# Fluorine-19 Nuclear Magnetic Resonance Studies of the Structure of 5-Fluorouracil-Substituted *Escherichia coli* Transfer RNA†

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ABSTRACT: $^{19}F$ nuclear magnetic resonance has been used to study fully active *Escherichia coli* $tRNA_1^{Val}$ in which 5-fluorouracil has replaced more than 90% of all uracil and uracil-derived modified bases. The $^{19}F$ spectrum of the native tRNA contains resolved resonances for all 14 incorporated 5-fluorouracils. These are spread over a 6 ppm range, from 1.8 to 7.7 ppm downfield of the standard free 5-fluorouracil. The $^{19}F$ resonances serve as sensitive monitors of tRNA conformation. Removal of magnesium or addition of NaCl produces major, reversible changes in the $^{19}F$ spectrum. Most affected is the lowest field resonance (peak A) in the spectrum of the native tRNA. This shifts 2–3 ppm upfield as the $Mg^{2+}$ concentration is lowered or the NaCl concentration is raised. Thermal denaturation of the tRNA results in a collapse of the spectrum to a single broad peak centered at 4.7 ppm. Study of the pH dependence of the $^{19}F$ spectrum shows that five incorporated fluorouracils with $^{19}F$ signals in the central, 4–5.5 ppm, region of the spectrum, peaks C, D, E, F, and H, are accessible to titration in the pH 4.5–9 range. All have $pK_a$'s close to that of free 5-fluorouridine (ca. 7.5). Evidence for a conformation change in the tRNA at mildly acidic pHs, ca. 5.5, is also presented. Four of the titratable 5-fluorouracil residues, those corresponding to peaks D, E/F, and H in the $^{19}F$ spectrum of fluorine-labeled $tRNA_1^{Val}$, are essentially completely exposed to solvent as determined by the solvent isotope shift (SIS) on transfer of the tRNA from $H_2O$ to $^2H_2O$. These are also the 5-fluorouracils that readily form adducts with bisulfite, a reagent that reacts preferentially with pyrimidines in single-stranded regions. On the basis of these results, resonances D, E, F, and H in the middle of the $^{19}F$ spectrum are attributed to 5-fluorouracils in non-base-paired (loop) regions of the tRNA. Evidence from the ionic strength dependence of the $^{19}F$ spectrum and arguments based on other recent studies with fluorinated tRNAs support earlier suggestions [Horowitz, J., Ofengand, J., Daniel, W. E., & Cohn, M. (1977) *J. Biol. Chem. 252*, 4418–4420] that the resonances at lowest field correspond to tertiary hydrogen-bonded 5-fluorouracils. Consideration of ring-current effects and the preferential perturbation of upfield $^{19}F$ resonances by the cyclophotoaddition of 4′-(hydroxymethyl)-4,5′,8-trimethylpsoralen, which is known to react most readily with pyrimidines in double-stranded regions, permits initial assignment of upfield resonances to 5-fluorouracils in helical stems. The results demonstrate the promise of $^{19}F$ nuclear magnetic resonance as a probe for the structure of tRNA in solution.

Nuclear magnetic resonance studies have provided a wealth of information on the structure, conformation, and dynamic properties of tRNA in solution (Reid, 1981; Reid & Hare, 1982). A majority of these investigations involve proton NMR spectroscopy and have been limited to observation of either the hydrogen-bonded imino protons of secondary and tertiary base pairs or to the methyl and methylene protons of modified bases. The elegant nuclear Overhauser experiments introduced by Redfield and now widely used (Reid, 1981; Roy & Redfield, 1983) have permitted major advances in assignment of the imino proton resonances in the low-field region of the $^1H$ NMR spectrum of tRNA, and two-dimensional NOE studies have resulted in the complete assignment of these resonances in the spectrum of yeast $tRNA^{Phe}$ (Hilbers et al., 1983; Heerschap et al., 1985) and *Escherichia coli* $tRNA_1^{Val}$ (Hare et al., 1985). Important information on the environment of methylated bases in tRNA has been gained from the high-field region of the $^1H$ NMR spectrum (Kastrup & Schmidt, 1975, 1978). In addition to $^1H$ NMR, $^{31}P$ and $^{13}C$ NMR have been used as probes of tRNA structure. Although $^{31}P$ NMR permits monitoring changes in the phosphodiester backbone of tRNA (Gorenstein, 1984; Gorenstein & Goldfield, 1982) and $^{13}C$ NMR has proved quite useful for investigating the environments of both the methylated bases (Kopper et al., 1983) and the major constituents of tRNA (Olsen et al., 1982), these methods suffer from a lack of sensitiviy and the $^{13}C$ experiments require the use of isotope-enrichment procedures.

It has been found that $^{19}F$ NMR of fluorine-substituted proteins and nucleic acids provides another powerful probe of macromolecular structure and dynamics in solution. The high sensitivity of fluorine-19 to detection by NMR, 83.3% that of $^1H$, its high (100%) natural abundance, the large range of its chemical shifts, and the extreme sensitivity of $^{19}F$ chemical shifts to the environment of the nucleus, are among the advantages of using a $^{19}F$ probe (Sykes & Hull, 1978; Gerig, 1978; Sykes & Weiner, 1980). Furthermore, because fluorine does not normally occur in proteins, $^{19}F$ NMR spectroscopy of fluorinated RNAs is ideally suited to investigations of

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RNA–protein interactions without interference from the protein.

Cells of *E. coli* readily incorporate 5-fluorouracil (FUra[1]) into their tRNAs to levels in excess of 90 mol % (Lowrie & Bergquist, 1968; Johnson et al., 1969; Kaiser, 1972). With few exceptions (Ramberg et al., 1978), these substituted tRNAs retain their functional capabilities (Horowitz et al., 1974; Ofengand et al., 1974; Chinalli et al., 1978; Hills et al., 1983) and thus represent ideal systems for investigation by $^{19}F$ NMR spectroscopy. Preliminary studies have demonstrated the feasibility of $^{19}F$ NMR as a probe of tRNA structure. The spectrum of FUra-substituted *E. coli* $tRNA_1^{Val}$ showed 11 well-resolved resonances for the 14 FUra residues in the molecule (Horowitz et al., 1977); the tRNA used, however, was known to be partially degraded. A more recent investigation of (FUra)$tRNA_f^{Met}$ resolved 9–10 of the 12 incorporated FUra residues (Hills et al., 1983). In the present report, we extend these earlier studies by examining in detail the $^{19}F$ NMR spectrum of active, intact *E. coli* (FUra) $tRNA_1^{Val}$ under various conditions of pH, solvent, ionic strength, and divalent cation concentration and correlate the $^{19}F$ resonances with sites of FUra incorporation. Further, we investigate the possible use of the bisulfite reaction (Schulman & Pelka, 1977; Sander & Deyrup, 1972) and the reaction with psoralens (Ou & Song, 1978; Bachellerie & Hearst, 1982) as bases for developing assignment procedures.

## Experimental Procedures

### *Materials*

Sephacryl S-200, DEAE-Sephadex A-50, and Sepharose 4B were obtained from Pharmacia. BD-cellulose was supplied by Bio-Rad Laboratories and DEAE-cellulose (DE-32) was a Whatman product. Polynucleotide phosphorylase (*Micrococcus luteus*) was from Boehringer-Mannheim. Radioactive amino acids were purchased bought from New England Nuclear or ICN. 4′-(Hydroxymethyl)-4,5′,8-trimethylpsoralen (HMT) was obtained from Calbiochem. 5-Fluorouracil was donated by Hoffmann-La Roche. 5-Fluorouridine 5′-diphosphate was purchased from Sierra Bioresearch and used in the preparation of poly(FU) with *M. luteus* polynucleotide phosphorylase as described by Szer and Shugar (1963). The polynucleotide was freed of protein by extraction with chloroform–isoamyl alcohol (Scheit & Gaertner, 1969) and purified by chromatography on Sephadex A-25 with a gradient of triethylammonium bicarbonate, pH 8, from 50 to 500 mM. Deuterium oxide (99.8%, Gold Label) and sodium cacodylate were from Aldrich. All other reagents were of analytical grade or higher.

