Uridine is uniquely conserved at position 8 in elongator tRNAs and binds to A14 to form a reversed Hoogsteen base pair which folds the dihydrouridine loop back into the core of the L-shaped molecule. On the basis of 1H NMR studies, Hurd and co-workers (Hurd, R. E., Robillard, G. T., and Reid, B. R. (1977) Biochemistry 16, 2095–2100) concluded that the interaction between positions 8 and 14 is absent in Escherichia coli tRNAs with only 3 base pairs in the dihydrouridine stem. We have taken advantage of the unique 15N chemical shift of N3 in thiouridine to identify 1H and 15N resonances for the imino units of s'US and s'U9 in E. coli tRNA^{Ser} and tRNA^{Thr}. Model studies with chloroform-soluble derivatives of uridine and 4-thiouridine show that the chemical shifts of the protons in the imino moieties move downfield from 7.9 to 14.4 ppm and from 9.1 to 15.7 ppm, respectively; whereas, the corresponding 15N chemical shifts move downfield from 157.5 to 162.5 ppm and from 175.5 to 180.1 ppm upon hydrogen bonding to 5'-O-acetyl-2',3'-isopropylidene adenosine. The large difference in 15N chemical shifts for U and s'U allows one to unambiguously identify s'U imino resonances by 15N NMR spectroscopy. E. coli tRNA^{Ser} and tRNA^{Thr} were selectively enriched with 15N at N3 of all uridines and modified uridines. Two-dimensional 1H-15N chemical shift correlation NMR spectroscopy revealed that both tRNAs have resonances with 1H and 15N chemical shifts characteristic of s'U pairs. The 1H shift is approximately 1 ppm upfield from the typical s'U8 resonance at 14.8 ppm, presumably as a result of local diamagnetic anisotropies. An additional s'U resonance with 1H and 15N shifts typical of interaction of a bound water or a sugar hydroxyl group with s'U9 was discovered in the spectrum of tRNA^{Thr}. Our NMR results for tRNAs with 3-base pair dihydrouridine stems suggest that these molecules have an U8A14 tertiary interaction similar to that found in tRNAs with 4-base pair dihydrouridine stems.

* This work was supported by Grant GM 32490 from the National Institutes of Health. Purchase of instruments in the NMR Center at the University of Utah was supported by shared instrumentation grants from the National Institutes of Health and the National Science Foundation. The Regional NMR Center at Colorado State University was supported by National Science Foundation Grant CHE 78-18581. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

To whom correspondence and requests for reprints should be addressed.

Richard H. Griffey, Darrell R. Davis, Ziro Yamaizumi, Susumu Nishimura, Bruce L. Hawkins, and C. Dale Poulter

From the Department of Chemistry, University of Utah, Salt Lake City, Utah 84112, and the Regional NMR Center, Department of Chemistry, Colorado State University, Fort Collins, Colorado 80523
ro sine synthetase catalyzes the exchange of tritium at C5 of s'tU8, indicative of a covalent linkage between the tRNA and the synthetase, as is found for other tRNAs (20). We now report the use of H, 15N-dimensional NMR spectroscopy to locate the imino resonances for s'tU in E. coli tRNA\textsuperscript{ tyr} and tRNA\textsuperscript{ p"er} (see Fig. 1). Both species have 5 base pairs in their dihydrouridine stems because of a 13–22 mismatch.

**EXPERIMENTAL PROCEDURES**

**Materials—[3-\textsuperscript{15}N]Uracil was prepared by the procedure of Griffee and Poulter (21).** H-Labeled E. coli tRNA\textsuperscript{ tyr} and tRNA\textsuperscript{ p"er} was obtained from strain S0187, an uracil auxotroph, as previously described (22). The level of incorporation was greater than 90% as determined by the absence of H peaks for unlabelled tRNA between H-\textsuperscript{15}N doublets in the H NMR spectra of the tRNAs.

