Journal of Magnetic Resonance **142,** 1–10 (2000)
Article ID jmre.1999.1931, available online at http://www.idealibrary.com on IDEAL®

The $H_2{}^{18}O$ Solvent-Induced Isotope Shift in ^{19}F NMR

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Received February 2, 1999; revised August 26, 1999

The $H_2{}^{16}O/H_2{}^{18}O$ solvent-induced isotope shifts (^{18}O SIIS) of the ^{19}F NMR signals of a number of fluorine compounds have been measured. These isotope shifts are observed to be upfield, downfield, or zero, depending on the specific compound and the precise solution conditions. At 25°C and with an ^{18}O enrichment of 86%, the ^{18}O SIIS of several fluorinated amino acids were in the range of 0.0014–0.0018 ppm downfield. 5-Fluorouridine displays a significantly wider range of ^{18}O SIIS values. A 5-fluorouridine-labeled 16-mer RNA also displayed observable ^{18}O SIIS values, but the characteristics of these were significantly modified from those of free 5-fluorouridine. The experimental observations are consistent with the ^{18}O SIIS being composed of upfield and downfield components, with the relative contributions of these determining the size and direction of the overall isotope shift. This is discussed in terms of a combination of van der Waals interactions between the fluorine atom and the solvent, electrical and hydrogen bonding effects, and the perturbations to these due to ^{18}O substitution in the solvent water. This isotope effect promises to be a highly useful tool in a range of ^{19}F NMR studies.

Key Words: **^{19}F; ^{18}O; solvent; isotope shift.**

INTRODUCTION

Fluorine can be a useful label for incorporation in macromolecules such as proteins and nucleic acids (*1, 2*). Among the advantages of fluorine-based techniques in biomolecular NMR are that it is a relatively sensitive NMR nucleus, there is a general absence of background signals, and it displays a wide range of chemical shifts. Flourine can be readily incorporated biosynthetically into proteins, using fluorinated amino acids (*1*). 5-fluorouracil can be incorporated into RNA and DNA via chemical synthesis, enabling site-specific incorporation (*1*). Alternatively, fluorine incorporation into RNA can be achieved by transcription in the presence of the appropriate triphosphate (*1*).

The ^{19}F chemical shift is highly sensitive to the nuclear environment, such that it demonstrates appreciable H_2O/D_2O solvent-induced isotope shifts (SIIS) (*1–3*), i.e., changes in chemical shift when solvent H_2O is exchanged for D_2O. For many water-soluble fluorine compounds, this shift is around 0.2 ppm upfield (*1*), though it is as much as 3 ppm for fluoride (*3, 4*). The H_2O/D_2O SIIS has been used to assess the solvent exposure of specific residues in proteins (*1, 2*) and nucleic acids (*5–8*). Of course the change of solvent from H_2O to D_2O also means that all the solvent-exchangeable protons of a biomolecule will be changed to deuterons. It has been remarked that the structure and conformational averaging of the deuterated molecule may be subtly different to the proton version, and the ^{19}F H_2O/D_2O SIIS may in part be a response to these effects (*1*). The deuterium effect on the dissociation constants of water, functional groups, and buffers are also potential complicating factors. Thus, the *quantitative* analysis of the H_2O/D_2O SIIS in terms purely of solvent access requires caution. The use of $H_2{}^{18}O$ as a solvent does not involve any such perturbation of the host molecular structure or internal dynamics, and it is far less perturbing of dissociation constants, so in principle any displacement of a ^{19}F signal induced by this isotopic water can be confidently attributed to interactions with the solvent. In a recent preliminary report, we noted the observation of $H_2{}^{16}O/H_2{}^{18}O$ SIIS (herein the ^{18}O SIIS) on the ^{19}F signals of fluoride and 5-fluorouridine and the absence of such an effect with 5-fluorouracil (*4*). Here, we further characterize the effect with the study of a number of other fluorinated molecules, several of which are potential or actual macromolecular fluorine labels. We also demonstrate the ^{18}O SIIS in a prototype macromolecular case; a fluorine-labeled RNA.

RESULTS AND DISCUSSION

The ^{18}O SIIS of a range of fluorinated molecules is shown in Table 1; the isotope shifts are all distinctly small, including upfield, downfield, and zero isotope shifts. Certain compounds were observed within the same NMR sample; the five fluorinated amino acids were mixed, as were 3-fluoro-3-deoxy-D-glucose and the 2-fluoro-adenine derivative. This mixing did not alter the ^{19}F chemical shifts, so intercompound effects were minimal. Concentrations of the fluoro-compounds were kept relatively low (1 mM) for each compound, to minimize any intermolecular effects. Some examples of the spectra are shown in Fig. 1. Although the isotope shifts are relatively small, they were resolvable from the experimental linewidths

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1090-7807/00 $35.00

TABLE 1
^{18}O SIIS Values of the ^{19}F Signals for a Selection of Fluorine Compounds

Compound	δ_F, ppm[a]	H_2O/D_2O SIIS 25°C, ppm[b]	^{18}O SIIS, 25°C ppm[c]	^{18}O SIIS, 37°C ppm[c]
3-fluoro-tyrosine	29.3	−0.254	+0.0014	−0.0033
5-fluoro-tryptophan	41.1	−0.226	0	+0.0018
2-fluoro-phenylalanine	47.9	−0.199	0	0
3-fluoro-phenylalanine	52.7	−0.186	+0.0014	+0.0023
4-fluoro-phenylalanine	50.3	−0.180	+0.0016	+0.0025
5-fluoro-cytosine	−2.18	−0.308	+0.0058	+0.0085
2-fluoro-adenine-9-β-D-arabinofuranoside	113.0	−0.130	[d]	[d]
fluoro-benzene	52.27	−0.175	−0.0023	−0.0059
2-fluoro-pyridine	95.25	−0.233	[d]	[d]
3-fluoro-3-deoxy-D-glucose:				
α-anomer	−28.7	−0.165	0	0
β-anomer	−33.6	−0.182	0	0

[a] Chemical shifts measured in D_2O, and are given relative to 5-fluorouridine in D_2O at 25°C.
[b] For 100% D_2O and 100% H_2O, estimated error span ±0.001 ppm.
[c] Estimated error span ±0.0002 ppm; for 86 atom % ^{18}O.
[d] ^{19}F signals were too broad to resolve ^{18}O isotope shifts.

