hydrogen. The approximations made in obtaining Equation (3-1-1) include (a) the assumption that the molecule or system which has the F-H dipolar interaction tumbles isotropically such that the motion can be characterized by means of a single correlation time, τ_c and (b) the assumption that the distance between the fluorine and the hydrogen, r_{FH}, does not change as the molecule tumbles.[4] A more complete discussion of fluorine-hydrogen dipolar relaxation requires consideration of the interactions of all spins, fluorines and hydrogen, of the system, not just a single pair of spins in isolation However, Equation (3-1-1) is a useful approximation for representing the effect of a single F-H interaction on fluorine relaxation.

$$\frac{1}{T_1} = \frac{1}{10} \cdot \frac{\gamma_F^2 \gamma_H^2 \hbar^2}{r_{FH}^6} \cdot \tau_c \cdot \left(\frac{3}{(1 + \omega_F^2 \tau_c^2)} + \frac{1}{(1 + (\omega_F - \omega_H)^2 \tau_c^2)} + \frac{6}{(1 + (\omega_F + \omega_H)^2 \tau_c^2)} \right) \quad \text{3-1-1}$$

The dipole-dipole mechanism for fluorine relaxation has a strong dependence on fluorine-hydrogen internuclear distances and reflects the dynamics of local molecular motions. To get some idea of the magnitude of the effects, consider the model of a fluorinated aromatic ring shown in the drawing on the next page. The ring could represent the side chain of a 4-fluorophenylalanine residue that has been incorporated into a protein or it could correspond to part of a small molecule that is bound within a receptor. The protons of the aromatic ring will produce fluorine relaxation and by

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[4] As a rough approximation, the rotational correlation time τ_c for spherical molecules in non-viscous solvents near room temperature is about 10^{-12} x M_w, where M_w is the molecular mass expressed in Daltons.
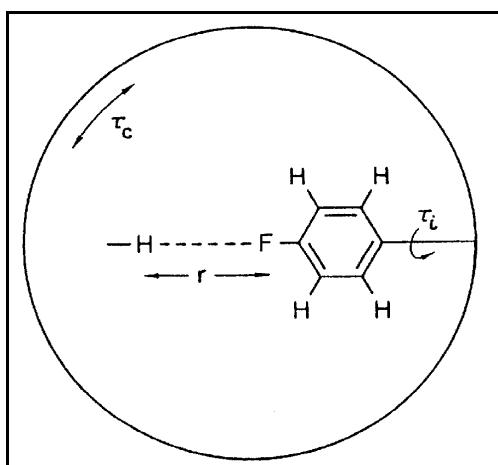
Model for a fluorinated aromatic ring within a biological macromolecule. The correlation time τ_c is used to describe overall motion of the large molecule, while the correlation time τ_i describes local motion of the fluorinated group. For the calculations done here, the effects of internal or local motion were neglected.

protons of the macromolecule that are close to the fluorine. Motions that could be significant for relaxation include overall reorientation of the fluorine-containing structure by Brownian motion and motions of groups within the structure as the structure reorients. Overall reorientation can be assumed to be isotropic and characterized by a rotational correlation time, τ_c. (Internal motions of the fluorinated ring and groups that interact with it would be characterized by additional correlation times, here symbolized by τ_i.) If we assume that the protons of the aromatic ring and a single other proton, representing all other F-H interactions that produce relaxation, are the sources of dipolar relaxation, and further assume that there are no internal motions present, Equation (3-1-1) predicts the fluorine T_1 relaxation times shown below when the fluorine NMR experiment is done in a magnetic field that corresponds to proton NMR at 500 MHz. It is seen that T_1 depends on the overall tumbling of the system containing the fluorinated aromatic ring and that the same value for T_1 potentially can be observed at different correlation times.
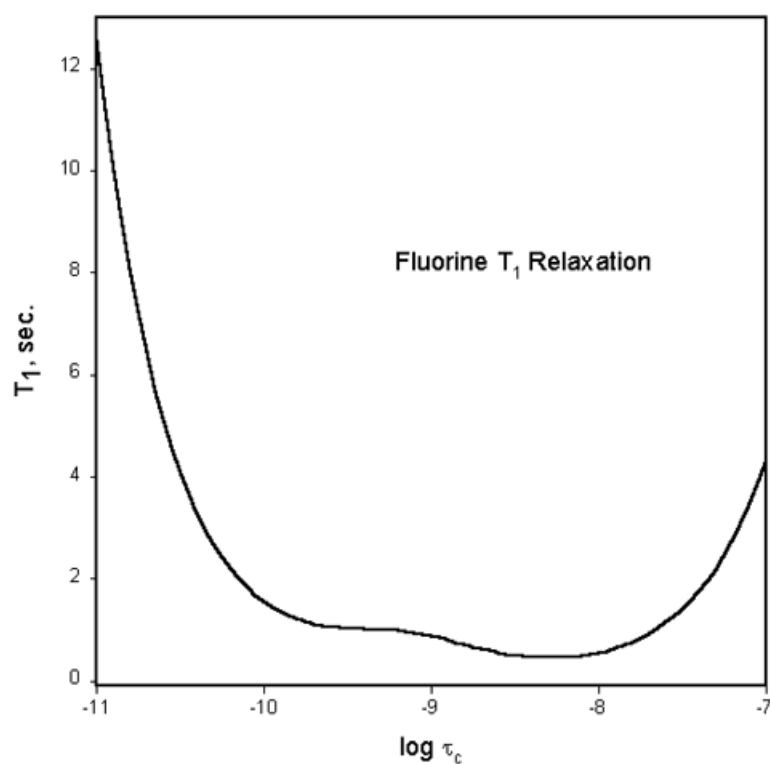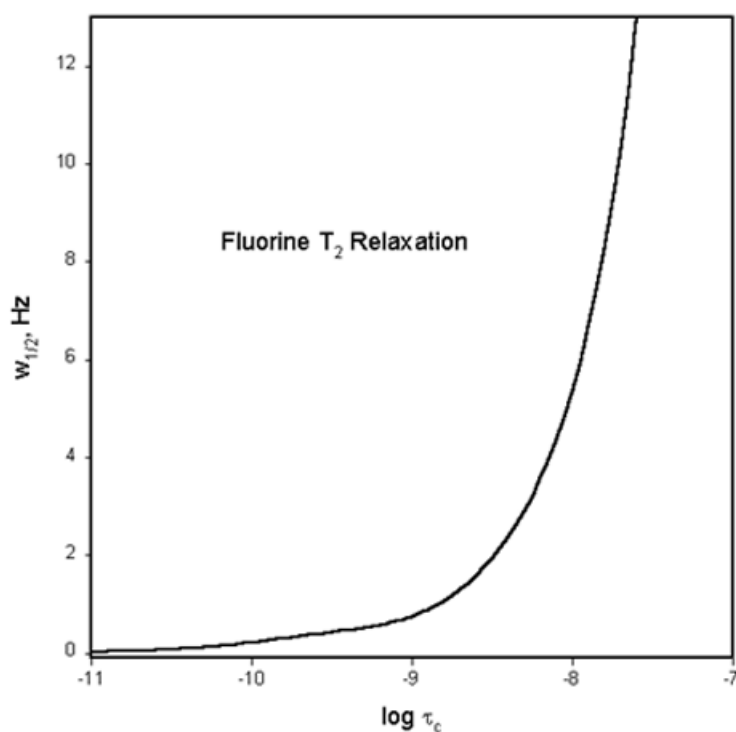

Predicted fluorine T_1 relaxation at 470 MHz for the model system described in the text. Only dipole-dipole relaxation is considered. The distance r was 2.4 Å.