5'-O-Acetyl-2',3'-O-isopropylidene adenosine was purchased from Sigma. Tri-O-acetyl-4-thiouridine was prepared according to the procedure of Fox et al. (23). The material was a pale yellow solid, \( R_F = 0.6 \) upon silica gel TLC when eluted with ethyl acetate; \( ^1\text{H} \) NMR (300 MHz, CDCl\textsubscript{3}) \( \delta 2.15 \) (s, 9H, acetate CH\textsubscript{3}), 4.38 (m, 3H, H at C4' and C5'), 5.45 (m, 2H, H at C2' and C3'), 6.00 (d, 1H, \( J = 7 \) Hz, H at C3'), 6.45 (d, 1H, \( J = 6 \) Hz, H at C1'), 7.25 (d, 1H, \( J = 7 \) Hz, H at C6), 10.20 (s, 1H, H at N3); \( ^1\text{C} \) NMR (75 MHz, CDCl\textsubscript{3}) \( \delta 20.43, 20.51, 20.78, 62.92, 69.93, 72.50, 79.96, 87.96, 113.78, 113.46, 147.29, 169.35, 169.40, 169.91, 189.50, 207.55 ppm downfield from its oxo counterpart.

**Preparation of NMR Samples—** A sample of tRNA\textsuperscript{ tyr} (8.5 mg) was dissolved in 300 \( \mu \)l of 10 mM cacodylate buffer, pH 7.0, that contained 50 mM sodium chloride, 10 mM magnesium chloride, 1 mM EDTA, and 8% deuterium oxide. tRNA\textsuperscript{ p"er} (8.2 mg) was dissolved in 400 \( \mu \)l of 10 mM phosphate buffer, pH 7.0, that contained 10 mM magnesium chloride, 10 mM sodium chloride, 5% deuterium oxide.

**NMR Measurements—**H chemical shifts were referenced to internal tetramethylsilane or 2,2-dimethyl-2-silapentane-5-sulfonate and \( ^1\text{H} \) chemical shifts, to an external 2.9 M solution of ammonium chloride in 1 M hydrochloric acid. The \( ^1\text{H} \) shifts are given relative to ammonia at 25 °C using a correction factor of 24.9 ppm as previously described (11, 15, 22). MQS data for tRNAs were obtained on a Varian XL-400 NMR spectrometer equipped with a \( ^1\text{H} \) observe, \( ^1\text{H} \) lock 5-mm probe and on the Nicolet system previously described (11, 22). Two-dimensional data sets consisting of 16 blocks of 5200 transients were collected for tRNA\textsuperscript{ p"er} and 64 blocks of 3600 transients, for tRNA\textsuperscript{ tyr}. After transformation, the data sets S (\( F_1 \), \( F_2 \)) were plotted as \( ^1\text{H} \) chemical shifts versus \( F_2 \) for tRNA\textsuperscript{ p"er} and \( ^1\text{H} \) for tRNA\textsuperscript{ tyr}. Software currently available for the Varian XL-400 does not allow us to perform the phase shearing operation required to plot the spectrum for tRNA\textsuperscript{ p"er} in the same manner as tRNA\textsuperscript{ tyr}.

**RESULTS**

**Model Studies—** Formation of hydrogen bonds to imino protons in nucleic acids is accompanied by downfield shifts in the NMR signals of the protons and nitrogens in the imino units (24, 25). Interactions that shift the protons and nitrogens to the lower field can be modeled with appropriately substituted nucleosides in chloroform. Model studies with a variety of hydrogen bond donors and acceptors have been very helpful for assigning \( ^1\text{H} \)-\textsuperscript{15}N resonances of related interactions in nucleic acids (1, 2, 10, 15, 22).