### *Methods*

*Preparation of 5-Fluorouracil-Substituted E. coli tRNA.* *E. coli* $tRNA_1^{Val}$ was purified from *E. coli* B cells grown in 5-fluorouracil (Horowitz et al., 1974) by minor modifications of the procedures described previously for the purification of FUra-substituted $tRNA_f^{Met}$ (Hills et al., 1983). In outline, the method involves an initial chromatography step on DEAE-cellulose at pH 8.9, to separate normal and FUra-substituted tRNAs. Subsequent purification is achieved by chromatography on BD-cellulose, followed by Sepharose 4B chromatography using a descending salt gradient from 1.5 to 0 M $(NH_4)_2SO_4$. Valine acceptance was measured according to Horowitz et al. (1974) and found to be 1400–1800 pmol [$^{14}C$]-L-valine per $A_{260}$ unit. Nucleoside analysis by the $^3H$ derivative method of Randerath et al. (1974), showed that more than 90% of the uridine and uridine-derived modified nucleosides in the tRNA were replaced by 5-fluorouridine. Preparations used in these experiments consisted predominantly of one of the two isoaccepting forms of the tRNA, the B form, which contains FUra at position 17.[2] The other isoacceptor (form A) has a ring-opened fluorodihydrouridine at this site (Horowitz et al., 1983).

*Bisulfite Modification of tRNA.* Transfer RNA was reacted with 2 M sodium bisulfite at pH 7.0 essentially as described by Schulman and Pelka (1977). Solid sodium sulfite (16.9 mmol) and sodium metabisulfite (1.6 mmol) were dissolved in 10 mL of 10 mM $MgCl_2$ to yield a solution of 2 M sodium bisulfite, pH 7.0. The tRNA sample, after ethanol precipitation, was dissolved in the bisulfite solution to a concentration of 30 $A_{260}$/mL and incubated at 22 °C. After 4 h, the sample was diluted to 10 $A_{260}$/mL with water and dialyzed twice against 500 volumes of 20 mM sodium acetate, pH 4.5, containing 0.15 M NaCl. Reversal of the bisulfite addition reaction was accomplished by incubating the modified tRNA in 100 mM Tris–HCl, pH 9, at 37 °C for 8 h.

*Photoreaction with 4′-(Hydroxymethyl)-4,5′,8-trimethylpsoralen (HMT).* Samples (30 mL) containing 200 μg/mL (FUra)$tRNA_1^{Val}$ and 20 μg/mL HMT in 10 mM Tris-HCl, pH 7.4, and 0.1 mM EDTA were put into Pyrex tubes. After deoxygenation by flushing with nitrogen for 1 h, the samples were irradiated for 5 min at 5 °C with RUL 3500-Å lamps in a Rayonet photoreactor. The reaction mixtures were extracted twise with two volumes of chloroform–isoamyl alcohol (24:1 v/v) and three times with ether, and the tRNA was then precipitated 3 times with ethanol to remove residual unreacted HMT.

*$^{19}F$ NMR Spectroscopy.* Transfer RNA samples (2.5–5.5 mg) were dissolved in the buffer used in each experiment and dialyzed against the same buffer in a flow-dialysis microcell (BRL Model 1200 MA) Ten percent $^2H_2O$ was then added to provide an internal deuterium lock. The sample (0.3 mL) was transferred to a Wilmad 529A-10 spherical NMR microcell, which was suspended in a 10-mm NMR sample tube and surrounded with sample buffer (pH 6.0) containing 1 mM FUra as an external standard. Most spectra were obtained on samples in 15 mM $Mg^{2+}$. To remove $Mg^{2+}$ for studies at low divalent cation concentrations, tRNA samples were dissolved in 10 mM EDTA, pH 7.0, and the solution was heated to 70 °C for 5 min. After the solution was cooled to room temperature, the tRNA was precipitated with Chelex 100 treated ethanol, dissolved in 50 mM sodium cacodylate, pH 6.0, containing 100 mM NaCl, and dialyzed against the same buffer (Chelex 100 treated). This procedure yielded tRNA containing less than 0.1 mol of magnesium/mol of tRNA as determined by atomic absorption measurements on a Perkin-Elmer 2000 AA spectrophotometer (kindly provided by Dr. E. Travis Littledike, National Animal Disease Center, Ames, IA). $Mg^{2+}$ or NaCl concns. were increased by the addition of appropriate volumes of 150 mM $MgCl_2$ or 3 M NaCl, respectively. When required, tRNA was transferred to $^2H_2O$ by lyophilyzing the sample twice and redissolving it in 99.996% $^2H_2O$ (Stohler Isotope Chemicals) under a nitrogen atmosphere.

[1] Abbreviations: FUra, 5-fluorouracil; (FUra)$tRNA_1^{Val}$, 5-fluorouracil-substituted *E. coli* $tRNA_1^{Val}$; DEAE, diethylaminoethyl; BD-cellulose, benzoylated DEAE-cellulose; EDTA, ethylenediaminetetraacetic acid; HMT, 4′-(hydroxymethyl)-4,5′,8-trimethylpsoralen; SIS, solvent isotope shift; Tris, 2-amino-2-(hydroxymethyl)-1,3-propanediol; ppm, parts per million; D, dihydrouridine; T, ribosylthymine; Ψ, pseudouridine; $s^4U$, 4-thiouridine.

[2] The numbering system used is that of Sprinzl et al. (1985).

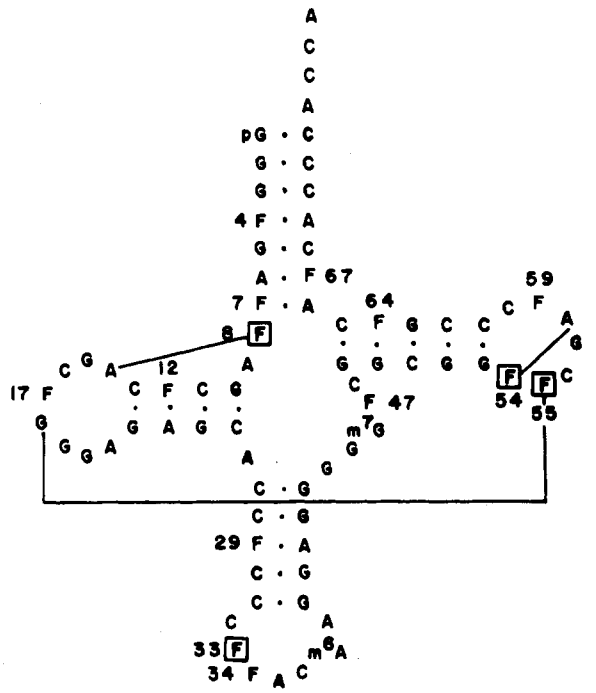

FIGURE 1: Cloverleaf structure of *E. coli* $tRNA_1^{Val}$ (Yaniv & Barrell, 1969) with uracil and uracil-derived bases replaced by 5-fluorouracil (F). Tertiary base pairs involving 5-fluorouracil are shown as solid connecting lines.

$^{19}$F NMR spectra were obtained on a Bruker WM300 pulsed FT NMR spectrometer operating at 282 MHz. Spectra were collected by using 8K data points, no relaxation delay, and a pulse width sufficient to optimize the Ernst condition (Shaw, 1976), except when making area measurements, in which case a 5-s relaxation delay and 90° pulse angle were employed to ensure complete relaxation. Unless otherwise indicated, the temperature was 20–24 °C and was controlled to ±1 °C by the Bruker temperature control unit with nitrogen gas as coolant. $^{19}$F chemical shifts are reported relative to free FUra; downfield shifts are defined as positive. After each set of experiments, the activity of the tRNA sample was checked by aminoacylation assay, and possible degradation was monitored by polyacrylamide gel electrophoresis (Hills et al., 1983).

## RESULTS

*$^{19}$F NMR Spectra.* The fluorouracil-substituted valine tRNA used in these studies is intact, as judged by polyacrylamide electrophoresis in denaturing (7 M urea) gels, and is fully active in protein synthesis in vitro (results not shown). It is nearly completely substituted, having more than 90% of all its uracil and uracil-derived modified bases replaced by FUra. There is a total of 14 FUra residues in the tRNA, and these are distributed throughout the molecule in both loop and stem regions of the cloverleaf structure (Figure 1). Each FUra residue represents a site-specific $^{19}$F NMR probe, which can serve as a reporter of structural changes in its vicinity.