(with a couple of exceptions noted below) and reproducible. The experimental linewidth imposed the smallest ^{18}O SIIS that could be detected. The ^{18}O-enriched samples required the use of concentric inserts in the NMR tube, and shimming in the presence of the extra surfaces present meant that the ^{19}F linewidths were slightly greater than the linewidths obtained with standard single-wall NMR tubes. ^{18}O SIIS values at 25 and 37°C are given in Table 1, showing that in most cases the magnitude of the observed ^{18}O SIIS increased with rising temperature, though the 3-fluorotyrosine value actually changed sign between these two temperatures. For comparison, the H_2O/D_2O SIIS values at 25°C are also given in Table 1; clearly there is no simple relation between this isotope shift and the ^{18}O-induced shift.

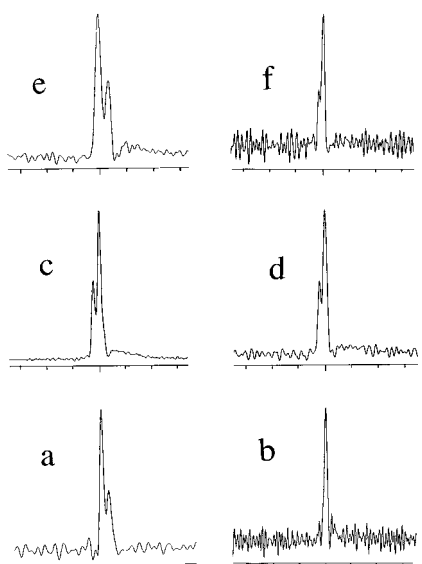

FIG. 1. ^{19}F spectra showing varying ^{18}O SIIS values among a selection of compounds. The samples were prepared as described in the Experimental section; the ^{18}O-shifted signals (using 86 atom% ^{18}O) are the smaller ones in each case. (a) fluorobenzene, (b) β-anomer of 3-deoxy 3-fluoro-D-glucose, which does not display an ^{18}O isotope shift, (c) 4-fluorophenylalanine, (d) 3-fluorophenylalanine, (e) 3-fluorotyrosine, and (f) 5-fluorotryptophan. The fluorobenzene spectrum was recorded with sample temperature of 25°C, the other spectra shown here were at 37°C. The tick marks on the axes are at 0.01 ppm intervals.

2-Fluoro-adenine-9-β-D-arabinofuranoside, a potent inhibitor of nucleic acid biosynthesis, displayed a relatively broad ^{19}F resonance under the conditions used here, with a linewidth of 6.1 Hz at 25°C, corresponding to 0.013 ppm at the field used, which is significantly larger in magnitude than most of the ^{18}O SIIS values in Table 1. No ^{18}O SIIS was discerned for this compound, which may be either because it is essentially zero, or it is much less than this linewidth. Similarly, the signal for 2-fluoropyridine displayed a linewidth of around 12 Hz (corresponding to 0.025 ppm). These linewidths were in the presence of proton decoupling, which produced adequately narrow signals for other fluorine compounds examined. It thus seems that an aromatic fluorine adjacent to one or two ring nitrogens is not conducive to sharp ^{19}F signals under the solution conditions used here.

Fluorinated amino acids were of particular interest in our study as they are useful reporters when incorporated into proteins. In our previous report we noted that the presence of 30 mM fluoride had a strong influence on the ^{18}O SIIS of 5-fluorouridine (*4*), but this was not found to be the case with the fluorinated amino acids examined here. The addition of 30 mM NaF to the fluorinated amino acids effectively quenched the ^{18}O SIIS values, so that they became

effectively zero. Addition of 30 mM NaCl had the same quenching effect.

Neither anomer of 3-fluoro-3-deoxy-D-glucose (whose ^{19}F signals have been assigned previously (*9*)) displayed an ^{18}O SIIS under the conditions employed, including the addition of 30 mM NaF. Apart from fluoride, this is the only nonaromatic fluorine compound we have so far investigated. While these observations for just one example of a fluorinated sugar are not necessarily representative of this entire class of compound, it may indicate that the ^{18}O SIIS will not be useful for studies of sugars and oligosaccharides.

So far we have only considered ^{18}O SIIS with an ^{18}O enrichment level of 86%. Given the small sizes of the isotope shifts, it was impractical to obtain reliable isotope shifts at lower enrichment levels as we have done previously with 5-fluorouridine (*4*). Indeed, the magnitudes of the ^{18}O SIIS values arising from the compounds surveyed here have all been smaller than we have previously seen in 5-fluorouridine. Thus, 5-fluorouridine is a particularly suitable molecule to use for more detailed characterization of this ^{18}O effect.

We had previously noted that the ^{18}O SIIS of 5-fluorouridine was strongly influenced by the presence of NaF (*4*). It was thus of interest to determine if this effect is due to the presence of fluoride specifically, or is it related to the increase in ionic strength, in which case NaCl should also be effective. Figure 2a shows the ^{18}O SIIS values of the ^{19}F signals of 5-fluorouridine and fluoride with increasing salt concentration; initially this was by addition of NaCl (to 46 mM), then by addition of NaF (up to 91 mM total salt). The plot suggests that up to moderate ionic strength the observed ^{18}O SIIS of fluorouridine has a linear downfield dependence on the salt concentration, irrespective of this being NaF or NaCl. The deviation from linearity at a salt concentration of 80–90 mM is not a result of mixing NaCl and NaF, but is due to a change in the dependence of the isotope shift on salt concentration. This is clear in Fig. 2b, which shows how at higher salt levels the ^{18}O SIIS becomes decreasingly downfield with added NaCl, the slope has changed sign (and is not as steep) from that in Fig. 2a. The ^{18}O SIIS of fluoride is somewhat less sensitive to the ionic strength at lower salt levels, and between ca. 75 and 230 mM salt its dependence seems to parallel that of 5-fluorouridine (Fig. 2b).