Fluorine-proton dipole-dipole interactions also give rise to spin-spin or transverse (T_2) relaxation. The symbols in the Equation 3-1-2 for T_2 relaxation have the same meaning as those in Equation 3-1-1. It may be recalled that T_2 relaxation is responsible for the observed width of NMR signals. The expected line widths for the fluorine signal from the 4-fluorophenyl model system that was previously described are shown in the plot below. Note that dipole-dipole relaxation leads to an increase in the fluorine signal width with increasing correlation time. At correlations times greater than ~10^{-8} sec, corresponding to systems with apparent molecular weights greater than 10 kDa, the fluorine resonances are expected to be quite broad.

$$\frac{1}{T_2} = \frac{1}{20} \cdot \frac{\gamma_F^2 \, \gamma_H^2 \, \hbar^2}{r_{FH}^6} \cdot \tau_c \cdot \left(4 + \frac{3}{(1 + \omega_F^2 \, \tau_c^2)} + \frac{6}{(1 + \omega_H^2 \, \tau_c^2)} + \frac{1}{(1 + (\omega_F - \omega_H)^2 \, \tau_c^2)} + \frac{6}{(1 + (\omega_F + \omega_H)^2 \, \tau_c^2)} \right)$$
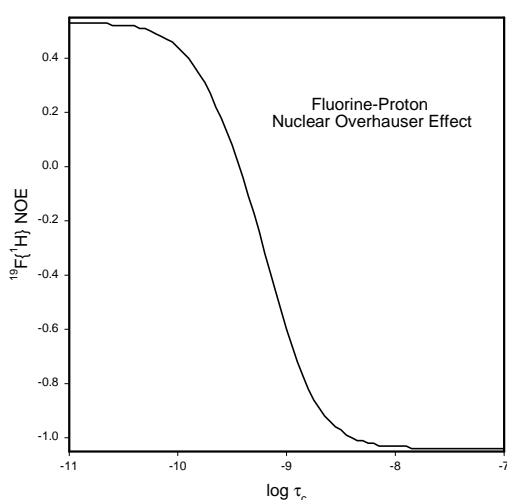
3-1-2

Fluorine T$_2$ Relaxation

Fluorine NMR line widths at 470 MHz predicted for the model system that was used to calculate the T_1 relaxation times shown earlier.

The nuclear Overhauser effect (NOE) is connected to dipolar relaxation processes. A variety of such effects can be determined experimentally and under the appropriate conditions can be used to estimate internuclear distances. A fluorine Overhauser effect in a fluorinated biological system can be produced by perturbing the proton spins of the systems by the application of RF pulses or continuous RF irradiation at the proton frequency. The figure on the next page shows the ^{19}F{^{1}H} NOE calculated for the system used to produce the theoretical T_1 and line width values shown earlier when the NOE experiment consists of continuous saturation of the proton spins. The calculations show that for this experiment, the fluorine signal will be enhanced by up to 53% when the molecules holding the fluorine and protons move rapidly (small τ_c). For systems with long correlation times the ^{19}F{^{1}H} NOE is -104%. These values are the largest magnitudes of the NOE possible for this experiment; the presence of other, non-dipolar relaxation processes will reduce the magnitude of the NOE

observed at any correlation time. It is important to notice that, like proton-proton NOES, there is a value of the correlation time at which the fluorine-proton NOE is zero. Under this condition the protons causing fluorine relaxation could be physically close to the fluorine, could have effects on fluorine T_1 and T_2 relaxation, and yet have no detectable effect on fluorine signal intensity when the proton spins are perturbed in an attempt to generate an NOE.

$^{19}F\{^{1}H\}$ nuclear Overhauser effects at 470 MHz predicted for the model system that was used to calculate the relaxation data shown previously.

3-2 FLUORINE RELAXATION THROUGH CHEMICAL SHIFT ANISOTROPY

A second important mechanism for fluorine spin-lattice and spin-spin relaxation is that produced by the anisotropy of the chemical shielding (CSA). The magnetic field experienced by a nucleus depends predominantly on the electronic structure of the molecule holding the nucleus and the ease with which electrons move in this structure when the molecule is placed in a magnetic field. Movement of electrons is more facile in some molecular orientations than in others. The shielding parameter σ is thus anisotropic, and the value observed for a specific nucleus in a particular molecule depends on how the molecule is oriented in the magnetic field. Molecules in liquids rapidly experience all possible orientations relative to the direction of a magnetic field so that the observed σ is an averaged value. However, the motions that produce this average value cause oscillations of the local magnetic field and these time-varying fields lead to relaxation.

Equations (3-2-1) and (3-2-2) show the contribution of fluorine CSA to T_1 and T_2 relaxation under simplifying conditions. These include the assumption that the bond attaching the fluorine to the system of interest is cylindrically symmetric so that the fluorine exhibits a shielding parameter $\sigma_{\parallel}$ when the molecule is oriented with the bond axis parallel to the direction of the magnetic field and $\sigma_{\perp}$ when the bond direction is perpendicular to the direction of the field. The shielding anisotropy $(\sigma_{\parallel} - \sigma_{\perp})$ of ^{1}H is relatively small, about 5 ppm, while the anisotropies of heavier elements such as ^{19}F are much larger, perhaps as much as 1000 ppm.

$$\frac{1}{T_1} = \frac{2}{15}(\sigma_{\parallel} - \sigma_{\perp})^2 \omega_F^2 \tau_c \frac{1}{(1 + \omega_F^2 \tau_c^2)} \qquad \text{3-2-1}$$

$$\frac{1}{T_2} = \frac{2}{15}(\sigma\| - \sigma\perp)^2 \omega_F^2 \tau_c \left(\frac{2}{3} + \frac{1}{2}\frac{1}{(1 + \omega_F^2 \tau_c^2)} \right)$$ 3-2-2

These equations show that the CSA relaxation mechanism depends on the square of the laboratory field used to make the NMR observation. Relaxation due to CSA, thus becomes more important relative to other relaxation processes, even for ^{1}H experiments as the magnetic field used for an NMR observation is increased.

Calculations using Equations 3-1-1 and 3-2-1 show that the CSA mechanism makes a minor contribution to fluorine T_1 relaxation. However, anisotropy of chemical shielding adds significantly to the fluorine signal width (T_2 relaxation) at all magnetic fields commonly used to obtain fluorine NMR spectra of biological systems. Calculations (shown below) done with the same model of a 4-fluorophenyl ring in a macromolecule as was used in producing the plots on pages 16 and 17 suggest that at magnetic fields corresponding to ^{1}H NMR frequencies between 200 and 300 MHz CSA relaxation can be responsible for about ½ of the observed fluorine line width, and becomes the major source of fluorine line width at higher magnetic fields.

The precise role of dipole-dipole and CSA relaxation effects in any situation depends on details of the system, including the arrangement of protons near the fluorine being observed, the anisotropy of the fluorine chemical shift, and the details of how the fluorinated structure moves. There will also be complications when processes such as ligand binding or conformational changes that exchange the observed fluorine nucleus between two or more environments are present since such exchange processes can produce additional line broadening.
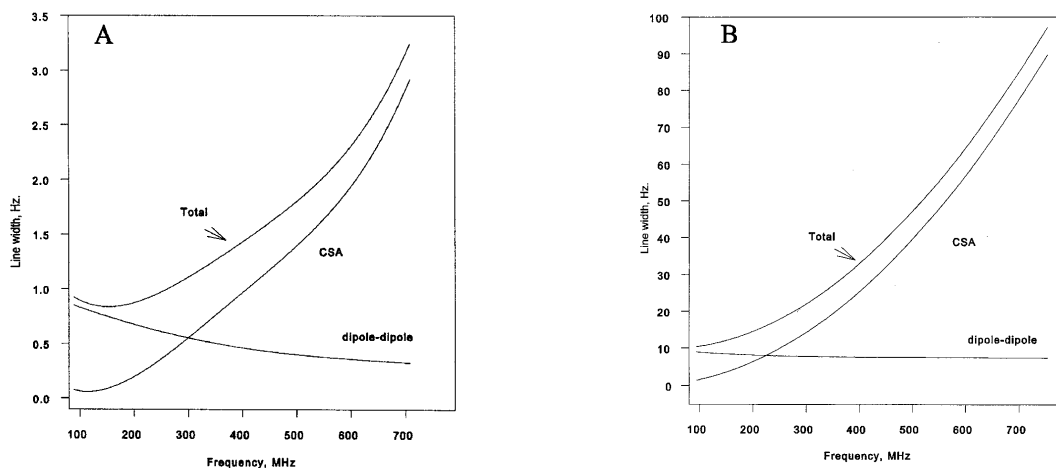
Predicted effects of chemical shift anisotropy on the line width of a fluorine signal from a fluorinated biological system. The calculations assume isotropic tumbling of a 4-fluorophenyl ring immobilized inside a macromolecule. Relaxation caused by dipole-dipole interactions with surrounding protons was calculated by the same procedures that were used in making the earlier figures. In panel A, the rotational correlation time (τ_c) for the macromolecule was 0.4 ns while for panel B the correlation time was 16 ns. The strong dependence of CSA relaxation on the magnetic field used for the NMR experiment is apparent.