A typical $^{19}$F NMR spectrum of fluorinated $tRNA_1^{Val}$, recorded in high $Mg^{2+}$ (15 mM)–NaCl (100 mM) buffer, pH 6.0, at 23 °C (standard conditions), is shown in Figure 2a. Twelve $^{19}$F resonances are resolved under these conditions; these span a 6-ppm range, from 1.8 to 7.7 ppm downfield of the standard, free FUra (0 ppm). Relative areas of each peak obtained by integration on the spectrometer are shown beneath the spectrum. The total area, normalized to that of peak B, corresponds closely to the expected number of 14 FUra residues. A fractional peak is often seen at 4.85 ppm. This is just visible in Figure 2a, but is more pronounced in other spectra; its significance is not clear at present. When the

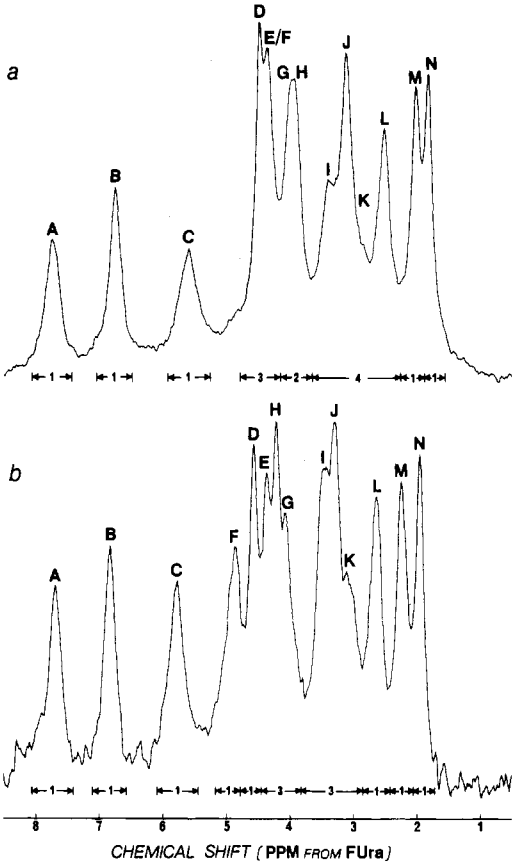


FIGURE 2: $^{19}$F NMR spectra of 5-fluorouracil-substituted $tRNA_1^{Val}$. The spectra were recorded at 282 MHz in the Fourier transform mode as described under Methods, with tRNA in 50 mM sodium cacodylate buffer, pH 6.0, containing 100 mM NaCl, 15 mM $MgCl_2$, 1 mM EDTA, and 10% $^2H_2O$ as an internal lock (standard buffer): (a) temperature 23 °C, 0.61 mM (FUra)$tRNA_1^{Val}$; (b) temperature 47 °C, 0.38 mM (FUra)$tRNA_1^{Val}$. Chemical shifts are given in ppm from 5-fluorouracil. Areas were obtained by integration on the spectrometer and are normalized relative to the area of peak B.

temperature is increased to 50 °C, most of the resonances in the $^{19}$F spectrum shift very little. However, downfield shifts of peaks F and H are observed, whereas peak K shifts slightly upfield. As a result, the spectrum recorded at 47 °C shows all 14 $^{19}$F peaks clearly resolved (Figure 2b). The spectrum of intact (FUra)$tRNA_1^{Val}$ closely resembles that of the partially degraded sample reported earlier (Horowitz et al., 1977), differing only in the central region of the spectrum (4–5 ppm).

The multiplicity of resonances in the $^{19}$F NMR spectrum of (FUra) $tRNA_1^{Val}$ and the large range of chemical shifts observed are due to the secondary and tertiary structure of the native molecule. When the higher order structure is disrupted by heating to 80 °C, all 14 fluorouracils have the same chemical environment, and the $^{19}$F NMR spectrum collapses to a single broad resonance (135 Hz at half-height) centered 4.7 ppm downfield from free FUra (results not shown), at a position corresponding to the central region of the native tRNA spectrum. Furthermore, when FUra is incorporated into poly(FU), which exists as a random coil under our experimental conditions (Szer & Shugar, 1963; Massoulie et al., 1963), the $^{19}$F spectrum contains a single peak (20 Hz at half-height) at 4.5 ppm, within 0.2 ppm of the position of the $^{19}$F signal in the spectrum of denatured tRNA.

*pH Effects on the $^{19}$F NMR Spectrum of FUra-Substituted $tRNA_1^{Val}$.* $^{19}$F NMR spectra were routinely recorded at pH 6.0 to minimize effects due to dissociation of the N(3)-H of incorporated 5-fluorouracil. 5-Fluorouridine has a $pK_a$ of 7.57 (Wempen et al., 1961), and the $pK_a$ of FUra in poly(FU) is

8.1–8.3 (Szer & Shugar, 1963; Massoulie et al., 1963). Major changes in $^{19}F$ chemical shifts occur at pHs near the p$K_a$. The $^{19}F$ resonance of 5-fluorouridine shifts more than 1.6 ppm downfield as a result of dissociation of the N(3) proton (Figure 3). Alderfer et al. (1983) have reported that ionization of FUra in poly(FU) produces a 2.7-ppm downfield shift of the $^{19}F$ signal.

We have examined the $^{19}F$ spectrum of (FUra)tRNA$_1^{Val}$ as a function of pH in the range 4.5–9 (Figure 3). As might be expected for FUra residues located in different environments within the tRNA, changes in pH produce differential effects on the chemical shifts of $^{19}F$ peaks in the spectrum. Five resonances in the central region of the $^{19}F$ spectrum, peaks C, D, E, F, and H, shift downfield 1.0–2.5 ppm as the pH is raised from 6.0 to 8.75 (Figure 3). This behavior is similar to that of free 5-fluorouridine (Figure 3) and indicates that the p$K_a$'s of these FUra residues are similar to that of FUrd (ca. 7.6). The chemical shift of one resonance in this part of the spectrum, either G or H, is independent of pH in the range studied (Figure 3). Because of peak overlap, it is difficult to differentiate G and H. We have, however, tentatively identified the unshifted peak as G because this peak exhibits anomalous behavior in several respects. It is the only peak in the central cluster of peaks that does not shift upfield when the tRNA is transferred from $H_2O$ to $^2H_2O$ (see Table I); it is also the one peak in this region of the spectrum that does not shift upfield as a result of reaction with sodium bisulfite (Figure 7). Most of the other $^{19}F$ resonances, including those in the high-field region of the spectrum, remain unperturbed with rising pH and presumably have p$K_a$'s greater than 9.

Two interesting chemical shift changes occur in the acid pH range. Peak C shifts upfield at pHs below 5.5 (Figure 3), and peak E or F (it is difficult to distinguish the two because they merge at pH 5.5–6) exhibits a marked downfield shift. From the pH range of these changes and the behavior of the chemical shifts, it is probable they are not due to dissociation of the N(3)–H of incorporated FUra, but may be the result of conformation changes in the tRNA. Such changes under mildly acid conditions have been reported for several tRNA species (Bina-Stein & Crothers, 1974, 1975), including *E. coli* tRNA$_1^{Val}$ (Steinmetz-Kayne et al., 1977).

*Magnesium Dependence of the (FUra)tRNA$_1^{Val}$ $^{19}F$ Spectrum.* Magnesium is known to stabilize the functional, native conformation of tRNA by binding at the loops and sharp turns of the tertiary structure (Kim, 1979). Changes in tRNA conformation that accompany variations in divalent cation concentration are readily monitored by $^{19}F$ NMR spectroscopy. This is seen in Figure 4, which shows the effects of magnesium on the $^{19}F$ NMR spectrum of (FUra) tRNA$_1^{Val}$. The spectrum in the absence of added $Mg^{2+}$ ($\leq$0.1 equiv of magnesium/tRNA) differs radically from that at 15 mM $Mg^{2+}$ (compare Figures 2a and 4a). By following the chemical shift changes as magnesium is added and assuming that the shift trends are continuous, it is possible to relate $^{19}F$ resonances at low $Mg^{2+}$ to those at high $Mg^{2+}$; these are labeled in Figure 4. Most of the $^{19}F$ resonances shift slightly downfield as magnesium is added. However, peaks A, J, and K undergo large downfield shifts. Peak K, which is visible only as a shoulder at 15 mM $Mg^{2+}$ (Figure 2a), is well resolved as the farthest upfield resonance in the absence of magnesium. Smaller downfield shifts occur in the central region of the spectrum as magnesium concentration increases. Peaks G and H start to shift at the lowest levels of added magnesium, whereas peak F shifts only after the $Mg^{2+}$/tRNA ratio reaches 30 (Figure 4). These shift changes are sometimes difficult to follow because of the clustering of $^{19}F$ resonances in the middle of the spectrum. However, peaks F and H can be identified because they shift as the result of trinucleotide and tetranucleotide codon binding