The ^{19}F signal of 5-fluorouridine was also used to investigate further the temperature dependence of the ^{18}O SIIS. As shown in Fig. 3, with increasing temperature this isotope shift is increasingly downfield, and a nonlinear relationship applies. The ^{18}O SIIS for fluoride (present in the same sample) changes from an upfield value at lower temperatures to zero at higher temperatures.

While it is clear that 5-fluorouridine may display a relatively strong ^{18}O SIIS, it remained to be investigated if this still held when 5-fluorouridine was embedded in an oligonucleotide. For this we used a 16-mer RNA, the sequence of which, 5′-GCA

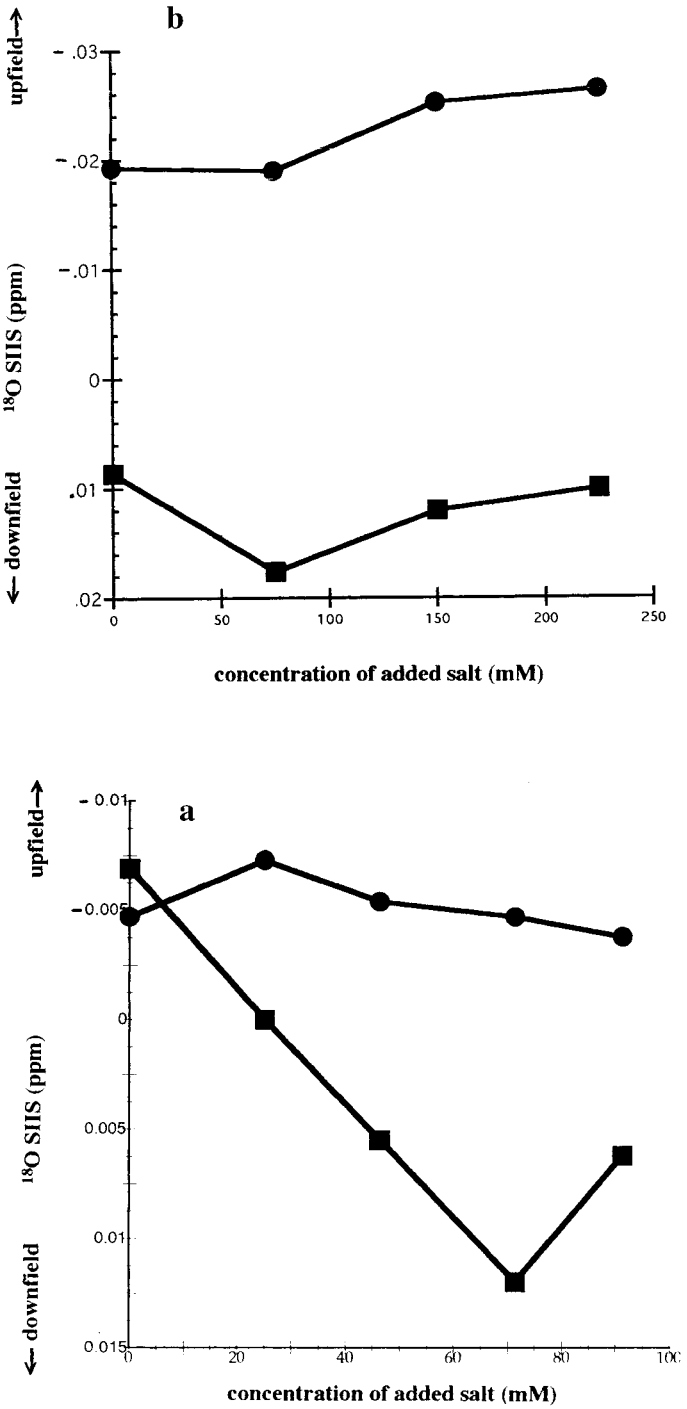

FIG. 2. The variation in the observed ^{18}O SIIS values at 25°C of ^{19}F signals of (■) 5-fluorouridine and (●) fluoride with the addition of monovalent salt, (a) this being NaCl (to 46 mM), and then NaF (to 91 mM). The sample contained 5-fluorouridine (1 mM), 5-fluorouracil (1 mM), and NaF (initially 5 mM), with ^{18}O enrichment of 53%. The ^{18}O SIIS value for 5-fluorouracil was observed to be zero throughout, for clarity this is omitted from the plot. (b) Addition of NaCl, to higher levels than applied in (a); sample contained 5-fluorouridine (5 mM), NaF (10 mM) and ^{18}O enrichment of 44%.