Proton chemical shifts for monomeric U and s'tU and U and s'tU base pairs are well characterized in nucleoside models. The \( ^1\text{H} \) chemical shifts for the imino protons in tri-O-benzoyluridine and tri-O-benzoyl-4-thiouridine at high dilution in chloroform at 21 °C are 7.9 and 9.0 ppm, respectively (26). At higher concentrations, the nucleosides self-associate and the signals move downfield. For 0.2 M solutions at 20 °C, both resonances have shifted by 1.4 ppm (26). Addition of 5'-O-acetyl-2',3'-O-isopropylidene adenosine to chloroform solutions of tri-O-benzoyluridine (24) and its 4-thio derivative causes large downfield shifts in the imino resonances. The effect is enhanced when the sample is cooled, and limiting values for U and s'tU of 14.4 and 15.7 ppm, respectively, are attained in the presence of 3 eq of purine at −35 °C and a total nucleoside concentration of 0.2 M. Thus, the changes in chemical shifts for U and s'tU imino protons are similar. The resonances move downfield by approximately 5–6 ppm from their intrinsic positions upon formation of base pairs with adenosine. Although the ranges for U and s'tU imino protons are similar, the resonance may move downfield by an additional 1 ppm at −35 °C. Thus, the imino \( ^1\text{H} \) chemical shifts in uridine (24) and 4-thiouridine also move downfield by approximately 5–6 ppm. With \( ^1\text{H} \) chemical shifts, however, the ranges do not overlap.

Two-dimensional \( ^1\text{H} \)-\textsuperscript{15}N experiments with tri-O-acetyl-4-thiouridine at natural abundance show related behavior for the imino nitrogens. The correlated \( ^1\text{H} \)-\textsuperscript{15}N chemical shift for the imino proton of a 0.2 M solution of tri-O-acetyl-4-thiouridine in chloroform is 10.2/175.5 ppm at 20 °C. Since the nucleoside self-associates at this concentration, the \textsuperscript{15}N chemical shift for monomeric 4-thiouridine should be upfield from 175.5 ppm by perhaps as much as 2 ppm. The \( ^1\text{H} \)-\textsuperscript{15}N correlated shift for tri-O-acetyl-4-thiouridine is 13.8/180.1 ppm in the presence of 1 eq of 5'-O-acetyl-2',3'-O-isopropylidene adenosine at 20 °C under conditions where the proton shift has reached 80% of its maximal downfield value. By analogy to the behavior of the directly attached proton in s'tU, the \textsuperscript{15}N resonance may move downfield by an additional 1 ppm at −35 °C. Thus, the imino \textsuperscript{15}N shifts in uridine (24) and 4-thiouridine also move downfield by approximately 5–6 ppm. With \textsuperscript{15}N chemical shifts, however, the ranges do not overlap.

\footnote{The abbreviations used are: MQS, multiple quantum spectroscopy; \( F \), frequency dimension; FINDS, fourier internuclear difference spectroscopy; \( f \), time dimension.}
The gap between the high field limit for the $^{15}$N resonances in s\textsuperscript{4}U and the low field limit for U is at least 10 ppm. This distance is too large to be bridged by shielding contributions from neighboring groups, and $^{15}$N chemical shifts can be used to distinguish between the imino units in s\textsuperscript{4}U and U in an unambiguous manner.

$^1$H-$^{15}$N Two-dimensional Spectra of $^{15}$N-Labelled E. coli tRNA\textsuperscript{Ser} and tRNA\textsuperscript{Phe}—$^1$H-$^{15}$N MQS spectra of tRNA\textsuperscript{Ser} and tRNA\textsuperscript{Phe} labeled regiospecifically with $^{15}$N at N3 of uridine and all modified uridines only have $^1$H-$^{15}$N correlated resonances for labeled imino units whose protons are not exchanging rapidly with solvent. Two-dimensional MQS maps for the two tRNAs are shown in Figs. 2 and 3. Both spectra have a cluster of partially resolved peaks with $^1$H shifts between 13.0 and 13.5 ppm and $^{15}$N shifts between 156 and 162 ppm. These signals have not yet been assigned to specific interactions, but are typical for Watson-Crick secondary UA base pairs in helical stems (10, 11), the tR54A58 reversed Hoogsteen tertiary pair (10, 11), and the anti-A31Ψ39 interaction in tRNA\textsuperscript{Phe} (10, 11, 22). Both NMR spectra also have unresolved resonances with $^1$H shifts near 11.5 ppm and $^{15}$N shifts between 155 and 158 ppm. These chemical shifts are typical for $Ψ65$-phosphate interactions expected in the rT\textsuperscript{W} loops and other stable U-phosphate contacts (10, 11). In addition, the cloverleaf structure of tRNA\textsuperscript{Ser} predicts a U6G67 wobble interaction which should generate a resonance in that area as well (10, 11).