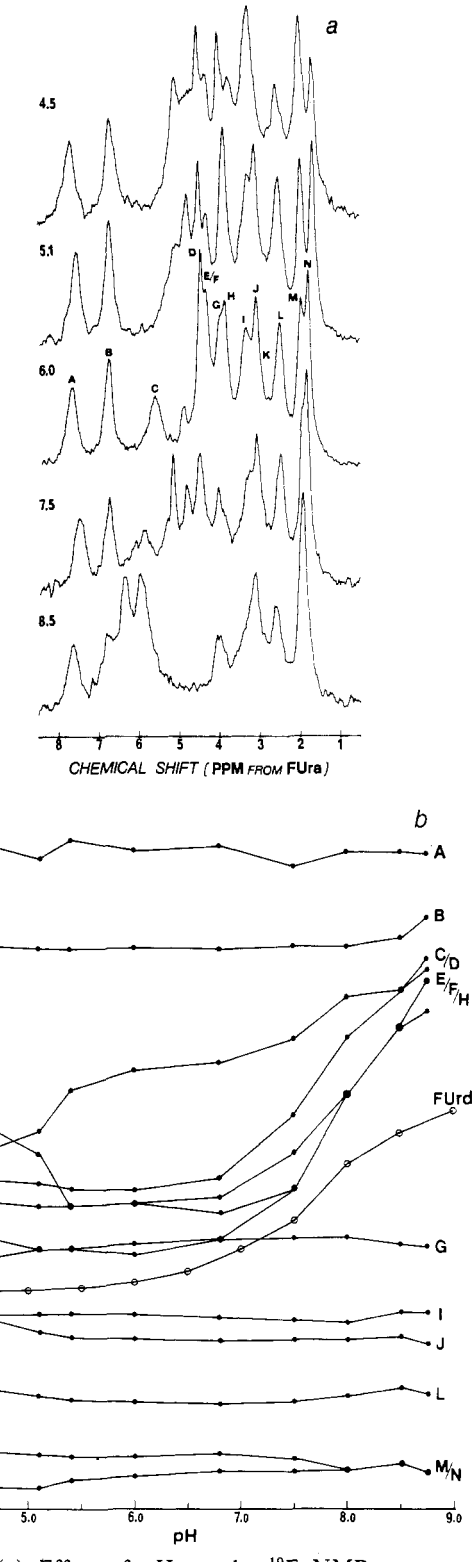


FIGURE 3: (a) Effect of pH on the $^{19}F$ NMR spectrum of (FUra)tRNA$_1^{Val}$. The tRNA (0.22–0.40 mM) was dialyzed against buffers of varying pH values. From top to bottom, spectra were taken at pH 4.5, 5.1, 6.0, 7.5, and 8.5. The buffers used were 50 mM sodium cacodylate containing 100 mM NaCl and 15 mM $MgCl_2$ (pH 4.5–7.5) and 5 mM Tris-HCl containing 150 mM NaCl and 15 mM $MgCl_2$ (pH 8.0–8.75). Both buffers also contained 1 mM EDTA and 10% $^2H_2O$ as an internal lock. (b) pH dependence of the chemical shifts of $^{19}F$ resonances identified in part a. For reference the chemical shift of free 5-fluorouridine (10 mM) at each pH value is also shown (open circles).

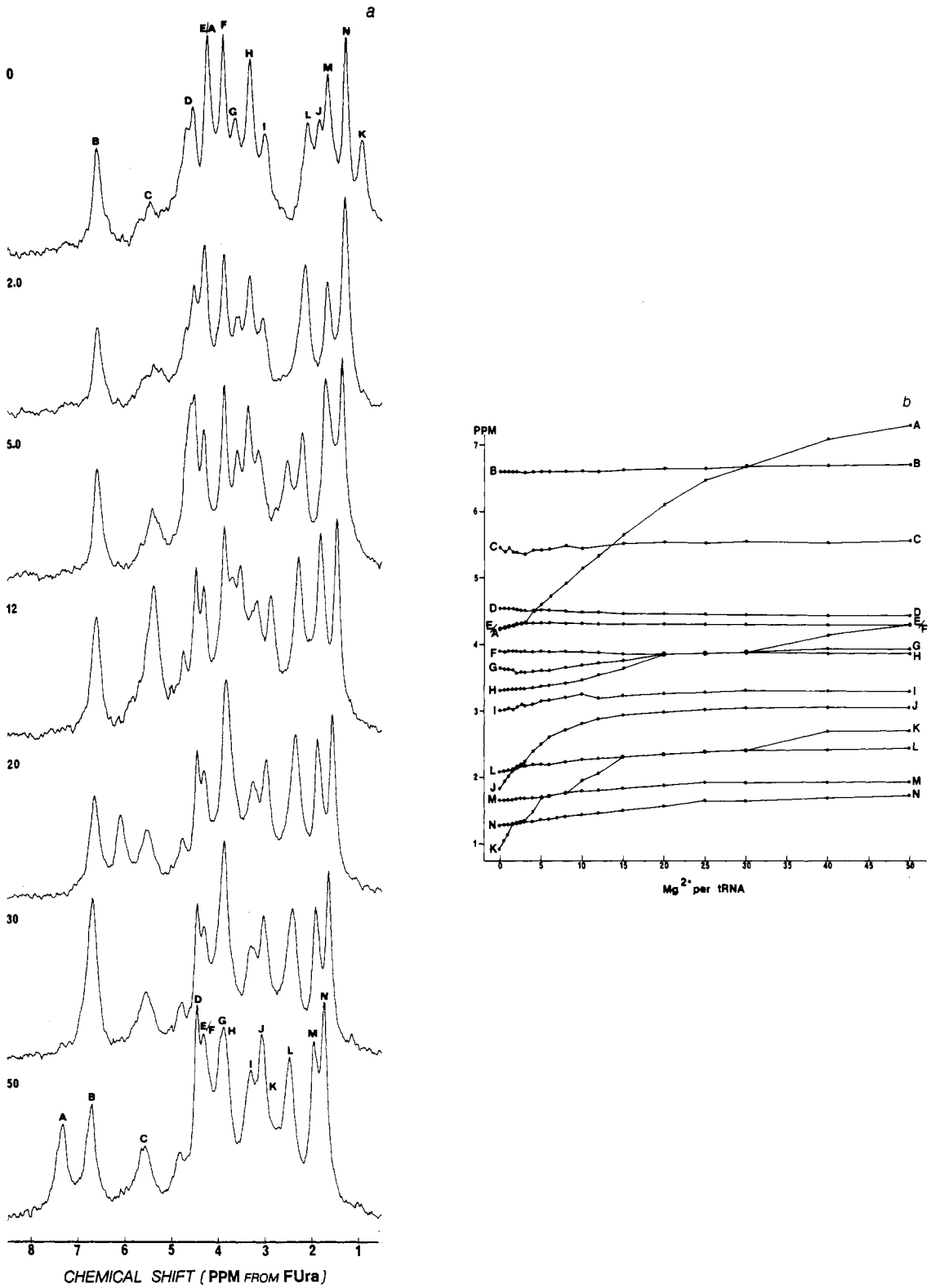


FIGURE 4: (a) Effects of magnesium on the $^{19}$F NMR spectrum of (FUra)tRNA$_1^{Val}$. Magnesium was removed from the tRNA as described under Methods; the tRNA sample (0.44 mM) was dissolved in standard buffer lacking $Mg^{2+}$, and varying amounts of $MgCl_2$ were then added. From top to bottom spectra were taken at molar ratios of $Mg^{2+}$/tRNA of 0, 2, 5, 12, 20, 30, and 50. (b) Magnesium ion dependence of the chemical shifts of $^{19}$F resonances identified in part a.

(Gollnick et al., 1984, 1986). The effects of magnesium depletion are fully reversible. Addition of $Mg^{2+}$ to levels above 50/1 $Mg^{2+}$/tRNA restores the spectrum to that observed at high (15 mM) magnesium (compare Figures 2a and 4a).