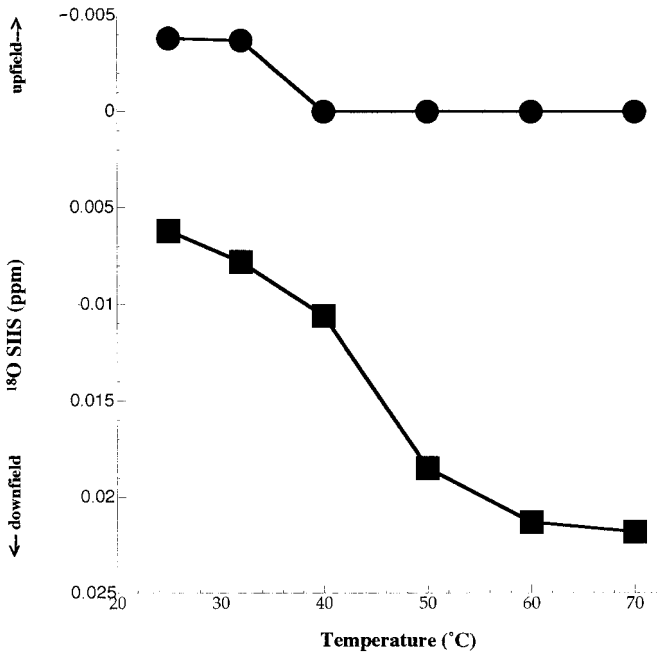

FIG. 3. Temperature dependences of the ^{18}O SIIS values observed for (●) fluoride and (■) 5-fluorouridine. The sample contained 5-fluorouridine (1 mM), 5-fluorouracil (1 mM), NaF (46 mM), and NaCl (45 mM), with ^{18}O enrichment of 53%. The ^{18}O SIIS of 5-fluorouracil was observed to be zero at each temperature; for clarity these points have not been included in the graph.

UCG GCU AAC UCU G-3′, has no distinct structure-forming properties (as we have previously demonstrated (*10*)), and so should adopt the RNA version of a random coil in solution. 5-Fluorouridine was incorporated at each of the four uridine positions in the sequence, and again to minimize intermolecular effects, ^{19}F studies were performed at a relatively low RNA concentration (0.1 mM). The ^{19}F spectrum of this RNA at 25°C shows four closely spaced signals (Fig. 4). That the fluorouridine sites do not interact with each other allowed us to assign the spectrum by comparison with a series of deletion versions of the sequence, the assignments are shown in Fig. 4. The spectrum evidently consists of two adjacent pairs of differing linewidths. This was borne out by relaxation time measurements; the ^{19}F T_1 and T_2 values are given in Table 2. In larger molecules at high field strengths, transverse ^{19}F relaxation is dominated by chemical shift anisotropy (*11*); with decreasing mobility, T_2 becomes shorter and the linewidth increases. In this regime, longitudinal relaxation becomes less efficient as mobility is reduced, and thus T_1 values become longer. From these considerations, it is clear (Table 2) that the fluorinated uridines at positions 4 and 9 are somewhat less mobile than those at positions 13 and 15, and this applies across the explored temperature range of 15–45°C, though the distinction is reduced at higher temperature. These relative mobilities may be quite local, referring only to the bases themselves. Alternatively, the segments of the molecule that includes these pairs of nucleotides may have differential mobility, which would mean that there is (on average) a distinct bend in the oligoribonucleotide somewhere around nucleotides 10–12. To distinguish between these possibilities will require relaxation or other dynamic data from other sites in the molecule. Nonetheless, the differing mobilities of bases 4 and 9 compared 13 and 15 constitute the distinctive features of the ^{19}F signals of our 16-mer RNA. As an "unstructured", single-stranded RNA, all the bases are expected to be significantly solvent exposed. At 25°C, the signals for fluorouridines 4 and 9 both display downfield isotope shifts, while the other two signals do not show an effect (Fig. 5a). Raising the temperature to 37°C and then 40°C quenched the isotope shifts as none were evident at these temperatures (data not shown), even though the ^{19}F signals are somewhat narrower than at 25°C. This behavior with increasing temperature contrasts with that of free 5-fluorouridine described above. Since we had previously observed that the addition of salt had a strong effect on the ^{18}O SIIS of 5-fluorouridine (*4*), 30 mM NaF was then added to the RNA sample, while the ^{18}O enrichment was decreased slightly to 75%. This addition of fluoride did not significantly alter the linewidths or chemical shifts of the 16-mer. Now, at 25°C all four ^{19}F signals display isotope shifts: fluorouridines 4 and 9 are downfield, while 13 and 15 are upfield (Fig. 5b). At 32°C the isotope shifts for nucleotides 4, 9, and 13 have altered slightly while that for position 15 has become undetectable (Fig. 5c). At 40°C fluorouridine 13 retains a decreased upfield isotope shift, while there is a suggestion of a small upfield shift for fluorouridine 9 due to a shoulder on that signal (Fig. 5d); alternatively (but less likely) this shoulder is due to a downfield isotopically shifted signal from position 4, while that for 9 has collapsed. The various ^{18}O SIIS values for the RNA are given in Table 3, along with H_2O/D_2O SIIS values at 25°C for comparison; again there is no simple relation between the two types of solvent isotope effect. The spectra of Fig. 5 show that despite ^{19}F signals which are relatively broad compared to those of smaller molecules, the ^{18}O SIIS can be detected in a fluorine-labeled RNA. Here the different fluorouridines in the molecule have similar degrees of solvent exposure, since the base sequence has no particular structure-forming tendencies. However, the dynamics of the fluorouridines are distinctive, at least in a pair-wise sense, and this seems related to the observed ^{18}O SIIS. The relatively less mobile bases 4 and 9 display downfield isotope shifts, which are slightly enhanced by the presence of sodium fluoride. The more mobile bases 13 and 15 show no isotope shifts in the absence the salt, but when it is added upfield isotope shifts become evident. It is thus evident that in RNA the ^{18}O SIIS cannot be used as a simple measure of solvent exposure; notably, a zero value does not necessarily mean that a base is well protected from the solvent. Furthermore, it can be anticipated that the conformation of a structured RNA will be both salt- and temperature-dependent, so variation of the solution conditions in these respects would