Any spin-lattice relaxation produced by fluorine CSA reduces the magnitude of the ^{19}F{^{1}H} NOE; it is often observed that the ^{19}F{^{1}H} NOE in fluorine-containing proteins is in the range of 70% to 90% of the value expected if fluorine-proton dipolar interactions were the only means of relaxation present.

4.0 PRACTICAL CONSIDERATIONS

It is usually no more difficult to obtain fluorine spectra of a sample than it is to get proton NMR data under the same sample conditions. Fluorine signals from a biological system may be broader than proton signals because of the CSA contribution to transverse relaxation and because of exchange effects on signal widths or shapes. Suppression of the H_2O solvent signal is usually a major consideration in the design of proton-observe NMR experiments but there is no such complication when observing fluorine.

The proton and fluorine resonance frequencies are similar and it is often possible to get fluorine NMR spectra by using a probe designed for proton spectroscopy. It is better to use a probe specifically designed for fluorine, both in terms of the selection of tunable components of the probe and in the use of fluorine-free materials in the construction of the probe. Fluorocarbon polymers are often used in the construction of proton-observe probes and these can give rise to large, broad, baseline-distorting signals in a fluorine spectrum. These can be particularly annoying and somehow manage always to appear in the spectral region of interest!

The instrument to be used for a fluorine NMR experiment with a biological system depends on what is available and what are the goals for a particular experiment. In proton NMR, and to a large extent in NMR experiments with other nuclei, it is usually advantageous to examine samples using the highest available resonance frequency. An increase in magnetic field increases the inherent sensitivity of the experiment. Moreover, the separation of two adjacent chemically shifted signals, measured in units of Hz, increases linearly as the magnetic field increases. If the line width of the signals does not change appreciably with magnetic field, the ability to resolve these signals is thus enhanced by a higher magnetic field.

It was indicated earlier that the CSA relaxation mechanism becomes increasingly important in determining the line width of fluorine-19 NMR signals as the resonance frequency increases. As the resonance frequency (magnetic field) used for a fluorine NMR experiment increases, it may turn out that the ability to resolve nearby resonances begins to suffer because their widths become such that they overlap too much. An increase in line width may also lead to a reduction in the signal-to-noise ratio in a spectrum since a wider line is not as tall, relative to the noisy baseline, as a sharper line of the same area. The optimum instrument for a given fluorine NMR experiment with a biological system in practice will depend on the parameters which define CSA and other contributions to the signal shape.

5. SOME EXAMPLES

5-1 FLUORINE NMR AS AN ANALYTICAL TOOL

5.1.1 Drug analysis. Presuming that the spins of the sample are at equilibrium in the magnetic field when the experiment starts, the intensity of an observed NMR signal is directly proportional to the number of spins present. The exact position (chemical shift) at which a signal appears in the

spectrum depends on the chemical environment of the spin. Samples can be recovered unchanged after an NMR experiment. Thus, NMR observations offer a powerful tool for qualitative and quantitative analysis of biological systems. Fluorine NMR observations are especially useful in this regard because of the sensitivity of fluorine chemical shifts to structural details. NMR experiments are relatively insensitive for chemical analysis when compared to analytical methods based on optical or mass spectral technologies and this can be a limitation. However, it is possible to do quantitative analysis by fluorine NMR of samples that have concentrations of fluorinated molecules in the range of 10μM on a convenient time scale (*ca.* 15 minutes). Detection of fluorinated materials at the ppb level by NMR has been demonstrated.
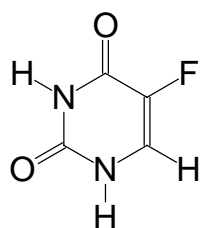
5-Fluorouracil

The potency of fluorine NMR as an analytical technique is demonstrated by observations made with the drug 5-fluorouracil. This material is a widely-used anti-cancer drug. Significant side effects of the drug have been noted, including cardiotoxicity. It was thought that the side effects could be the result of impurities in the drug that arise during its manufacture or during storage. Figure 5.1.1 shows the fluorine NMR spectrum of a sample of the drug in common use before 1994. The presence of many fluorine-containing compounds at trace levels is indicated by the spectrum. Subsequent identification of some of these led to improved procedures for storage and use of the drug (Malet-Martino, 1997).

Fluorine NMR studies of the metabolism of 5-fluorouracil *in vivo* have been summarized recently (Bachert, 1998; Wolf, Presant and Waluch (2000).
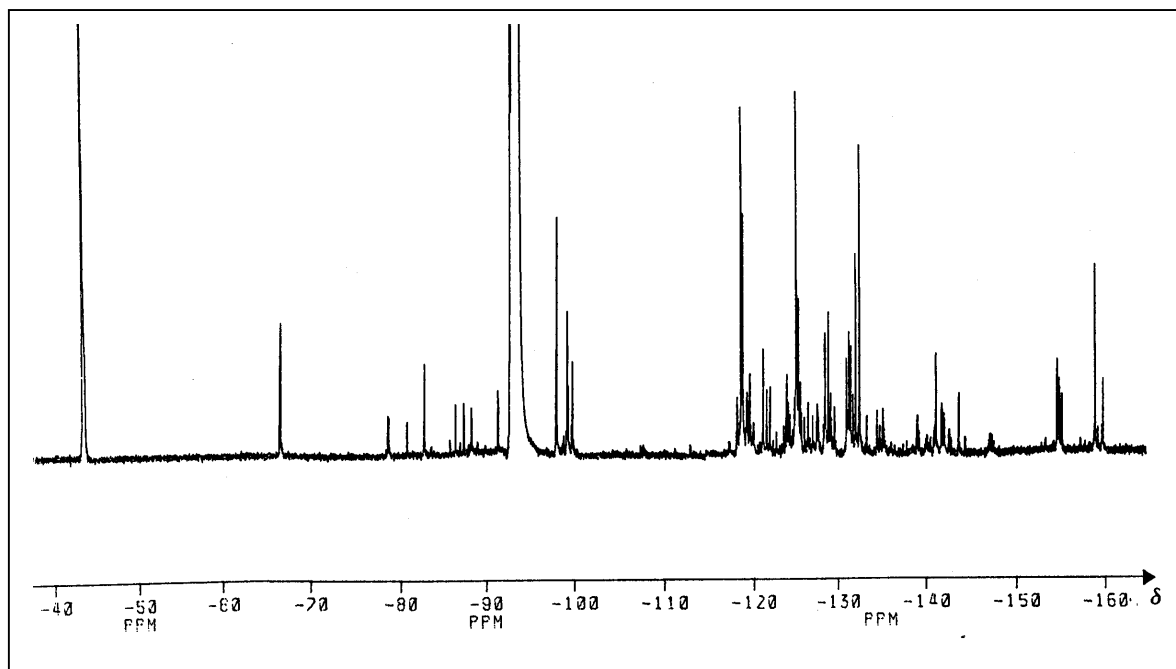