*Effects of Sodium Ion on the $^{19}$F Spectrum of (FUra)-tRNA$_1^{Val}$.* Monovalent cations also cause significant chemical shift changes as shown in Figure 5, which depicts the $^{19}$F NMR spectrum of (FUra)tRNA$_1^{Val}$ as a function of NaCl concentration at 15 mM $Mg^{2+}$. As the ionic strength increases, the most pronounced change is the 2–3 ppm upfield shift of the farthest downfield $^{19}$F resonance (peak A). This is the same resonance that exhibits a large downfield shift with increasing magnesium concentration (Figure 4). These inverse shifts suggest that, at high $Na^+$ concentrations, an ion competition exists for sites occupied by weakly bound divalent cations. One of the $^{19}$F signals in the composite peak D/E/F also shifts

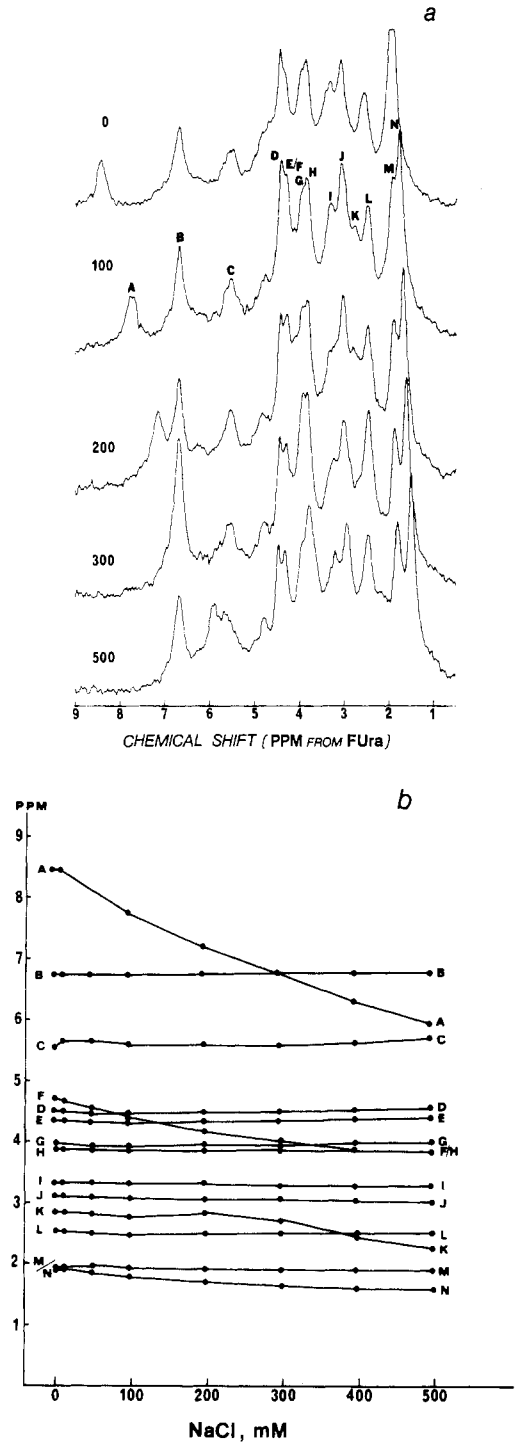


FIGURE 5: (a) Effects of NaCl on the $^{19}F$ NMR spectrum of (FUra)$tRNA_1^{Val}$ at high $Mg^{2+}$ concentration. The tRNA (0.44 mM) was dissolved in standard buffer (15 mM $Mg^{2+}$) containing varying concentrations of NaCl. From top to bottom, spectra were taken at 0, 100, 200, 300, and 500 mM NaCl. (b) Sodium chloride concentration dependence of the chemical shifts of $^{19}F$ resonances identified in part a.

upfield as NaCl concentration increases. Although it is not possible to clearly define which of the three peaks shifts, we presume it is peak F, on the basis of the downfield shift of this peak with increasing magnesium (see Figure 4). Other notable effects of increasing NaCl concentration include a small (0.3 ppm) upfield shift of peak N that resolves the two highest field $^{19}F$ resonances; peak K also shifts upfield at high NaCl concentration.

In the absence of divalent cations, relatively high salt concentrations are required for tRNA to retain a structure approximating its native state (Crothers & Cole, 1978). The effects of $Na^+$ on the $^{19}F$ spectrum of (FUra)$tRNA_1^{Val}$ at room temperature (21–24 °C) and in the absence of added $Mg^{2+}$ are shown in Figure 6. As the NaCl concentration decreases below 75 mM, the farthest downfield $^{19}F$ resonance, peak B, splits in two, the upfield component becoming more prominent with decreasing salt concentration. At lower NaCl levels, peak B broadens and splits further and diminishes in intensity. Peak C shifts upfield into the middle of the $^{19}F$ spectrum as the NaCl concentration drops. In general, with decreasing NaCl, the relative intensity of the cluster of resonances in the central region of the $^{19}F$ spectrum increases at the expense of the upfield and downfield resonances (Figure 6). This is undoubtedly due to unfolding of the tRNA at low ionic strength in the absence of $Mg^{2+}$ (Crothers & Cole, 1978), with a resulting shift of the $^{19}F$ signals to the 4–5-ppm range characteristic of FUra in unstructured polynucleotides. Below 20 mM NaCl, a new signal appears upfield at 0.4 ppm, its intensity increasing as the NaCl concentration decreases (not shown in Figure 6). Increasing NaCl also shifts peak H upfield and peak G downfield. Even at the highest NaCl concentration used (400 mM), the low-$Mg^{2+}$ $^{19}F$ spectrum of the tRNA differs significantly from the spectrum at high (15 mM) $Mg^{2+}$ (compare Figures 2 and 6). It would seem that, as determined by $^{19}F$ NMR spectroscopy, $Na^+$ cannot fully substitute for $Mg^{2+}$ in maintaining the structure of tRNA.

*Solvent Accessibility of 5-Fluorouracil in (FUra)$tRNA_1^{Val}$.* In $^{19}F$ NMR studies with proteins labeled with fluorinated amino acids, chemical shifts downfield from the position of the free fluoroamino acid have been interpreted as indicating amino acid residues in a hydrophobic (buried) microenvironment (Robertson et al., 1977; Sykes & Weiner, 1980). To determine the relationship between chemical shift and the degree of exposure to solvent in fluorinated nucleic acids, we have compared the $^{19}F$ NMR spectrum of (FUra) $tRNA_1^{Val}$ in $^2H_2O$ and $H_2O$ buffers. As a result of the interaction of exposed fluorines with solvent, $^{19}F$ resonances from accessible FUra residues will be shifted to lower field when the solvent is changed from $^2H_2O$ to $H_2O$ because of a H–D isotope effect (Hull & Sykes, 1976). The difference, termed the solvent isotope shift (SIS), provides a quantitative estimate of the degree of exposure to solvent of individual FUra residues in the tRNA. Table I summarizes the chemical shift measurements of fluorinated $tRNA_1^{Val}$ in $H_2O$ and $^2H_2O$ buffers at 15 mM $Mg^{2+}$. The results permit grouping peaks in the $^{19}F$ NMR spectrum into three classes. Peaks D, E/F, and H in the central region of the spectrum, 3.6–4.3 ppm downfield from FUra, have the largest SIS values (0.2–0.3 ppm), comparable to that of free 5-fluorouridine, and might correspond to FUra residues that are completely exposed to solvent. An exception in this chemical shift range is peak G with a SIS value close to zero, indicating a fluorine with essentially no contact with solvent. The upfield peaks I–N (1.8–3.4 ppm), with SIS values between 0.1 and 0.15, could represent FUra residues only partially accessible to solvent, whereas the downfield resonances B and C (4.5–6.7 ppm), SIS $\leq$0.1, are from fluorines that are essentially buried within the tRNA structure. Peak A, however, which is hypersensitive to changes in ionic strength and $Mg^{2+}$ (see Figures 4 and 5) has a large SIS value (0.26–0.38) and is, by this criterion, highly exposed.

*Bisulfite Modification of FUra-Substituted tRNA.* Reaction of tRNA with sodium bisulfite at pH 7 produces a stable uridine–bisulfite adduct by addition across the 5,6 double bond of the pyrimidine (Furuichi et al., 1970; Shapiro et al., 1970). The product is unstable at pH 9 and reverses