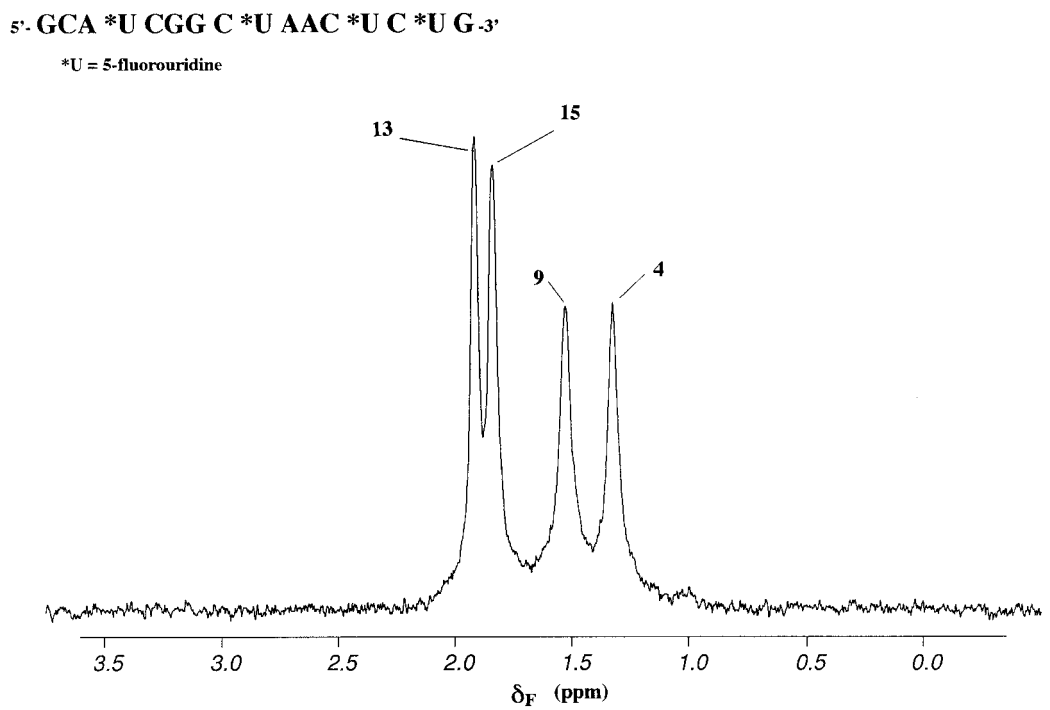

FIG. 4. Base sequence and ^{19}F spectrum of the fluorine-labeled 16-mer RNA at 25°C, with assignments. The spectrum was processed using modest line broadening (3 Hz) in order to preserve the different lineshapes present.

not be possible with the same freedom we have had here (since the addition of 30 mM NaF did not alter the relative linewidths of the ^{19}F signals of our 16-mer). This further complicates the possible use of the ^{18}O SIIS for macromolecular studies.

It is clear from this work (particularly as shown by Fig. 2) and our previous report that the observed ^{18}O SIIS for 5-fluorouridine is the sum of upfield and downfield contributions; the overall isotope shift may then be upfield, zero, or downfield depending on circumstances. The ^{18}O SIIS for fluoride, however, has not been observed to be downfield in any of our investigations. The origins of these effects presumably lie in the solvent contribution to the ^{19}F chemical shift; here we discuss various possible contributions to the observed isotope shift.

TABLE 2
16-mer RNA ^{19}F Relaxation Times

Fluorouridine position[a]	Temperature ^{19}F relaxation time[b]	15°C		25°C		45°C	
		T_1	T_2	T_1	T_2	T_1	T_2
4		900	5	890	14	720	20
9		940	5	880	14	730	22
13		830	12	780	20	690	22
15		790	6	720	17	670	22

[a] Position numbered from the 5′ end of the sequence.
[b] T_1 and T_2 values are given in milliseconds.

In general, a ^{19}F chemical shift may be represented as the shift change on transfer from a gas to dissolution in a given solvent, and it has previously been expressed by Lau and Gerig as the sum (*12*)

$$\delta_{obs} = \delta_{vdW} + \delta_E + \delta_H, \quad [1]$$

where δ_{vdW} arises from van der Waals interactions between the solvent and the fluorine compound, δ_E is from solvent-induced electric fields at the fluorine nucleus, and δ_H arises from specific solute–solvent interactions such as hydrogen bonds. (The contributions due to the bulk magnetic susceptibility and the magnetic anisotropy of the solvent are much less significant than these terms (*12*)). The observed ^{18}O SIIS ($\Delta\delta_{obs}$) may then result from changes (Δ) in one or more of the terms in Eq. [1], attributable to this isotopic substitution,

$$\Delta\delta_{obs} = \Delta\delta_{vdW} + \Delta\delta_E + \Delta\delta_H. \quad [2]$$

Since the observed ^{18}O SIIS shows clear signs of being due to competing upfield and downfield components, we use the premise that there are both upfield and downfield terms among the terms on the right-hand side of Eq. [2].

A movement of chemical shift downfield to what would otherwise be expected is indicative of van der Waals interactions (*13*). The gas-to-solvent chemical shifts for fluorobenzene have been calculated by Lau and Gerig for a range of