Figure 5.1.1. *Proton-decoupled fluorine NMR spectrum of a commercial sample of the drug 5-fluorouracil. Nearly 100 fluorinated compounds are indicated, although at levels less than 1% that of the drug (represented by the strong signal at -94 ppm). The spectrum was kindly provided by Prof. M. Malet-Martino.*

5.1.2 Enzyme product identification. Fluorine NMR observations have been used to characterize the regiochemistry of a desaturase enzyme important in fatty acid biochemistry (Buist, *et al.*, 1996). Biological desaturation of fatty acids, represented in reaction 5.1, shows remarkable enzymatic selectivity. The enzyme which achieves this reaction has an iron atom at its active site and this is involved in hydrogen abstraction from the substrate through an iron oxo intermediate. Such species are capable of oxidizing a sulfide to a sulfoxide. Oxidation of 4-(trifluoromethyl)phenyl substituted
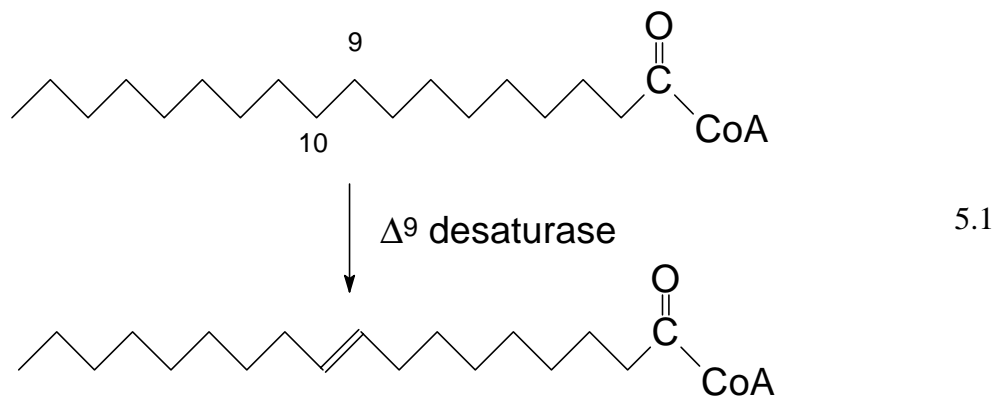
sulfides is accompanied by an easily detected ~0.4 ppm change in fluorine chemical shift. Growing cultures of the yeast *S. cerevisiae* were exposed to trifluoromethylated sulfides **X** and **XI**. ^{19}F NMR spectra of the supernatants showed that sulfide **X** was much more readily oxidized, thus supporting the conclusion that the yeast Δ^9-desaturase initiates oxidation of stearoyl CoA at carbon 9 of the fatty acid.
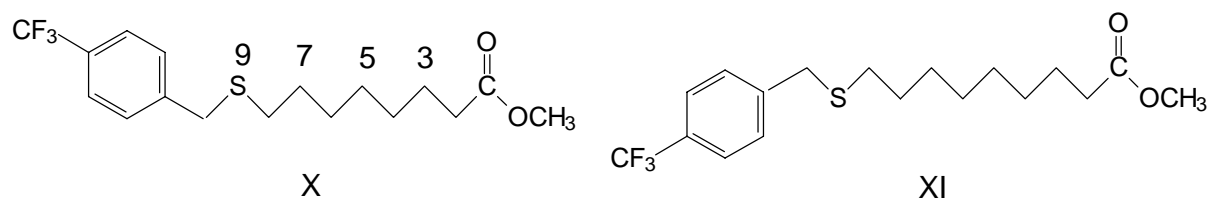
5.1.3 Cellular calcium ion concentration. Calcium ion is a nearly universal messenger in cellular communication and the intracellular concentration of free Ca^{++} is tightly regulated. Metal cations such as Cd^{++} or Zn^{++} can compete with Ca^{++} for calcium ion binding sites and are thus potentially toxic when present at inappropriate concentrations. The fluorinated analog of EDTA (5F-BAPTA) shown on the next page coordinates a variety metal ions. The metal ion dissociates slowly from the complexes and separate fluorine signals are observed for free 5F-BAPTA and a metal ion complex. Importantly, the fluorine chemical shift of each metal ion-5F-BAPTA complex is distinctive (Figure 5.1.2). Benters, *et al.* (1997) have used fluorine NMR observations of 5F-BAPTA to examine the uptake of cadmium and zinc ions by rat neuroblastoma cells and the modulation of intracellular Ca^{++} levels by these cations.
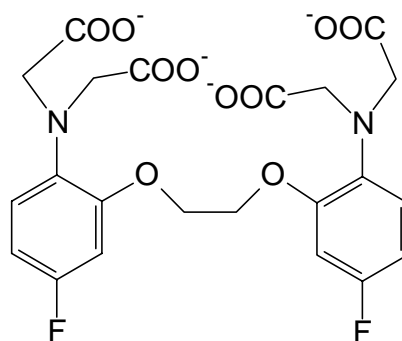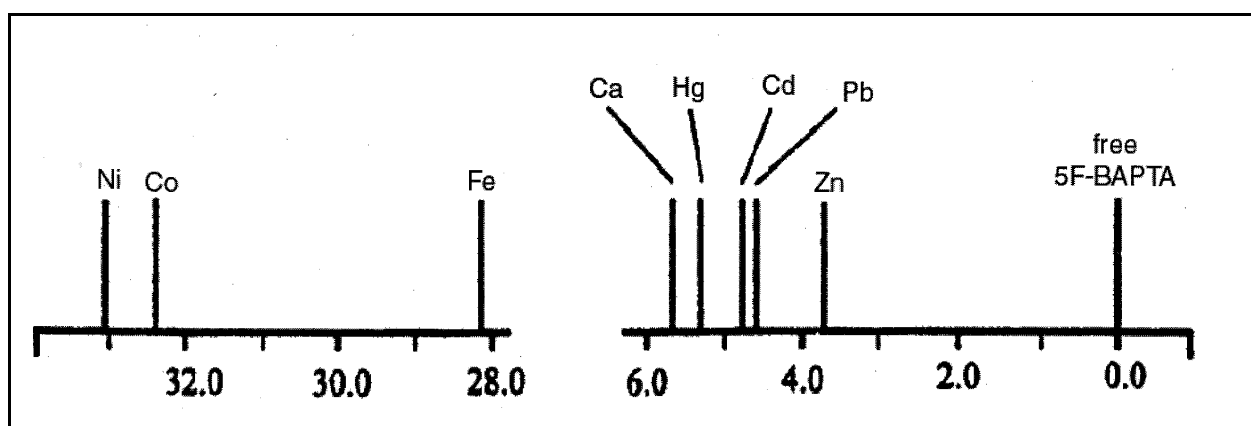
5F-BAPTA

Figure 5.1.2 *Fluorine chemical shifts of various metal ion complexes with 5F-BAPTA compared to the shift of the free ligand. Figure adapted from Benters, et al., 1997.*

5.2 FLUORINE NMR STUDIES OF LIGAND-RECEPTOR COMPLEXES

5.2.1. Inhibition of Carbonic Anhydrase. Carbonic anhydrase (CA) is a metalloenzyme that catalyzes the hydration of CO_2. Virtually all plant and animal tissues contain one or more carbonic anhydrase isozymes. Aromatic and heterocyclic sulfonamides are particularly potent competitive inhibitors of both the hydration of CO_2 by carbonic anhydrase and the opposing reaction, the dehydration of carbonic acid. Sulfonamide inhibitors of carbonic anhydrase provided the first modern oral diuretics; aryl and heteroaryl sulfonamides have shown promise as anti-epileptic agents, have been used to lower intraocular pressure in the treatment of glaucoma, and have been used to promote healing of gastric and duodenal ulcers.