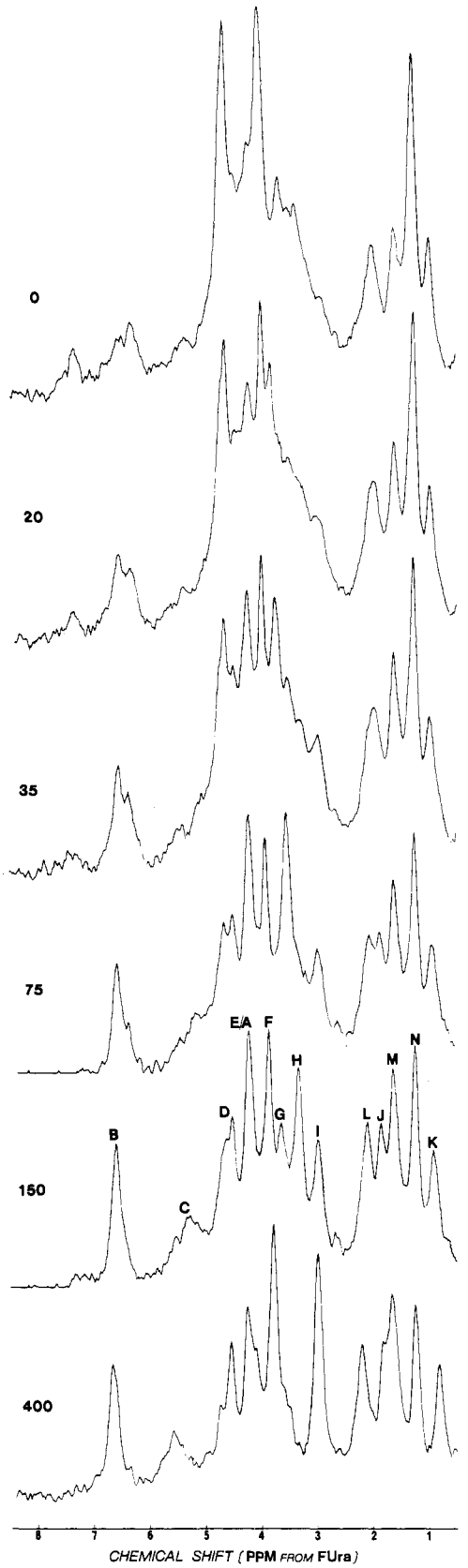


FIGURE 6: Effects of NaCl on the $^{19}$F NMR spectrum of (FUra)-tRNA$_1^{Val}$ in the absence of $Mg^{2+}$. The tRNA (0.40 mM) was dissolved in 50 mM sodium cacodylate buffer, pH 6.0, containing varying NaCl concentrations. From top to bottom, spectra were taken at 0, 20, 35, 75, 150, and 400 mM NaCl.

to uridine. Cytidine also reacts, but the adduct dissociates on removal of reagent to reform cytidine. Because the bisulfite reaction occurs preferentially with uracil residues in single-

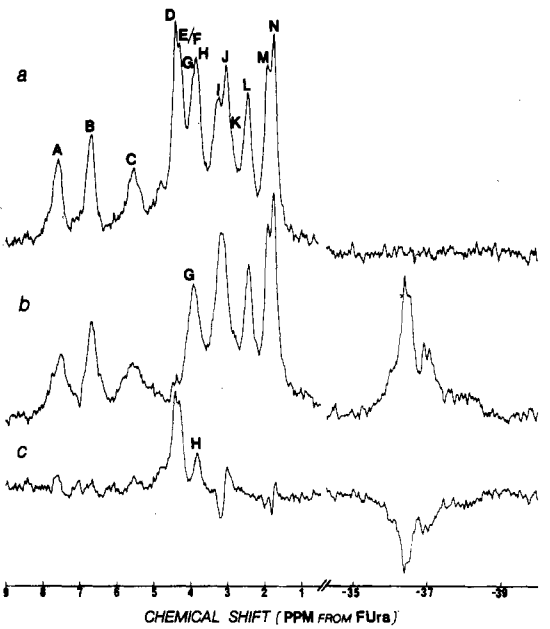


FIGURE 7: $^{19}$F NMR spectra of (FUra)tRNA$_1^{Val}$: (a) spectrum of unmodified (FUra)tRNA$_1^{Val}$ (0.17 mM); (b) spectrum of bisulfite-modified (FUra)tRNA$_1^{Val}$ (0.17 mM); (c) the difference spectrum obtained by subtracting curve b from curve a. The reaction with sodium bisulfite was carried out as described in Methods. Spectra were recorded in standard buffer.

stranded regions, it has been widely used as a probe of structure–function relationships in tRNA [e.g., see Schulman and Pelka (1977)].

5-Fluorouracil undergoes an analogous reaction with bisulfite, and saturation of the 5,6 double bond leads to large, 35–40 ppm, upfield $^{19}$F chemical shifts (Sander & Deyrup, 1972). Because this reaction presents the possibility of identifying the reactive residues in FUra-substituted tRNAs and thus assigning one or more resonances in the $^{19}$F spectrum, we have carried out preliminary $^{19}$F NMR studies of the bisulfite reaction with (FUra)tRNA$_1^{Val}$. The $^{19}$F spectrum of bisulfite-modified tRNA is presented in Figure 7b. Comparison with the spectrum of unreacted tRNA (Figure 7a) shows that the intensity of four $^{19}$F resonances, peaks D, E, F, and H, in the central region of the spectrum (3.9–4.5 ppm) decreases dramatically after the reaction. These $^{19}$F signals are shifted upfield, and several new peaks are observed between −36 and −37 ppm (Figure 7b), at the chemical shift position expected for a bisulfite adduct of FUra (Sander & Deyrup, 1972). Preliminary kinetic studies indicate that the relative rates of modification of the reactive FUra residues differ, and correlation of the reaction rate at each site with the shift of $^{19}$F resonances should permit peak assignments. Note that peak G does not react with bisulfite under our conditions. An additional effect of bisulfite treatment is a decreased resolution between peaks I and J (Figure 7b); the significance of this is not clear. Reversal of the bisulfite addition reaction by incubation at pH 9 restores the $^{19}$F spectrum to that of unreacted tRNA (result not shown).

*Photoaddition of 4′-(Hydroxymethyl)-4,5′,8-trimethylpsoralen to FUra-Substituted tRNA.* To identify $^{19}$F resonances from FUra residues in double helical regions of fluorinated tRNA, we have photoreacted (FUra)tRNA$_1^{Val}$ with 4′-(hydroxymethyl)-4,5′,8-trimethylpsoralen (HMT). Psoralen derivatives are photoreactive compounds that intercalate into double-stranded nucleic acids and, upon irradiation with long-wavelength ultraviolet light, react with the 5,6 double bond of pyrimidines to form stable monoadducts and diadducts

Table I: Solvent Isotope Shift (SIS) for $^{19}F$ Resonances in NMR Spectra of FUra-Substituted $tRNA_1^{Val}$ [a]

| $^{19}F$ resonance[b] | 10 mM NaCl | | | 100 mM NaCl | | |
|---|---|---|---|---|---|---|
| | $\delta(^2H_2O)$ | $\delta(H_2O)$[c] | SIS[d] | $\delta(^2H_2O)$ | $\delta(H_2O)$[c] | SIS[d] |
| A | 8.08 ± 0.04[e] | 8.34 ± 0.03 | 0.26 | 7.24 ± 0.17 | 7.62 ± 0.14 | 0.38 |
| B | 6.53 ± 0.01 | 6.60 ± 0.11 | 0.06 | 6.54 ± 0.02 | 6.65 ± 0.06 | 0.11 |
| C | 5.54 ± 0.04 | 5.57 ± 0.06 | 0.03 | 5.57 ± 0.06 | 5.57 ± 0.03 | 0 |
| D | 4.28 ± 0.01 | 4.48 ± 0.01 | 0.20 | 4.25 ± 0.02 | 4.46 ± 0.05 | 0.21 |
| E/F | 4.09 ± 0.01 | 4.35 ± 0.01 | 0.26 | 4.07 ± 0.03 | 4.32 ± 0.02 | 0.25 |
| G | 4.00 ± 0.01 | 3.98 ± 0.02 | −0.02 | 3.98 ± 0.03 | 3.94 ± 0.03 | −0.04 |
| H | 3.65 ± 0 | 3.89 ± 0.03 | 0.24 | 3.66 ± 0.04 | 3.86 ± 0.05 | 0.20 |
| I | 3.22 ± 0 | 3.37 ± 0 | 0.15 | 3.19 ± 0.04 | 3.32 ± 0.02 | 0.13 |
| J | 2.89 ± 0.04 | 3.10 ± 0.03 | 0.21 | 2.91 ± 0.02 | 3.06 ± 0.02 | 0.15 |
| K | ND[f] | ND | ND | ND | ND | ND |
| L | 2.41 ± 0.01 | 2.54 ± 0.01 | 0.13 | 2.37 ± 0.03 | 2.49 ± 0.02 | 0.12 |
| M | 1.83 ± 0.03[g] | 1.94 ± 0.01 | 0.11 | 1.85 ± 0.03 | 1.96 ± 0.03 | 0.11 |
| N | | | | 1.64 ± 0.06 | 1.79 ± 0.04 | 0.15 |
| 5-fluorouridine | | | | 3.24 | 3.52 | 0.28 |

[a] Spectra were recorded in 50 mM sodium cacodylate, pH 6.0, containing 15 mM $MgCl_2$ and 10 or 100 mM NaCl as indicated. [b] $^{19}F$ Resonances are labeled as shown in Figure 2. [c] Buffers prepared in $H_2O$ contained 10% $^2H_2O$ as an internal lock. [d] SIS values are defined as $(\delta(H_2O) - \delta(^2H_2O))$. [e] Standard deviation of two to six determinations. [f] ND, not determined; peak K is not resolved under these conditions. [g] Peaks M and N overlap at 10 mM NaCl.