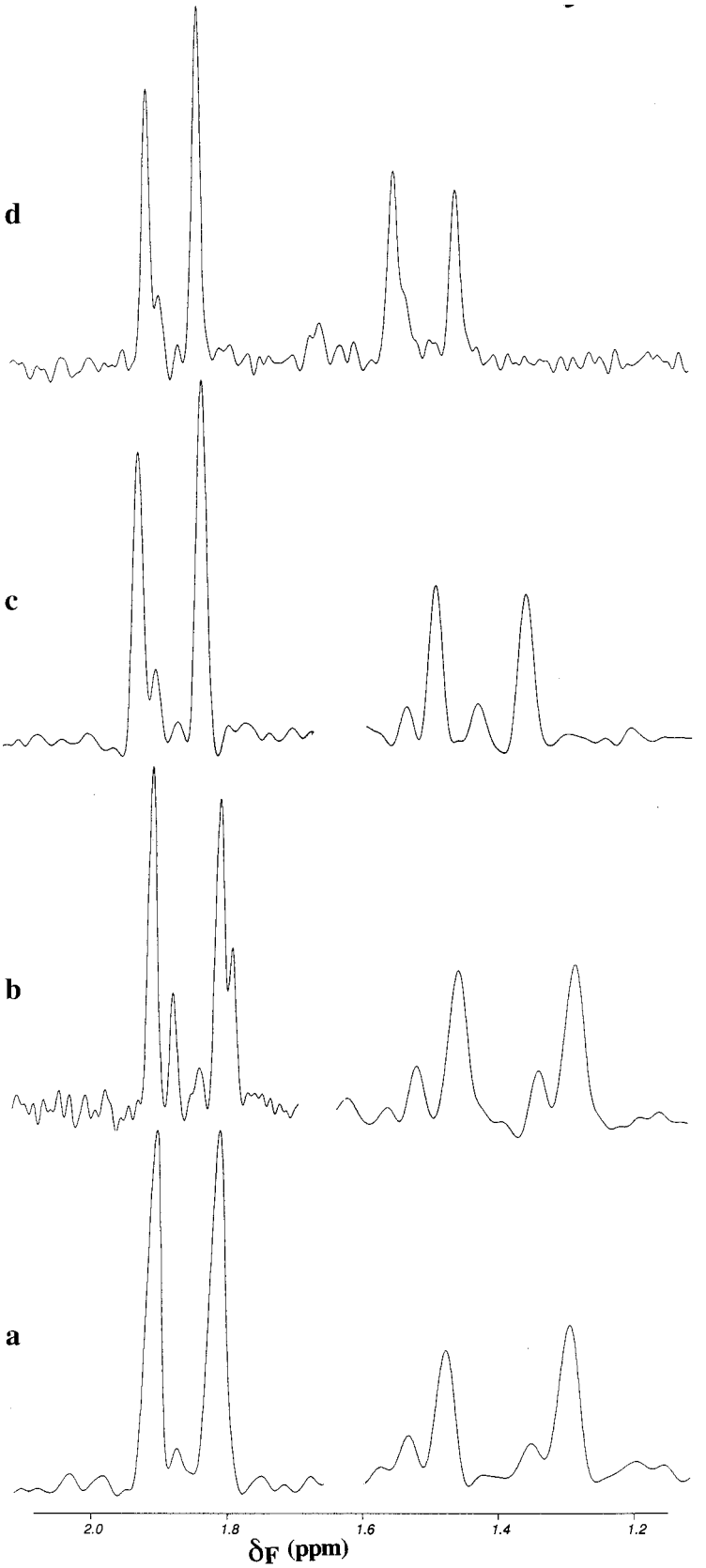
d
c
b
a
2.0
1.8
1.6
1.4
1.2
δF (ppm)

TABLE 3
Solvent-Induced Isotope Shifts of the ^{19}F Signals of the 16-mer RNA

Fluorouridine position	^{18}O SIIS					H_2O/D_2O SIIS[c]
	25°C[a]	40°C[a]	25°C[b]	32°C[b]	40°C[b]	
4	+0.057	0	+0.052	+0.070	0	−0.165
9	+0.054	0	+0.062	+0.041	−0.018	−0.165
13	0	0	−0.032	−0.027	−0.018	−0.140
15	0	0	−0.020	0	0	−0.160

[a] With ^{18}O enrichment of 86%.
[b] With ^{18}O enrichment of 75% and in the presence of 30 mM NaF.
[c] At 25°C, in absence of NaF; 100% D_2O compared to 90% H_2O 10% D_2O.

solvents as being the sum of the van der Waals and electric field shift contributions; with appropriate weighting, the shifts calculated in this way are in encouragingly close agreement with experimentally determined values (*12*). The van der Waals term, which is downfield, was calculated using

$$\delta_{vdW} = 3B_1 I_S I_F \alpha_S / \{2(I_S + I_F) r^6\}, \quad [3]$$

where I_S and I_P are the first ionization potentials of the solvent atom and fluorine atom respectively, α_S is the static polarizability of the solvent atom, r is the distance between the two atoms, and B_1 is a term which includes the polarizability of fluorine. For B_1 and I_F, values for neon were used, since this is isoelectric with covalent fluorine. The van der Waals shift for solvent water was calculated to be 7.94 ppm downfield, subsequently weighted by 1.07 in the expression for the overall shift (*12*). It is important here to note that Eq. [3] predicts that a slight increase in I_S will give rise to increased deshielding, i.e., the fluorine resonance being shifted downfield. So, for present purposes we assume that r and α_S are unchanged between $H_2{}^{16}O$ and $H_2{}^{18}O$, so that I_S is the most relevant term in Eq. [3]. Ionization energies of $H_2{}^{16}O$ and $H_2{}^{18}O$ have been compared previously using high-resolution valence electron spectra (*14*). These show that the ionization potentials of $H_2{}^{16}O$ and $H_2{}^{18}O$ are similar at lower vibronic levels (i.e., at the level of experimental uncertainty), but diverge toward higher levels. For the $2a_1$ orbital, the ionization potential of the $1v_2$ vibrational level is 0.006 eV greater in $H_2{}^{18}O$ than $H_2{}^{16}O$; applying this value as a change in I_S in Eq. [2] and the value of δ_{vdW} calculated by Lau and Gerig (*12*) suggests an ^{18}O-induced change in the van der Waals shift term of +0.0034 ppm (for 100 atom% ^{18}O). This is of course a simplistic treatment; perhaps the most serious limitations being that the ^{18}O isotope effects on the ionization potentials are inconveniently uncertain, and the vibronic level used here is not quite the lowest (it is about 1.2 eV above $1b_1$, in the gaseous state (*14*)). However, in principle an increase (even if relatively small) in ionization energy *is* to be expected on heavy isotope substitution, and so van der Waals effects can cause a *downfield* contribution to the overall isotope shift, if no other term in Eq. [3] is significantly altered by the isotopic substitution (which is a reasonable assumption). The value calculated above is indeed comparable to the range of ^{18}O SIIS values noted here and previously (*4*). A more definitive calculation is limited by the precision to which properties of water isotopomers have been measured.