The interaction of 4-fluorobenzenesulfonamide (**XII**) with the two isozymes of human carbonic anhydrase found in the red blood cell (CA I and CA II) has been examined by fluorine NMR. A titration of CA I with **XII** followed by fluorine NMR (spectrum on next page) demonstrated that the stoichiometry of interaction of this inhibitor with CA I is 2:1. This titration is feasible because the

dissociation of the sulfonamide-enzyme complex is slow enough that separate signals for free and enzyme-bound inhibitor molecules are observed (Dugad and Gerig, 1988). At low concentrations of the inhibitor relative to the enzyme concentration only the fluorine spectrum of the enzyme-bound inhibitor is observed. When a stoichiometric excess of inhibitor has been added, signals for both free and bound inhibitor molecules are apparent.

SO_2NH_2 SO_2NH_2

XII   XIII
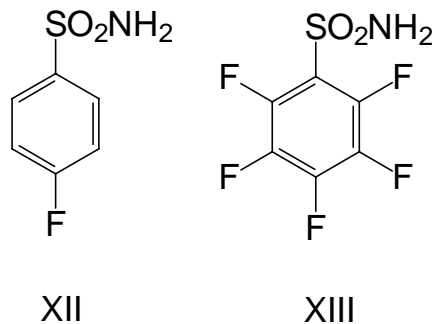
Interestingly, the fluorine spectrum for carbonic anhydrase-bound **XIII** consists of five signals of equal intensity (Gerig and Moses, 1987). The molecular environments of the fluorines of the pentafluorophenyl ring in these complexes must on average be different, implying that rotation of this aromatic ring within the complex is slow. Measurements of the rate of pentafluoroaromatic ring rotation by fluorine NMR suggest that the ring rotation rate in the complex of **XII** with CA I is about 4 times per second. However, in the complex with CA II the ring rotation is about 6000 times faster than this.
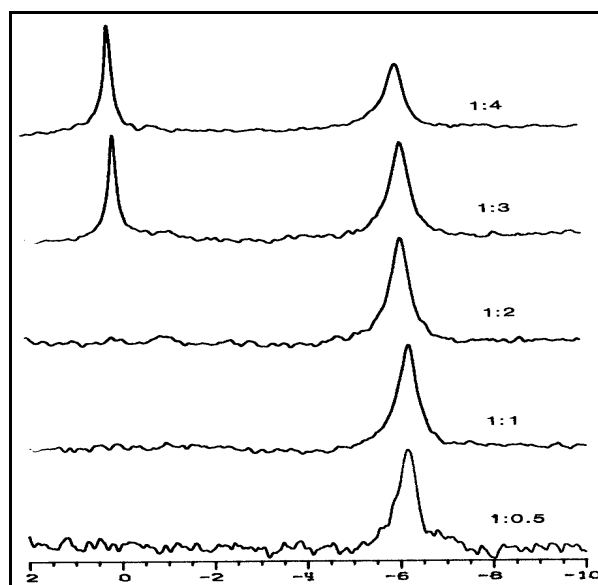

Figure 5.2.1 *Fluorine NMR spectra of the 4-fluorobenzene-sulfonamide-human CA I complex at various ratios of inhibitor to enzyme concentrations, indicated on the traces.*

5.2.2 Retinol trafficking. Vitamin A, shown below as its all-*trans*-retinol form, is an essential nutrient in all mammals. All forms of the vitamin are highly insoluble in water and are moved from tissue to tissue by interacting with specific intracellular carrier proteins. Quantitative studies of these interactions present some experimental difficulties because of the low solubility of the vitamin and other lipids.

Rat cellular retinol-binding protein (CRBP) and cellular retinol-binding protein II (CRBP II) are highly homologous cytoplasmic proteins that bind all-*trans*-retinol with high affinity. They are
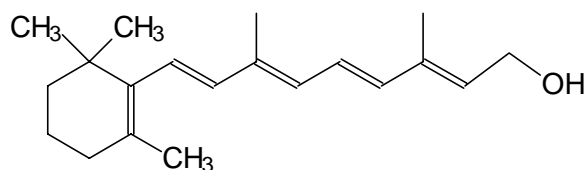
All-*trans*-retinol (Vitamin A)
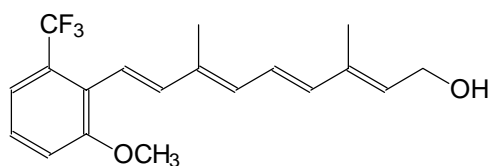
XIV

found at varying levels in a wide variety of rat tissues where they are involved in the intracellular trafficking and metabolic processing of retinol. Each of these proteins is small (15.6 kDa) and contains four tryptophan residues. Li and coworkers have achieved 85-93% replacement of the tryptophan residues by 6-fluorotryptophan (Rong, *et al.*, 1997). Fluorescence studies showed that incorporation of 6-fluorotryptophan into the protein does not significantly affect retinol binding. Some fluorine spectra of 6-FTrp-containing CRBP II in the presence of varying amounts of retinol are shown in Figure 5.2.3. The first trace (A) shows the fluorine NMR spectrum of fluorine-containing CRBP-II in the absence of any lipidic materials. The assignments of the signals observed to specific tryptophans of the receptor were made by site-specific mutations. The resonances for 6-FTrp-9 and 6-FTrp-107 are nearly coincident.[5] Complexation of all-*trans*-retinol to CRBP-II changes the fluorine spectrum to that shown in the second trace (B). In the complex the fluorine signals for residues 9 and 107 are distinctively changed while there are only small shifts of the signals from the other fluorotryptophans. Thus, a structural change in the receptor sufficient to alter appreciably the local environment of tryptophans 9 and 107 likely accompanies binding of the retinol ligand. The remainder of Figure 5.2.3 shows the results of associating the protein-retinol complex with phospho-lipid vesicles. The reappearance of the spectrum of the apo-CRBP-II at high concentrations of vesicles in spectrum D makes it clear that some of the ligand is transferred to the vesicular phase under these conditions.

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[5] Two signals are observed for 6-FTrp-89. These arise because some of the protein retained the amino terminal initiator (Met) from the bacterial synthesis of the fluorine-labeled protein, while the remainder had the Thr residue expected as the second residue of the translation product. Apparently some post-translational processing of the protein by aminopep-tidases present in *E. Coli* takes place to remove the Met residue.

Transfer of ligand from the receptor protein to the vesicles may also be examined by placing a fluorine "label" in the ligand. A trifluoromethylated analog all-*trans*-retinol (**XIV**) was prepared. The fluorine NMR spectrum of this ligand in phosphatidylcholine vesicles is shown in trace A of Figure 5.2.4. When bound to CRBP-II the fluorine resonance of the analog is shifted about 0.6 ppm. As increasing amounts of phospholipid are added to the CRBP-ligand complex, the reappearance of the signal for vesicle-associated ligand shows that transfer of the trifluoromethylated retinol analog from the receptor protein to the vesicles has taken place.
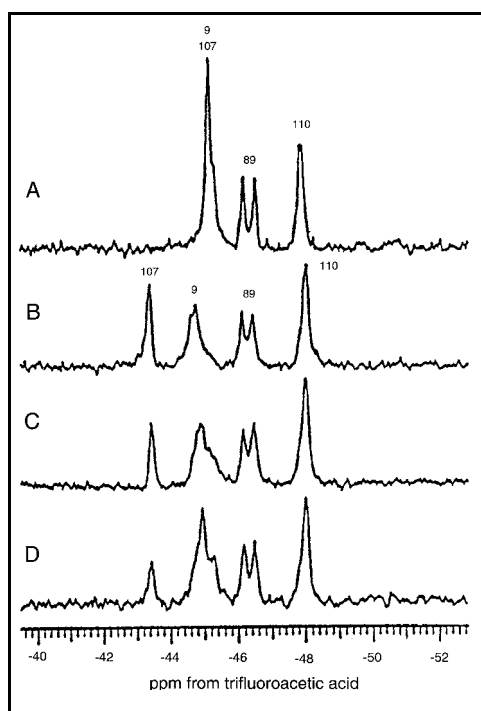