A FINDS spectrum (see Fig. 4) of tRNA\textsuperscript{Ser} has a low intensity resonance at 9.8/150 ppm not observed above the noise in the MQS spectrum of the molecule. Based on recent studies from our laboratory with E. coli tRNA\textsuperscript{Ser}, tRNA\textsuperscript{Phe}, and tRNA\textsuperscript{Phe}, we assign this peak to the imino unit in a dihydrouridine (15). The $^1$H and $^{15}$N chemical shifts indicate that the imino proton interacts with a nearby sugar hydroxyl or an immobilized water molecule. tRNA\textsuperscript{Ser} has two dihydrouridines, D20 and D20A. Although the FINDS spectrum only shows a single peak in that region, the width of the resonance in the $^1$H dimension is significantly broader than those of other peaks at both 15 and 25 °C. Thus, it is possible that the 9.8/150 ppm peak is composed of overlapping resonances for D20 and D20A. A similar coincidence of $^1$H and $^{15}$H shifts was found for dihydrouridine in E. coli tRNA\textsuperscript{Phe}.

tRNA\textsuperscript{Phe} does not contain dihydrouridine and, as expected, has no resonance with $^1$H and $^{15}$N shifts typical of the D imino unit.

Finally, both tRNAs have $^1$H resonances that correlate with low field $^{15}$N chemical shifts. E. coli tRNA\textsuperscript{Ser} contains a single s\textsuperscript{4}U moiety at position 8 and the MQS spectrum of the molecule has a peak at 13.4/177.5 ppm with $^1$H and $^{15}$N chemical shifts consistent with a s\textsuperscript{4}UA14 pair. We assign this peak to a s\textsuperscript{4}U8A14 tertiary interaction in the molecule. tRNA\textsuperscript{Phe} has two s\textsuperscript{4}U bases, one at position 8 and the other at position 9. Interestingly, the MQS spectrum of the molecule has two peaks with $^1$H chemical shifts correlated with low field $^{15}$N resonances. One resonance occurs at 13.8/175.4 ppm which, by analogy with tRNA\textsuperscript{Ser}, we assign to s\textsuperscript{4}U8 in a s\textsuperscript{4}U8A14 pair. The peak at 9.3/176.5 ppm in the MQS spectrum of tRNA\textsuperscript{Phe} is at the high end of the $^1$H and $^{15}$N imino chemical shift ranges. We assign this resonance to s\textsuperscript{4}U9. The $^1$H chemical shift is characteristic of an unassociated imino proton or one that interacts with a hydroxyl group in a nearby ribose or a tightly bound molecule of water.

FIG. 2. $^1$H chemical shift versus $F_1$ two-dimensional spectrum at 25 °C for E. coli tRNA\textsuperscript{Ser}. Sixty-four increments of $t_1$ were acquired with 3600 transients per increment. The diagonal line is at the position of the $^{15}$N carrier, 177.0 ppm. $^{15}$N chemical shift increases from left to right.

FIG. 3. $^{15}$N versus $^1$H chemical shift two-dimensional spectrum at 15 °C for E. coli tRNA\textsuperscript{Ser}. Sixteen increments of $t_1$ were acquired with 3200 transients per increment.

FIG. 4. FINDS spectrum of E. coli tRNA\textsuperscript{Ser} at 25 °C. $^{15}$N decoupler at 150.0 ppm. Two thousand transients were acquired on and off resonance.
mentioned, similar 1H chemical shifts are seen for D in several tRNAs (15).