The treatment of the electric field term of Eqs. [1] and [2] is more complicated, but previous chemical shift calculations have involved the dielectric constant and dipole moment of the solvent (*12*). Heavy atom substitution would be expected to cause a variation in the dipole moment of water due to anharmonicity in bending modes (*15*), but spectroscopic measurements of ^{18}O-enriched water species indicate that this is minimal if indeed distinguishable from experimental error (*16, 17*). Thus even if in principle the ^{19}F chemical shift is of high enough sensitivity to detect such an ^{18}O isotope-related perturbation of the solvent dipole moment, it is not certain if such a perturbation is actually present. However, with more confidence we can predict that the addition of salt will alter the electrostatics as sensed by the fluorine nucleus: certainly the dielectric constant will effectively increase. For this to effect the observed isotope shift there must be a distinction between $H_2{}^{16}O$ and $H_2{}^{18}O$; this would seem to be the case (Fig. 2). For 5-fluorouridine this presumably means with added salt the δ_E contribution to the overall isotope shift ($\Delta\delta_E$ in Eq. [2]) diminishes, so that the van der Waals term becomes relatively more important. This would explain the downfield behavior of the ^{18}O isotope shift observed at low salt levels. At higher salt levels other considerations must apply; from Fig. 2b it appears that under these conditions another weaker salt dependence holds for the ^{18}O SIIS value of 5-fluorouridine, and this is shared with the value for fluoride.

The third term of Eq. [1] involves specific interactions between solvent water and fluorine atom, of which hydrogen bonding is one example. Fluoride would be expected to be strongly hydrogen bonded, but the participation of aromatic fluorine in hydrogen bonding has recently been a matter of some controversy. There have been claims that difluorotoluene

FIG. 5. ^{19}F spectra showing the ^{18}O SIIS of the ^{19}F resonances of the fluorine-labeled 16-mer RNA under various solution conditions: at (a) 25°C, with 86 atom% ^{18}O, (b) 25°C in the presence of 30 mM NaF and 75 atom% ^{18}O, (c) as spectrum (b) but at 32°C, and (d) as spectrum (b) but at 40°C. Due to different intrinsic linewidths, three of the spectra are displayed in two segments as necessitated by differing optimum Gaussian parameters used in processing. As in Fig. 4, from right to left the signals are from nucleotides 13, 15, 9, and 4.

does not participate in hydrogen bonding to any significant extent (*18*), but also that in such circumstances the fluorine atom acts as a hydrogen bond acceptor about half as effectively as oxygen (*19*). A specific interaction between fluorine and solvent water will mean that the alteration of water internal vibrational modes by heavy isotope substitution will generally cause an upfield ^{19}F isotope shift; the librational modes are expected to be most influenced by ^{18}O enrichment (*20*), so this can be anticipated to provide an upfield contribution to the ^{18}O SIIS. While clearly there is an upfield component to the ^{18}O SIIS of 5-fluorouridine, on the basis of the data here we cannot necessarily attribute this entirely to the $\Delta\delta_H$ of Eq. [2], nor distinguish between hydrogen bonding and a less intimate interaction. Further work, including H_2O/D_2O investigations, should resolve this. Interactions between the fluoride ion and solvent water are likely to be dominated by hydrogen bonding; effective models have assumed that the ion is coordinated by six hydrogens disposed octahedrally (*21*). Therefore the ^{18}O SIIS of F^- would be expected to have a relatively strong upfield contribution due to water vibrational effects, and so the resulting large upfield $\Delta\delta_H$ term in Eq. [2] would then exceed in magnitude the downfield $\Delta\delta_{vdW}$ term. This would explain why we only see upfield values for the ^{18}O SIIS for fluoride.

The temperature dependence of the ^{18}O SIIS of 5-fluorouridine can be expected to contain a number of contributions. For example, there will be water vibrational effects, in both a direct and indirect sense. The population distribution among the various vibrational levels of water is temperature-dependent, and the ^{18}O frequency shift will vary between different vibrational states. Also, at higher vibronic levels the ionization energy differences between $H_2{}^{16}O$ and $H_2{}^{18}O$ become more marked (*14*), which may alter the van der Waals contribution to the isotope shift. The ionization energy of Eq. [3] is used an approximation to the average excitation energy (*22*), and so need not be confined to consideration of only the lowest vibronic level. Higher levels would be expected to start to make a contribution as they become populated. This may explain the overall trend (downfield with increasing temperature) shown in Fig. 3 for 5-fluorouridine, while other vibrational effects may be involved to produce the curvature of this plot.

The ^{18}O SIIS behavior of 5-fluorouridine is evidently modified when it is embedded in an oligoribonucleotide. The slower molecular tumbling requires that various quantities which contribute to the ^{18}O SIIS must be treated as tensor quantities, with the strong influence of chemical shift anisotropy taken into account; the significant assumption of isotropic values fails (*22*). Indeed, anisotropies of one or more of various terms on the right-hand side of Eq. [2] would seem to have become dominant. This accounts for the apparent link between the observed ^{18}O SIIS and the fluorine mobility, and further studies and analysis are necessary. However, it is obvious that the ^{18}O SIIS is too complicated to be used as a *simple* complement to the H_2O/D_2O SIIS in biomolecular studies.

Although 5-fluorouridine has proved to be useful in probing this isotope effect, it is still perplexing that the closely related 5-fluorouracil did not display a detectable ^{18}O SIIS under any of the sample conditions that we investigated (i.e., salt and temperature variation).