[reviewed by Cimino et al. (1985)]. Single-stranded nucleic acids also react but to a lesser extent than double-stranded structures (Thompson et al., 1982). Uracil is the major photoreactive base in tRNA (Bachellerie & Hearst, 1982), FUra is even more reactive than uracil (Harter et al., 1974; Ou & Song, 1978).

The effects of 5 min of irradiation at 350 nm in the presence of HMT on the $^{19}F$ NMR spectrum of (FUra)$tRNA_1^{Val}$ are shown in Figure 8b. Comparison with the spectrum of tRNA irradiated in the absence of HMT (Figure 8a) indicates that photoaddition of HMT decreases the intensity of several $^{19}F$ resonances, most noticeably of peaks I through L in the upfield region of the spectrum. The intensity of the farthest downfield resonance, peak A, is also clearly diminished. Longer irradiation times, up to 3 h, result in progressively greater decreases in the intensities of all $^{19}F$ resonances in the spectrum, including those in the central region that are not affected at shorter times.

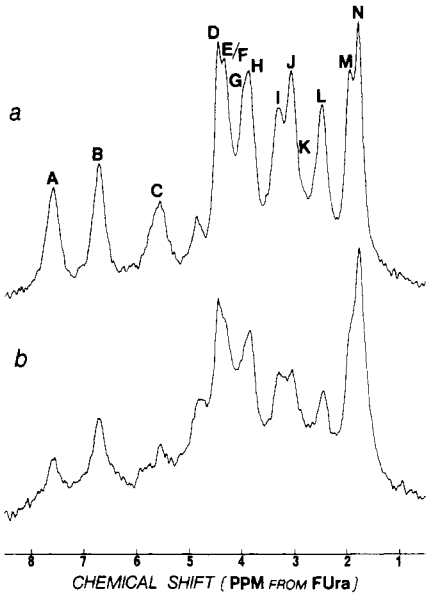


FIGURE 8: $^{19}F$ NMR spectra of (FUra)$tRNA_1^{Val}$ after photoreaction with and without 4′-(hydroxymethyl)-4,5′,8-trimethylpsoralen (HMT): (a) (FUra)$tRNA_1^{Val}$ (0.31 mM) irradiated at 350 nm in the absence of HMT; (b) HMT-modified (FUra)$tRNA_1^{Val}$ (0.31 mM). Reaction with HMT was performed as described under Methods. Spectra were recorded in standard buffer.

## Discussion

Our results demonstrate that $^{19}F$ NMR spectroscopy of tRNA labeled in vivo with 5-fluorouracil can provide important information on the solution structure and dynamic properties of tRNA. *E. coli* $tRNA_1^{Val}$ is well suited to these studies because its structure and function are not significantly affected by introduction of the $^{19}F$ label (Horowitz et al., 1974; Ofengand et al., 1974). Furthermore, the incorporated fluorouracils are distributed throughout the tRNA (Figure 1), each located in a different microenvironment as indicated by the resolution of $^{19}F$ resonances for all 14 FUra bases (Figure 2). Each resonance in the $^{19}F$ spectrum separately monitors structural transitions in its neighborhood, and together, they serve as sensitive probes of conformational changes in every loop and stem of the tRNA. To relate observed spectral shifts to specific changes in molecular conformation, it is necessary to assign the $^{19}F$ resonances to individual FUra bases in the polynucleotide sequence. This represents a formidable undertaking, worth approaching from several viewpoints and in stages. The results presented here and those reported earlier (Horowitz et al., 1977) provide a preliminary basis for making assignments.

The *E. coli* valine acceptor molecule, a class 1 ($D_4V_5$) tRNA with a total of 76 nucleotides, closely resembles yeast $tRNA^{Phe}$ and might resonably be expected to possess a similar solution structure (Sigler, 1975). By analogy to the crystal structure of $tRNA^{Phe}$ (Rich and RajBhandary, 1976) it is possible to classify the 14 fluorouracils in (FUra)$tRNA_1^{Val}$ into three groups: (1) five non-base-paired fluorouracils located in loops (17, 33, 34, 47, and 59), (2) three tertiary hydrogen-bonded bases (8, 54, and 55), and (3) six secondary (helical) hydrogen-bonded bases (4, 7, 12, 29, 64, and 67). We noted earlier (Horowitz et al., 1977) that the well-defined peaks in the $^{19}F$ spectrum of (FUra)$tRNA_1^{Val}$ as well as the broadened peaks observed in the spectrum of unfractionated FUra-substituted tRNA, fall into three groups, and suggested that these groupings may correspond to FUra bases in the three structural environments in the tRNA.

Heat-denatured (FUra)$tRNA_1^{Val}$ and poly(FU), which exists as a random coil at the temperature and ionic strength used in our experiments (Szer & Shugar, 1963; Massoulie et al., 1963), both give $^{19}F$ NMR spectra with a single peak at 4.5–4.7 ppm. This is presumably the chemical shift position corresponding to FUra in a random coil polynucleotide, and it seems likely that non-base-paired (loop) FUra bases in native

(FUr)tRNA$_1^{Val}$ will also give $^{19}$F resonances in the 4–5 ppm range. Support for this assignment comes from several experiments, indicating that changes in pH and solvent, as well as reaction of the tRNA with single strand-specific reagents, preferentially affect resonances in the middle (4–5 ppm) of the $^{19}$F spectrum of (FUra)tRNA$_1^{Val}$.

A pH titration of (FUra)tRNA$_1^{Val}$ shows that five FUra bases are susceptible to titration in the pH 7–9 range. These correspond to peaks C, D, E, F, and H, all in the central (4–5.5 ppm) region of the $^{19}$F spectrum (Figure 3). Although FUra C is included in the group of titratable FUra residues, the extent of its downfield shift is not as great as that of the other resonances in this group (Figure 3). Furthermore, the SIS values (Table I) indicate that it is not exposed to solvent nor does it react with sodium bisulfite (Figure 7). The possibility that the pH-dependent shift of peak C in the 7–9 pH range is due to a conformation change in the tRNA cannot be dismissed. It should be noted that one $^{19}$F resonance in the 4–5.5 ppm chemical shift range, tentatively identified as peak G, is not sensitive to pH (Figure 3).

Measurement of the degree of solvent exposure by determination of the solvent isotope shift (SIS) on transferring the tRNA from $H_2O$ to $^2H_2O$, indicates that FUra bases D, E/F, and H, with $^{19}$F resonances in the 4–5 ppm range, have SIS values near that of free 5-fluorouridine (Table I). By this criterion, these fluorouracils are completely exposed. Fluorouracil A, which gives the lowest field peak in the $^{19}$F spectrum of native (FUra)tRNA$_1^{Val}$, also has a large SIS value (see later discussion). However, FUra C (5.5 ppm in $H_2O$) and FUra G (3.9 ppm in $H_2O$) both show SIS values close to zero, indicating no significant contact with solvent.

Four $^{19}$F resonances in the spectrum of (FUra)tRNA$_1^{Val}$, peaks D, E, F, and H (4–5 ppm), shift upfield as a result of reaction of the tRNA with the single-strand-specific reagent sodium bisulfite (Figure 7), again locating the corresponding fluorouracils in non-base-paired regions of the tRNA. Once more, peak G is an exception among resonances in the 4–5 ppm chemical shift range and does not shift upfield when the tRNA reacts with bisulfite.

These results enable us to assign four of the five non-base-paired (loop) FUra bases in (FUra)tRNA$_1^{Val}$ to peaks D, E, F, and H. The chemical shift position of peak G suggests that it may correspond to the fifth such FUra even though it is shielded from solvent effects and chemical reaction. A possible candidate for FUra G is the base at position 59 in the T-loop. Chemical modification studies show that this position has little reactivity with a variety of reagents (Goddard, 1977) and that, in general, the T-loop is buried in the tertiary structure of the tRNA [e.g., see Vlassov et al. (1981)].

The remaining resonances in the $^{19}$F spectrum of (FUra)tRNA$_1^{Val}$ are shifted upfield and downfield of the cluster of peaks in the central portion of the spectrum, representing the most exposed FUra residues. Because our knowledge of the theoretical basis of $^{19}$F shifts is far from complete, it is difficult to interpret the observed shifts in terms of the local environments of individual fluorouracils. Several factors could be responsible for these shifts, including ionization of the N(3)–H of the FUra base or hydrogen bonding of this group, ring-current effects, and van der Waals or hydrophobic interactions. Dissociation of the N(3)–H of FUra shifts the $^{19}$F resonance 1.5–2.7 ppm downfield [Figure 3 and Alderfer et al. (1983)]. Although it is possible that such an ionization is at least partly the cause of the downfield shift of peaks in the $^{19}$F spectrum of (FUra)tRNA$_1^{Val}$, it seems unlikely because a pH as low as 4.5 fails to produce the upfield shift anticipated on protonation of the ring nitrogen (Figure 3). Peak C does exhibit an upfield shift as the pH decreases below 5.5 (Figure 3). This may be due to protonation of the FUra base but is more likely a result of the conformational change observed in *E.coli* tRNA$_1^{Val}$ at mildly acidic pH (Steinmetz-Kayne et al., 1977).