**DISCUSSION**

The U8A14 tertiary base pair is thought to be important for maintaining the local structure of tRNAs in the region of the dihydrouridine stem (4) and is a specific site for recognition of tRNAs by their cognate aminoacyl synthetases during charging (6). In many *E. coli* tRNAs, U8 is modified by thiolation at C4 in the pyrimidine ring. Replacement of the oxygen at C4 in uridine by sulfur results in a modest downfield shift of greater than 20 ppm for the 15N signal of the imino nitrogen. While it is not possible to conclusively distinguish between 1H imino signals in U and s·U in a tRNA on the basis of chemical shifts because of anisotropies from neighboring groups, the separation of 15N resonances is so large that assignments based on nitrogen chemical shifts are unambiguous.

Reid and co-workers (1, 2, 17) concluded that tRNAs without a secondary interaction involving the base at position 13 in the dihydrouridine stem do not have the 8-14 reversed Hoogsteen interaction, because the typical low field 1H resonance for s·U8 was absent in their NMR spectra. The possibility that the tertiary interaction exists but is unstable and cannot be detected by NMR due to rapid exchange of the imino proton with solvent was deemed unlikely because of the long lifetimes of other tertiary interactions. A third possibility exists. The absence of a fourth Watson-Crick base pair in the dihydrouridine stem may slightly alter the local conformation of the tRNAs near the 8-14 locus and induce an upfield shift in the resonance of the s·U8 imino proton without major changes in folding. Our data with 15N-labeled tRNAs indicate that this is indeed the case. We previously reported 1H-15N chemical shift correlations for several *E. coli* tRNAs with short variable loops and 4-base pair dihydrouridine stems (10, 11, 15). In all cases, the 1H-15N two-dimensional spectra gave correlated peaks at 14.8 ± 0.1/178 ± 0.5 ppm for the s·U8 imino units. The 1H-15N correlated peak at 13.4/177.5 ppm in the two-dimensional spectrum of tRNA_phe (4) and *E. coli* tRNA_1le (5) show that the bases at the terminus of the dihydrouridine stem; whereas, tRNA_phe has a G13A22 mismatch at the terminus of the dihydrouridine stem; whereas, tRNA_phe has a G13A22 mismatch. A slight adjustment which places A22 directly over the s·U8 imino proton in tRNA_phe (28) or twists the s·U8A14 pair from planarity (10) would be sufficient to produce the shielding necessary for an upfield shift from 14.8 to 13.4 ppm.

tRNA_phe also has a G13A22 mismatch, and the two-dimensional resonance at 13.8/179.4 ppm is most likely from the s·U8 imino proton in an environment similar to that found in tRNA_1le. Redfield and co-workers (4) detected a peak with similar 1H and 15N correlated shifts from the s·U8A14 tertiary pair in *E. coli* tRNA_phe. This species, with a small variable loop, has a G13A22 mismatch at the end of the dihydrouridine stem. Thus, it appears that the absence of the 14.8 ppm signal in *E. coli* tRNAs results from local perturbations in conformation which generate an upfield shift in the resonance, rather than a disruption of the 8-14 interactions accompanied by a large change in the folding of those regions of the tRNAs.

Our results do not allow us to unambiguously assign the s·U8 imino resonances in tRNA_phe and tRNA_phe to a reversed Hoogsteen interaction with A14. For example, the chemical shift data are also consistent with a s·U8A14 Watson-Crick structure. Two observations, however, cause us to strongly favor the former explanation. The U8 and A14 bases are highly conserved and, in all instances reported to date, form reversed Hoogsteen structures. Also, a recent nuclear Overhauser effect study by the Redfield group (2) using unlabeled and C8-adenine deuterated *E. coli* tRNA_phe which strongly resembles tRNA_phe and tRNA_phe in the dihydrouridine stem, supports the presence of a s·U8A14 reversed Hoogsteen pair in that molecule (29).