Here we have presented a range of experimental observations of the ^{18}O SIIS in ^{19}F NMR, together with a general framework within which the effect may be understood. Here the sensitivity of the ^{19}F nucleus means that we encounter the limits of the precision with which various properties of solvent isotopic water have been determined, which means an element of speculation is inevitably present. However, with further theoretical analysis and parameterization, this isotope effect has a number of potentially important applications, since the ^{18}O SIIS can probe the precise nature of solvent interactions with fluorine. This may be useful, for example, to explore and resolve the hydrogen bonding characteristics of aromatic fluorine. Also, the calculation of solvent-induced chemical shifts is of special interest as a means to interpret chemical shifts of fluorine-labeled proteins. From an analysis of 5-fluorotryptophan labelled *E. coli* galactose binding protein, it has been claimed that the fluorine shifts are dominated by electrical interactions, with van der Waals interactions not being significant (*23*). Subsequently, from the same protein, a strong correlation between calculated shifts due to van der Waals contacts (using an expression similar to Eq. [3] above) has also been demonstrated (*24*). It is thus of considerable interest to determine experimentally a fluorine chemical shift (or part thereof) which can be attributed to van der Waals contacts. The ^{18}O SIIS offers the prospect of this.

CONCLUSIONS

We have shown that an ^{18}O SIIS can be observed in the ^{19}F signals of a number of molecules. For small molecules this isotope shift is generally small (often less than 0.01 ppm) and can be either upfield or downfield depending on the specific sample conditions and the identity of the compound. The effect is likely to involve a balance between van der Waals interactions and other effects and clearly requires further theoretical analysis and parameterization. In a macromolecule, an ^{18}O SIIS value of zero may mean that a fluorine atom is considerably shielded from the solvent, but can also arise from a balance between upfield and downfield isotope effects to the chemical shift when fully solvent exposed. The 16-mer RNA suggests that at least with significant solvent exposure, the observed macromolecular ^{18}O SIIS is related to the local mobility of the individual fluorine sites. These various factors mean that there is no simple relation between an ^{18}O SIIS and its H_2O/D_2O counterpart. However, the more complicated nature of the ^{18}O SIIS means that it is highly sensitive to solution conditions and

is potentially a useful probe of both solvent exposure and the *nature* of interactions with solvent molecules, and of the various contributions to the ^{19}F chemical shift. While prospects for the effect being of use with fluorinated proteins do not seem strong, fluorinated nucleic acids hold much stronger promise.

EXPERIMENTAL

Fluorinated amino acids (as racemic DL mixtures) were obtained from Lancaster Synthesis. 5-fluorocytosine, 5-fluorouridine, 5-fluorouracil, 3-fluoro-3-deoxy-D-glucose, $H_2{}^{18}O$ (95 atom%), $H_2{}^{16}O$-(^{17}O,^{18}O-depleted), and D_2O were obtained from Aldrich. Fluorobenzene and 2-fluoropyridine were obtained from Fluka. The 16-mer RNA with 5-fluorouridine incorporated at 4 sites was prepared as described previously (*10, 24*).

The general procedure for NMR sample preparation with nonvolatile fluorine compounds was as follows: a solution containing the desired solutes at their appropriate concentrations and a trace of EDTA was divided into portions which were then lyophilized to dryness. The residues were redissolved in appropriate volumes of either $H_2{}^{18}O$ or $H_2{}^{16}O$, with 10% (by volume) of D_2O in each. Volatile compounds (fluorobenzene and 2-fluoropyridine) were dissolved in D_2O to appropriate levels before addition to the $H_2{}^{16}O/H_2{}^{18}O$ portions. In this way the concentrations of all solutes were identical in the ^{16}O and ^{18}O portions of the samples. The solutions were placed in an NMR tube containing a concentric insert (Wilmad, Buena, NJ); usually around 60–80 μl of ^{18}O-enriched solution was placed in the central chamber, with 340–400 μl of ^{16}O-solution in the outer part. Reduction of the level of ^{18}O enrichment was achieved by dilution with the ^{16}O-portion. Addition of NaF or NaCl was achieved by evaporating aliquots of NaF or NaCl solutions to dryness and then dissolving the residues in the ^{16}O and ^{18}O solutions as appropriate. The supplied ^{18}O-enriched water was 95 atom%, so with the addition of 10% D_2O for locking purposes the maximum enrichment level in an NMR sample was 86%. The buffer used throughout was 20 mM sodium phosphate pH 6.8. For the H_2O/D_2O SIIS measurements of the fluorine-labeled RNA, ^{19}F spectrum recorded in D_2O was compared to that recorded in 90% H_2O 10% D_2O.

^{19}F spectra were acquired on a GE-Omega 500 spectrometer operating at 470.5 MHz using a 5-mm $^{19}F/^1H$ probe. For all ^{19}F spectra, broadband ^1H decoupling was applied by Waltz-16 modulation of the decoupler. FIDs were usually multiplied by Gaussian functions and zero-filled prior to Fourier transformation. Digital resolution of spectra were *at least* 0.1 Hz (0.0002 ppm) per point. All quoted ^{18}O SIIS values are averages of at least two determinations, which for small molecules were generally reproducible to within $\pm$0.0004 ppm, and $\pm$0.0002 ppm for the smaller isotope shifts of 0.002 ppm or less. ^{19}F T_1 measurements were made using an inversion-recovery pulse sequence and nonselective pulses. T_2 determinations were made from the half-height linewidth (ν) using the relation $\nu = 1/(\pi T_2)$. ^{19}F chemical shifts are given relative to 5-fluorouridine in D_2O buffer at 25°C. For solvent isotope shifts, a negative value indicates an upfield shift in the heavier isotope solvent; downfield is denoted by a + symbol.

ACKNOWLEDGMENTS

This work was supported by the Wellcome Trust; JRPA is a Wellcome Senior Fellow. We thank A. K. Collier for assistance with RNA preparation.

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