Figure 5.2.3. *Fluorine-19 NMR spectra of 6-fluorotryptophan-containing rate cellular retinol binding protein (CRBP) in the absence of ligand (A), with all-trans-retinol bound (B). In spectra C and D, protein with ligand bound is exposed to increasing amounts of vesicles formed from phosphatidylcholine.*
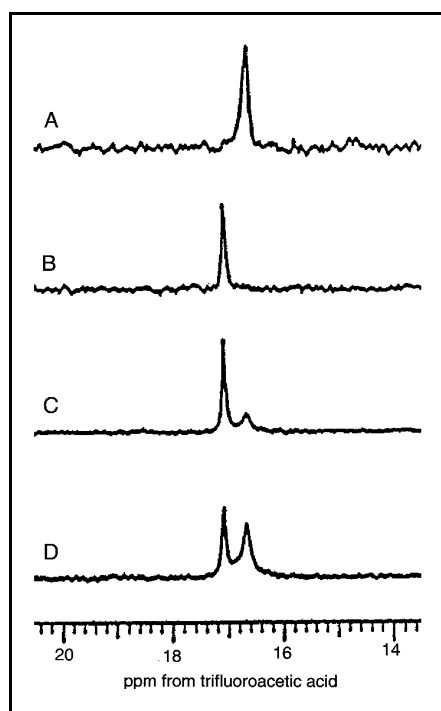

Figure 5.2.4 *Fluorine-19 NMR spectra of ligand XIV with rat cellular retinol binding protein (CRPB) and vesicles prepared from phosphatidylcholine. In spectrum A on the vesicles are present while in B only the protein in present. Spectra C and D are from samples containing vesicles and protein.*

5.3 PROTEIN DENATURATION

Proteins are special among polymeric molecules in that they have the ability to assume carefully tailored three-dimensional or "tertiary" structures. Understanding those processes which lead to folding of a protein or other biological macromolecule into its native, functional three-dimensional structure is fundamental, yet the forces which determine tertiary structures and the mechanisms by which proteins get to them remain poorly understood. Experimental evidence indicates that the

folding-unfolding process for many proteins involves just two states of the biopolymer, a folded state in which the native tertiary structure is present and an unfolded (denatured) state in which the polypeptide chain appears to be random.

Hoeltzli and Frieden (1994, 1996) have replaced the five tryptophan residues of the enzyme dihydrofolate reductase by 6-fluorotryptophan. The enzyme uses NADPH as a co-factor and is able to bind a good inhibitor of the enzymic activity, methotrexate, at the same time that it binds NADPH. Fluorine NMR spectra of 6-fluorotryptophan-containing dihydrofolate reductase with no ligands bound, with NADPH bound, with methotrexate bound, or with both ligands bound each exhibit five signals (Figure 5.3.1). The observed resonances have been assigned to specific fluorotryptophan residues in the sequence by site-specific mutations.

Crystallographic studies of these various forms of dihydrofolate reductase show that the basic three-dimensional fold of the protein is essentially the same for the native enzyme and the various enzyme-ligand complexes. However, it is clear from the distinctive fluorine NMR spectra that the local structures around each tryptophan are different in the different complexes. Moreover, the variations in the signal line widths that are observed may indicate the presence of interconversions between conformations that take place at different rates in the various complexes.

Native dihydrofolate reductase or any of the complexes mentioned unfold when they are dissolved in concentrated urea solutions. The unfolding process removes local tertiary structures that are responsible for the differences in fluorine shielding parameter observed and the fluorine spectrum changes to a collection of closely spaced lines near -46.5 ppm on the shift scale used. There are thus distinct fluorine NMR signatures for the native, folded protein and for the denatured protein.
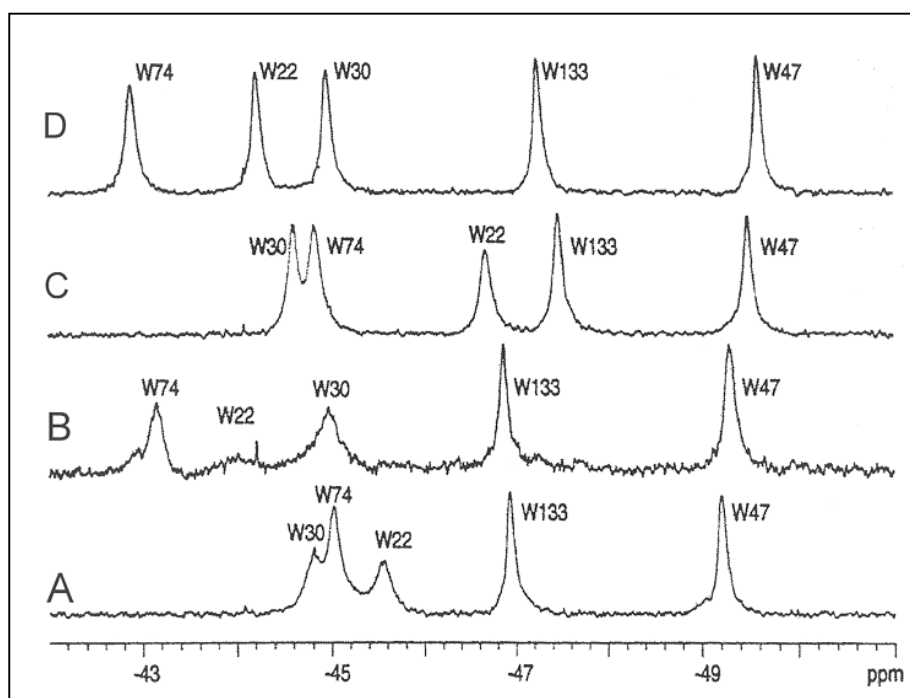

Figure 5.3.1. *Fluorine-19 NMR spectra of 6-fluorotryptophan-containing dihydrofolate reductase (E. Coli). (a) Native enzyme in the absence of any ligands; (B) NADPH-enzyme complex; (C) methotrexate-enzyme complex; (D) NADPH-methotrexate-enzyme complex.*

Hoeltzli and Frieden (1996) have used rapid mixing methods coupled with fluorine NMR spectroscopy to follow the rates of the folding-unfolding processes. In one series of experiments the 6-fluorotryptophan-containing protein was dissolved in 4.6 M urea, conditions under which dihydrofolate reductase is completely unfolded. Using the rapid mixing procedure the concentration of urea was quickly reduced to 2.3 M, a urea concentration where the enzyme is folded into its correct tertiary structure. Fluorine NMR spectra could be obtained in as little as 1.5 s after the dilution had been accomplished. Collection of additional spectra at intervals after mixing provided a means to quantify the rate at which the unfolded enzyme disappeared from the spectra, as well as the rate at which the native enzyme appeared. Under the conditions indicated, disappearance of unfolded enzyme was biphasic, but was largely over within 20 s. However, appearance of the native enzyme spectrum was significantly slower, requiring about 500 s for completion. This behavior indicates that one or more intermediate protein forms must be present in the folding-unfolding reaction(s) of dihydrofolate reductase.

5.4 THE ROLE OF SPECIFIC AMINO ACIDS IN PROTEIN FUNCTION

Nature commonly uses a palette of 20 amino acids in designing proteins. Understanding the role of particular amino acids in defining protein tertiary structure is an important component of attempts to relate protein structure to function. Examination of the effects of mutating one amino acid to another is an essential tool in these efforts. However, because of differences in spatial requirements or side chain electrical properties, simple replacement of one of the "natural" amino acids by another may be too large a perturbation of a protein structure to provide meaningful conclusions. Fluorine-for-hydrogen substitution on the natural amino acids offers the ability to change the electronic structure of an amino acid while not greatly altering its shape or spatial requirements.