A second s·U imino resonance which we assign to s·U9 is seen at 9.3/176.5 ppm in the two-dimensional spectrum of tRNA_phe. The high field 1H and 15N chemical shifts are inconsistent with a s·UA pair or a s·UG wobble interaction. The most likely possibilities are a hydrogen bond to a hydroxyl group in a nearby ribose or an immobilized water molecule. We don't know which specific interaction is responsible for the s·U9 resonance. One interesting possibility is a 9-12-23 base triple. Yeast tRNA_phe folds so that A9 hydrogen bonds to the U12A23 base pair as shown in Fig. 5A (4). A similar orientation for s·U9, C12, and G23 in tRNA_phe would not place s·U9 within direct hydrogen bonding distance of the C12G23 pair without a contraction of the phosphodiester.
15N-labeled tRNA

backbone, but an indirect association could occur through a bridging water as shown in part B of Fig. 5. We recently proposed similar interactions for dihydrouridine with neighboring groups through water bridges (15).

3H NMR spectroscopy is a powerful tool for studying biopolymers in solution. One of the most serious limitations of the method is the assignment of resonances in the spectrum to specific protons in the molecule. This is a major problem because protons have a narrow range of chemical shifts and spectral densities in regions of interest are often too high to see cleanly resolved peaks for individual nuclei. We have used a combination of site-specific labeling and two-dimensional NMR chemical shift correlation spectroscopy to overcome these hurdles for several tRNAs. Typically, uracil bearing a single 15N label at positions 1 or 3 was synthesized (21) and incorporated into the uridine-related bases in E. coli tRNA with high specificity using an uracil auxotroph (10, 11, 15, 22). 1H-15N two-dimensional chemical shift correlation spectroscopy of the labeled material then gave spectra which only had peaks for protons directly attached to labeled nitrogens (11, 15, 22). Thus, all other signals, including the interfering imino protons in guanosine, disappeared. The simplification of the spectra, especially in the critical imino region between 9 and 15 ppm was dramatic. In addition, dispersion was enhanced because 1H chemical shifts were simultaneously correlated with those of the directly attached nitrogens.

Detection and assignment of resonances for s'U imino protons in E. coli tRNA15N and tRNA15N were only possible by 1H-15N two-dimensional NMR spectroscopy with 15N-labeled material. The s'U8A14 resonances in tRNAs with a 3-base pair dihydrouridine stem were missed in previous studies because the 14.8 ppm peak typical for that base pair in tRNAs was shifted to higher field by over 1 ppm into a region of the spectrum occupied by G imino protons in GC pairs. Our assignments for imino protons in several uridine-related bases correlated with those of the directly attached nitrogens.

The simplification of the spectra, especially in the critical imino region between 9 and 15 ppm was dramatic. In addition, dispersion was enhanced because 1H chemical shifts were simultaneously correlated with those of the directly attached nitrogens.

Detection and assignment of resonances for s'U imino protons in E. coli tRNA15N and tRNA15N were only possible by 1H-15N two-dimensional NMR spectroscopy with 15N-labeled material. The s'U8A14 resonances in tRNAs with a 3-base pair dihydrouridine stem were missed in previous studies because the 14.8 ppm peak typical for that base pair in tRNAs was shifted to higher field by over 1 ppm into a region of the spectrum occupied by G imino protons in GC pairs. Our results demonstrate that the important U8A14 tertiary interaction in E. coli tRNAs does not require 4 base pairs in the dihydrouridine stem.

Site-specific labeling, in conjunction with two-dimensional chemical shift correlation, permitted us to make unambiguous assignments for imino protons in several uridine-related bases in E. coli tRNAs. The technique is especially useful for the modified bases s'U, D (15), and Ψ (22). The increasing availability of recombinant plasmids should further enhance the utility of this approach by facilitating the in vivo synthesis of labeled molecules.

REFERENCES

27. Deleted in proof