Hull and Sykes (1974, 1975a, 1975b, 1976) in their intensive investigation of 3-fluorotyrosine-substituted bacterial alkaline phosphatase demonstrated a correlation between the $^{19}$F chemical shift of an incorporated fluorotyrosine and its chemical environment in the protein. They found that the $^{19}$F resonances of buried fluorotyrosines appear downfield of those of fluoroamino acids in a solvent-accessible environment. This downfield shift was ascribed to a greater van der Waals contact interaction between buried fluorines and neighboring protons. We noted earlier (Horowitz et al., 1977) that the downfield resonances, especially peaks A and C, are generally broader than most of the other signals in the $^{19}$F spectrum of (FUra)tRNA$_1^{Val}$ (see Figure 2). Such a correlation between chemical shift and dipolar relaxation is to be expected if the mechanism of the shift involves van der Waals interactions.

From the relative areas of the downfield resonances in the $^{19}$F spectra of both unfractionated FUra-substituted tRNA and (FUra)tRNA$_1^{Val}$, we had earlier suggested (Horowitz et al., 1977) that the three farthest downfield resonances correspond to fluorouracils 8, 54, and 55, which replace the invariant bases U or s$^4$U8, T54 (or other derivatives of U), and Ψ55 that form tertiary hydrogen bonds in all unsubstituted tRNAs. A number of recent observations support such assignments. $^{19}$F{$^{19}$F} nuclear Overhauser studies have shown a reciprocal NOE between the downfield peaks A and B in the spectrum of (FUra)tRNA$_1^{Val}$ (Gollnick et al., 1985). If we assume the fluorine-labeled tRNA has a structure similar to the crystal structure of yeast tRNA$^{Phe}$, only FUra54 and -55 should be close enough to each other (4–5 Å) to give an appreciable $^{19}$F homonuclear NOE.[3]

Peak A, the farthest downfield resonance (at 15 mM $Mg^{2+}$) in the $^{19}$F spectrum of fluorinated valine tRNA is especially sensitive to change in monovalent and divalent cation concentrations (Figures 4 and 5). The same is true for the farthest downfield resonance in the $^{19}$F spectra of each of the other two fluorine-labeled *E. coli* tRNAs thus far purified, tRNA$_f^{Met}$ and tRNA$_m^{Met}$ (Gollnick et al., 1984). This could very well be characteristic of all fluorinated tRNAs and indicate that peak A corresponds to one of the invariant bases involved in maintaining the tertiary structure of the tRNA. In this connection, Redfield and co-workers (Johnston & Redfield, 1981; Tropp & Redfield, 1981) observed a resonance in the 10.6–10.9 ppm region of the $^1$H NMR spectra of several tRNAs that also exhibited a hypersensitivity to changes in $Mg^{2+}$ concentration. Tropp and Redfield (1981) assigned this resonance to the imino proton at N(1) of Ψ55 on the basis of an observed NOE from the methyl group of T54. In fluorine-labeled tRNAs, the fluorine of FUra55 is located at the position normally occupied by the N(1)-H of Ψ55.

In some tRNAs the tertiary structure is the most thermally labile (Crothers & Cole, 1978). Examination of the temperature dependence of the $^{19}$F spectra of (FUra)tRNA$_1^{Val}$ and of unfractionated fluorinated tRNA has shown that, as temperature increases, it is the lowest field resonances that are affected first, shifting upfield to the middle of the spectrum (Gollnick et al., 1984). A similar effect is observed at room

[3] Distance measurements were carried out with the program DISPLA written by P. Briley, Department of Biochemistry, University of Iowa, Iowa City, IA.

temperature as the tRNA unfolds at low $Na^+$ concentrations in the absence of magnesium (Figure 6). Below 75 mM NaCl, the lowest field resonance (peak B) broadens and shifts to the central region of the spectrum before the other peaks are affected.

Ring-current shielding effects on FUra residues located in helical regions of the tRNA, due to the neighboring magnetically anisotropic base pairs, could, at least partly, account for the upfield shifts of resonances in the $^{19}F$ spectrum of (FUra)$tRNA_1^{Val}$. For stacked FUra residues, this mechanism could produce upfield chemical shifts as large as 2 ppm (Arter & Schmidt, 1976). Coleman and his collaborators noted 0.3–0.6 ppm upfield chemical shifts for the $^{19}F$ signals of both m-fluorotyrosine (Coleman & Armitage, 1978) and 5-fluoro-2′-deoxyuridine-5′-phosphate upon binding of the nucleotide to fluorotyrosine-modified gene 5 protein from bacteriophage fd (O'Connor & Coleman, 1982). They attributed these shifts to ring-current effects produced by partial intercalation of the bound nucleotide between the stacked aromatic residues at the protein polynucleotide-binding site. We have observed a similar upfield shift due to base-pair formation involving the anticodon loop of the tRNA. Peak H in the $^{19}F$ NMR spectrum of (FUra)$tRNA_1^{Val}$ shifts almost 0.7 ppm upfield as a result of binding the trinucleotide codon GpUpA to the anticodon (Gollnick et al., 1984, 1986).

The reaction of HMT with (FUra)$tRNA_1^{Val}$ is also consistent with the idea that the upfield resonances correspond to FUra bases in helical arrays. Following a relatively short 5-min rection, the primary effect of the photocycloaddition of HMT to (FUra)$tRNA_1^{Val}$ is a marked reduction in the intensities of peaks I–L in the upfield portion of the $^{19}F$ spectrum (Figure 8). Psoralen derivatives are known to react most readily with pyrimidines in double stranded regions (Cimino et al., 1985). Furthermore, the major photoreaction sites on tRNA for several psoralen derivatives have been located in the T-stem (Bachellerie & Hearst, 1982; Nielsen & Leick, 1985). As a result, we suggest that the FUra bases corresponding to the upfield peaks are located in base-paired helical stems.

The results presented permit us tentatively to correlate various regions of the $^{19}F$ spectrum of 5-fluorouracil-labeled tRNAs with FUra bases in different structural environments. Because of the resolution obtainable and the evident sensitivity of the $^{19}F$ spectra to changes in the environment of the fluorine nuclei, it seems clear that $^{19}F$ NMR will provide a valuable probe of conformational changes in tRNA as they relate to function, especially after assignments of individual peaks have been made.

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**Registry No.** (FUra)$tRNA^{Val}$, 103958-83-4; Mg, 7439-95-4; NaCl, 7647-14-5.

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# Asparagine-Linked Sugar Chains of Fetuin: Occurrence of Tetrasialyl Triantennary Sugar Chains Containing the Galβ1→3GlcNAc Sequence†

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ABSTRACT: Asparagine-linked sugar chains were quantitatively released from fetuin by hydrazinolysis. Structural analysis of the sugar chains by sequential exoglycosidase digestion in combination with methylation analysis and Smith degradation revealed that most of them have typical biantennary (8%) and triantennary (74%) structures containing different amounts of *N*-acetylneuraminic acid residues. In addition, an unusual tetrasialyl triantennnary sugar chain (17%) containing the Galβ1→3GlcNAc sequence in the outer chain moiety was detected, and its structure was elucidated as NeuAcα2→3Galβ1→3(NeuAcα2→6)-GlcNAcβ1→4(NeuAcα2→6Galβ1→4GlcNAcβ1→2)Manα1→3(NeuAcα2→3Galβ1→4GlcNAcβ1→-2Manα1→6)Manβ1→4GlcNAcβ1→4GlcNAc.

Fetuin, one of the major glycoproteins found in fetal calf serum, has been shown to contain three mucin-type sugar chains (Spiro & Bhoyroo, 1974) and three asparagine-linked sugar chains in one molecule (Spiro, 1973). Both types of sugar chains have been extensively investigated; however, the structures of asparagine-linked sugar chains reported by Baenziger and Fiete (1979) and by Nilsson et al. (1979) were different. The sugar chains proposed by the two groups were the same with respect to having a trisialyl triantennary structure with 2,4-branched outer chains but were opposite in regard to the location of the outer chain branch. Later, Krusius and Finne (1981) presented the data that the 2,4 branch was located on the Manα1→3 side in accordance with the result obtained by Nilsson et al. (1979). While studying

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