Methionine appears rarely in proteins but plays a critical role in processes such as small molecule recognition. Methionine has a hydrophobic side chain that is more flexible than the sidechains of residues such as isoleucine, and because of the large sulfur atom, is more polarizable. Duewel, *et al*. (1997) have demonstrated that the trifluoromethyl analog of methionine (L-S-(trifluoromethyl)homocysteine) can be incorporated into proteins produced by *E. Coli*. This amino acid is a potent growth inhibitor in this and other organisms but, under appropriate conditions, up to 70% replacement of native methionine by the trifluoromethyl analog can be obtained.

$$H_3\overset{+}{N} \quad \quad H$$
$$^{-}OOC \quad \quad \quad S \diagup CF_3$$
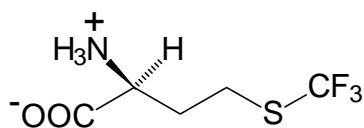
L-S-(Trifluoromethyl)homocysteine

The fluorine NMR spectrum of phage lysozyme isolated from their system exhibited four signals (Figure 5.4.1). The primary sequence of the enzyme contains only three methionines and it appears from studies of signal intensities that the two resonances at highest field in the spectrum represent different conformations of the trifluoromethylated sidechain of a single methionine. Tertiary structures of lysozymes exhibit a pronounced saccharide binding cleft that is commodious enough to bind five or more sugar residues of a polysaccharide. All fluorine signals from the trifluo-

romethylmethionine-containing lysozyme shift (slightly) when an oligomer of N-acetylglucos-amine binds to the protein. There are also line width changes upon binding. Detailed interpretation of these spectral changes, however, will have to await the assignment of the fluorine resonances observed to specific amino acids.
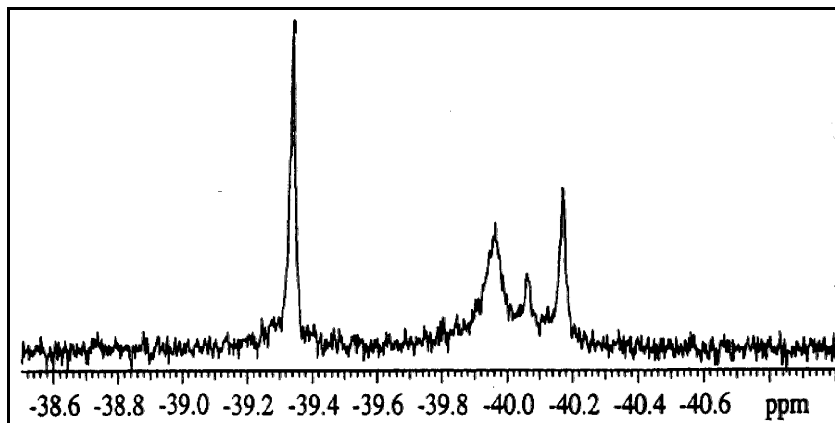

Figure 5.4.1. *Fluorine NMR spectrum of trifluoromethylmethionine-containing phage lysozyme with a pentamer of N-acetylglycosamine bound to it. The total intensity of the signals at -40.05 and -41.15 ppm (relative to CFCl$_3$) is equal to the intensities of each of the remaining signals, suggesting that one trifluoromethyl-methionine side chain is present in two conformations.*

5.5 STRUCTURAL MOBILITY

It was indicated earlier that nuclear spin relaxation of fluorine in biological systems depends on the interaction of nuclear magnetic dipoles (the dipole-dipole relaxation mechanism) and the anisotropy of fluorine chemical shifts (the CSA relaxation mechanism). A critical aspect of both relaxation processes is the local mobility of the structure that contains the fluorine nucleus. Measurement and analysis of fluorine relaxation behavior can therefore provide details of molecular motions.

The bacterium *E. Coli* has a periplasmic receptor protein for glucose and galactose. London and co-workers have examined fluorine relaxation in a version of this protein in which the five tryptophan residues have been replaced by 5-fluorotryptophan (Luck, *et al*., 1996). Detailed analysis of fluorine T_1 and T_2 relaxation data for this system showed that the fluorotryptophan rings reorient with a correlation time (τ_c) of about 23 ns. This is essentially the same correlation time that is expected for a globular protein of the size (33 kDa) of the glucose/galactose receptor protein. However, the analysis also showed that there are additional, highly restricted motions of the fluorotryptophans that take place with a correlation time of about 10^{-2} ns. The picture that emerges is that the tryptophan rings are not able to reorient in a major way, presumably because such motion would require large reorganizations of protein tertiary structure to permit such motion. However, small scale motions wherein three-dimensional structures change locally in relatively minor ways appear to be facile and rapid.

A clever aspect of this work was simultaneous incorporation of 5-fluorotryptophan in which the hydrogens of the tryptophan indole ring had been replaced by deuterium. Because the fluorine

chemical shift is sensitive to this isotopic substitution, for each tryptophan position separate signals were observed for fluorotryptophan rings holding hydrogen (^{1}H) and holding deuterium (^{2}H). The magnetic dipole of ^{1}H is much larger than that of ^{2}H, so comparison of the relaxation behavior of the two signals provided an incontrovertible indication of the contribution of indole ring hydrogens to the overall relaxation of a fluorine.

Fluorine relaxation has been used to study protein mobility in intact cells. Brindle and co-workers have developed methods for selectively incorporating 5-fluorotryptophan into three glycolytic enzymes (hexokinase, phosphoglycerate kinase, and pyruvate kinase) of yeast (Williams, Haggie, and Brindle, 1997). Fluorine relaxation behavior was examined for these fluorinated proteins within the intact organism, for preparations in which the cells had been broken, and for the proteins *in vitro* as isolated and purified materials. Recall that fluorine transverse (spin-spin) relaxation becomes more efficient as the molecular correlation time increases, leading to an increase in fluorine NMR signal line width. Reasonably sharp fluorine signals are observed for hexokinase and phosphoglycerate kinase in intact yeast cells, indicating that these enzyme molecules tumble in the cytoplasm of these cells at nearly the rate they do *in vitro*. (An analysis suggested that the viscosity of the cytoplasm is about twice the viscosity of pure water.) However, signals for fluorinated pyruvate kinase were barely detectable using intact cells, although experiments with disrupted cells showed that the enzyme was indeed present. There must be interactions of this enzyme with other cellular constituents in intact cells that are not present in disrupted cells. They are strong enough to increase the correlation time of pyruvate kinase significantly, leading to broadened signals that are difficult to detect above baseline noise.

Formation of organized, multi-enzyme complexes within cells is thought to offer kinetic and regulatory advantages to the cells. These advantages cannot be explored reliably using broken cell preparations or isolated proteins. The results of these researchers show that fluorine NMR methods will likely have utility in unraveling details of these multi-protein complexes as they exist *in vivo*.
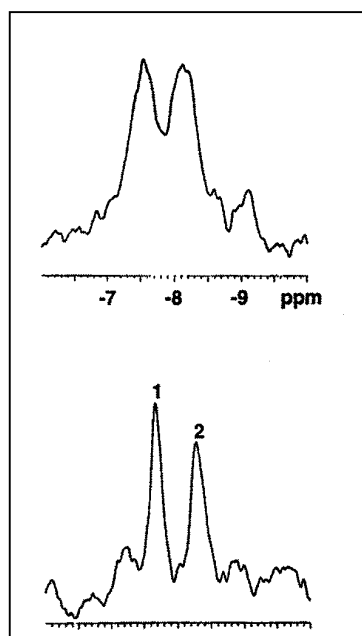

Figure 5.5.1 *Fluorine NMR spectra of 5-fluoro-tryptophan-containing phosphoglycerate kinase in intact yeast cell (top) and as a purified in vitro sample.*
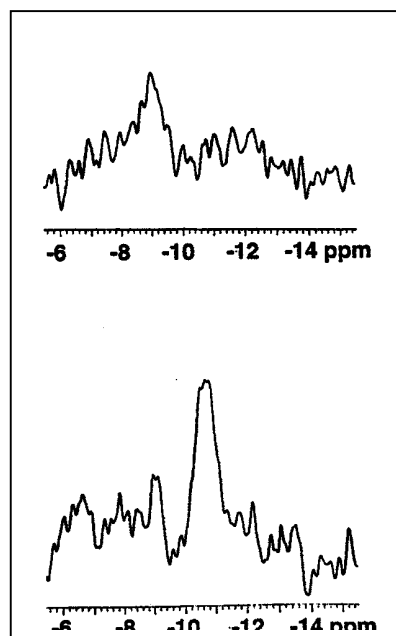

Figure 5.5.2 *Fluorine NMR spectra of 5-fluoro-tryptophan-containing pyruvate kinase in intact yeast cells (top) and in a cell lysate (bottom).*

5.6 STRUCTURAL EQUILIBRIA IN NUCLEIC ACIDS

Structure and conformation in small RNAs that contain 5-Fluorouridine have been explored by fluorine NMR experiments (Arnold and Fisher, 2000). These spectra provide evidence of different conformational states and how the populations and interconversion of these states are altered by the presence of metal ions. Standard $^1H\{^1H\}$ NOESY experiments have been used to explore the structural effects of 5-fluorouridine into short RNA duplexes (Sahasrabudhe and Gemeiner, 1997).

Klimasauskas, *et al.* (1998) have examined conformations of the base targeted for methylation in a complex of a 12mer DNA duplex bound to the methyl transferease M.*Hha* I. The target cytosine was replaced in the complex by 5-fluorocytosine. The molecular weight of the resulting complex was 46 kD, a sufficiently large system that structural studies by standard proton NMR methods would be problematic. Fluorine NMR data, in conjunction with gel shift assays, showed that three conformations of the 5-fluorocytosine ring were present, including structures in which the ring was flipped out of the stack of DNA base pairs, a conclusion important for understanding the mechanism of action of this enzyme.

Fluorine-proton nuclear Overhauser effects have been used in structural studies of DNA duplexes in which a thymidine has been replaced by a difluorotoluene nucleoside analog that is believed to mimic the hydrogen bonding abilities and steric requirements of the natural nucleoside (Guckian, Krugh and Kool, 1998). These and other NMR observations confirmed that the structure of the difluorotoluene-adenine pair closely resembles the natural thymine-adenine Watson-Crick base pair.
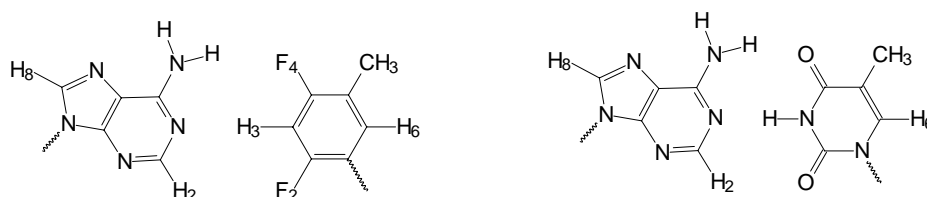

Schematic of the adenine-2,4-difluorotoluene pair (left). The adenine-thymine (A-T) base pair is shown on the right.

5.7 DEPTH PROFILING IN MEMBRANES

Molecular oxygen is quite soluble in highly fluorinated materials. Fluorocarbon-based systems that have been developed as blood substitutes take advantage of this property (Reiss and Krafft, 1998). Molecular oxygen is paramagnetic and its presence has appreciable effects on T_1 and T_2 relaxation times of spin 1/2 nuclei that it contacts, including fluorine (Parhami and Fung, 1983; Endo, Yamamoto, and Kado, 1993). Prosser and co-workers (2000, 2001) have incorporated the fluorinated detergent TFOM into bicellar dispersions and lipid bilayer systems, organized structures that are models for membrane systems. By increasing the partial pressure of O_2 to 100 atm or more they showed that the paramagnetic effects of molecular oxygen on fluorine relaxation varied with position along the perfluoroalkyl chain of the detergent. Oxygen had significantly larger effects on

the relaxation times of the fluorines of the terminal trifluoromethyl group of the detergent than on the fluorines of the difluoromethylene groups near the head group of the detergent. Fluorine chemical shifts were also found to be diagnostic of relative immersion depth of a given fluorine of the detergent in these systems. Oxygen is known to accumulate toward the hydrophobic interior of a lipid bilayer and the shift and relaxation effects observed are consistent with the insertion of the nearly fully extended detergent molecule into the bicellar and bilayer structures such that the sugar part of the molecule is on the solvent-exposed surface of these structures.

TFOM
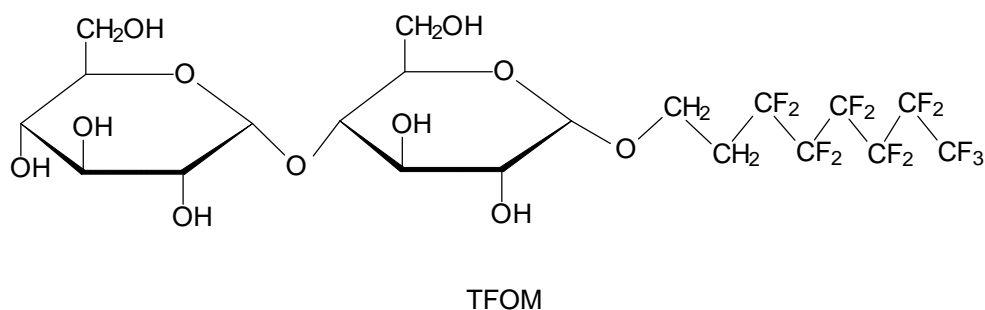
6. EFFECTS OF FLUORINE ON STRUCTURE AND DYNAMICS IN BIOLOGICAL SYSTEMS

The importance of fluorine-substituted materials in pharmacology is based on several aspects of the chemical nature of the carbon-fluorine bond. (1) Chemical bonds between carbon and fluorine are stronger than carbon-hydrogen bonds in corresponding situations. (2) Covalent fluorine is stereochemically similar to covalent hydrogen and to the hydroxyl group. Replacement of either H or OH in a structure should not have major consequences as regards steric considerations related to conformation or "fit" in a receptor site. (3) Fluorine is the most electronegative element and bonds between carbon and fluorine are highly polar. (4) While covalent fluorine cannot be a hydrogen-bond donor it potentially is a hydrogen-bond acceptor. (5) Fluorine substitution increases the hydrophobicity of an organic molecule.

Substitution of H or OH by F in a biologically important molecule could have substantial effects because a new set of chemical interactions are introduced. Should these be strong enough or directional enough they could produce changes in structure in the vicinity of the fluorine or in the rates at which processes such as conformational change or ligand dissociation take place. Crystallographic studies of glutathione transferase in which all four tryptophans have been replaced by 5-fluorotryptophane or in which all 14 tyrosine residues have been replaced by 3-fluorotyrosine have been reported (Parsons, *et al.*, 1998; Xiao, *et al*, 1998). The presence of fluorotryptophan does not alter the rates of substrate association or dissociation from the enzyme, but does increase the rate of substrate turnover. The crystal structure shows that the presence of fluorine causes local conformational changes that are consistent with the observed acceleration of product release from the enzyme. The presence of 3-fluorotyrosine produces local conformational changes at most of the tyrosine positions of the enzyme. At this time there seems to be no summarizing conceptualization of how covalent fluorine within or on the surface of a biological macromolecule alters local tertiary structure or the dynamics. Thus, some care in extending conclusions drawn from fluorine NMR

studies of a fluorine-containing system to the corresponding native or non-fluorinated system is appropriate.

7. CONCLUSIONS

There are several advantages of fluorine NMR in the context of exogenous "reporter group" or "spin label" kinds of experiments, of which the most important derive from the high sensitivity of the fluorine chemical shift to local environment. Numerous recent examples show how fluorine chemical shift sensitivity facilitates study of structure and dynamics in biological systems. There is a high probability that the shifts of a fluorinated ligand free in solution and bound to a receptor will be distinguishable so that NMR methods can be used to probe details of a receptor-ligand complex. The processes that lead to relaxation of fluorine nuclei in biological materials are well-understood and analysis of fluorine relaxation data can provide indications of structural mobility.

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9. ACKNOWLEDGMENTS