Complete Nuclear Magnetic Resonance Signal Assignments and Initial Structural Studies of [^{13}C]Methyl-Enriched Transfer Ribonucleic Acid†

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ABSTRACT: The carbon-13 NMR spectra of [^{13}C]methyl-enriched, unfractionated, *Escherichia coli* tRNA exhibited nine prominant high-field signals with chemical shifts between 11 and 60 ppm (relative to Me_4Si). These signals have been assigned to the methyl carbons of ribothymidine, 2-(methylthio)-N^6-(2-isopentenyl)adenosine, 2-methyladenosine, N^6-methyladenosine, 1-methylguanosine, 5-[(methylamino)methyl]-2-thiouridine, 7-methylguanosine, methyl ester of uridine-5-oxyacetic acid, and 2′-*O*-methylribose nucleosides. The natural abundance carbon-13 NMR signals of ribose and base ring carbons have also been assigned, with particular attention to those of the modified nucleosides. Low-temperature thermal transitions were exhibited by some of the ^{13}C-enriched methyl carbon resonances. The methyl resonance of ribothymidine exhibited a downfield shift of 0.6 ppm (15 Hz) between 8.9 and 30 °C but not between 30 and 46 °C. This lower temperature shift corresponds to the temperature at which the tertiary interactions that place the TΨCG loop in close proximity to the h_2U loop would be expected to dissolve. Three methylated nucleosides located adjacent to the 3′ end of tRNA anticodons also exhibited low-temperature downfield shifts of their methyl resonances: 2-methyladenosine (0.3 ppm) and 1-methylguanosine (0.4 ppm) between 8.9 and 30 °C and N^6-methyladenosine (0.2 ppm) between 30 and 46 °C. These chemical shift changes were attributed to the loss of base stacking of anticodon bases with the 3′-adjacent methylated base.

Transfer RNA is unique among RNA species because of its very high content of modified nucleosides, several of which occur at specific, nonvariable locations in the cloverleaf structure of the molecule (Agris & Söll, 1977). It is consequently an appealing thought that specific modified nucleosides may somehow be involved in regulating the molecule's biological activities, such as recognition by its cognate aminoacyl-tRNA synthetase or interaction with the ribosome and mRNA in protein synthesis. Results from many biochemical investigations support the involvement of modified nucleosides in the functioning of tRNA (Agris & Söll, 1977). It remains unclear, however, as to how structural conformation, especially with regard to modified nucleosides, is correlated to biological function and, in particular, whether this structure is constant or changing for the molecule's role in protein synthesis. Indeed, it is likely that tRNA conformation does change (Rich & RajBhandary, 1976).

Carbon-13 nuclear magnetic resonance (^{13}C NMR) spectroscopy might be an extremely useful tool for probing this dynamic behavior of tRNA in solution, for specific changes in molecular conformation may be readily monitored. The assignments of many ^{13}C NMR signals of ribose and base ring carbons have been accomplished with spectroscopy of the naturally abundant carbon-13 of tRNA (Komoroski & Allerhand, 1972, 1974; Chang & Lee, 1976). Signal assignments have also been made for those carbons occurring in the most prevalent modifications of tRNA nucleosides (Komoroski & Allerhand, 1974; Chang & Lee, 1976).

The nucleoside modifications are of interest not only because they represent minor bases of singular locations in tRNA and therefore can serve as intrinsic probes of molecular structure but also because their resonance signals occur in spectral regions free from ribose and major base interference (Agris et al., 1975b). Modified nucleosides usually occur in structural regions of tRNA that contain only a few of the approximately 10 tertiary-structure hydrogen-bonded base pairs and none of the approximately 20 hydrogen-bonded base pairs contributing to the secondary structure (Kim et al., 1974). Proton NMR studies of tRNA have exclusively dealt with these hydrogen-bonded protons (Reid & Robillard, 1975; Hurd et al., 1977; Reid et al., 1977; Römer & Varadi, 1977) with the exception of some recent studies of methyl protons (Koehler & Schmidt, 1973; Kan & Ts'o, 1974; Kastrup & Schmidt, 1975; Kan et al., 1977; Schmidt & Kastrup, 1978). Hydrogen-bonded protons produce signals that are relatively easy to assign in contrast to signals from non-hydrogen-bonded protons and are of interest because of their importance to the basic structure of the molecule. Therefore, ^{13}C NMR investigation of modified nucleosides supplements the proton NMR research of tRNA regions which have not yet been assessed with that technique. However, natural abundance signals cannot be obtained for modifications occurring in less than 20% of all tRNA molecules nor can structural information be obtained readily from the study of even the most prevalent modifications when natural abundance ^{13}C is used as a probe.

We have previously demonstrated the feasibility of ^{13}C enrichment of all methylated nucleosides in tRNA of a relaxed strain of *Escherichia coli* auxotrophic for methionine (Agris et al., 1975b; Fujiwara et al., 1978). We report here complete assignments of resolved resonances of ^{13}C-enriched methylated nucleosides in unfractionated *E. coli* tRNA in proton-decoupled ^{13}C spectra and summarize signal assignments for all other tRNA carbons. Furthermore, we report initial NMR structural studies by low-temperature "melting" of [^{13}C]-methyl-enriched tRNA tertiary structure. Through observing changes in chemical shifts and line widths of the signals upon thermal denaturation, we were able to examine sequential melting of certain molecular regions.

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Experimental Procedure

Preparation of [^{13}C]Methyl-Enriched tRNA. *E. coli* C6 rel$^-$met$^-$cys$^-$ (Harris et al., 1969) was grown in 15-L batches at 37 °C in a minimal medium composed of 22.2 mM KH_2PO_4, 42.4 mM Na_2HPO_4, 18.7 mM NH_4Cl, 1.91 mM $MgSO_4$, 0.0022 mM $FeSO_4$, 0.496 mM $CaCl_2$, 0.10 mM cysteine, 0.10 mM [^{13}C]methylmethionine (Merck, Canada), 27.7 mM glucose, 0.01 mM 6-mercaptopurine, 0.18 mM uracil, 0.18 mM cytosine, 0.11 mM guanine, and 0.054 mM adenine. The drug 6-mercaptopurine was added to block de novo purine biosynthesis (Balis, 1968; J. G. Tompson, unpublished experiments). Bulk tRNA was extracted and purified by DEAE-cellulose column chromatography as described previously (Agris et al., 1973) and checked for 5S rRNA content by polyacrylamide gel electrophoresis (Agris et al., 1975c). The *E. coli* bulk tRNA, concentrated by ethanol precipitation, was dialyzed extensively against glass-distilled water (pH 6.8). This unfractionated tRNA had an A_{260}/A_{280} ratio of 2.0. Its total amino acid acceptor activity (1400 pmol/A_{260}), based on individual tritiated amino acid acceptance assays for 16 amino acids, was comparable to that of similarly extracted bulk tRNA of *E. coli* cultures grown in the presence of [^{12}C]methylmethionine.

Purified ^{13}C-labeled unfractionated tRNA was dissolved in 2.2 mL of distilled H_2O (10% D_2O, pH 6.8) to give a final concentration of 1.52 mM. NMR sample tubes had inside diameters of 11.0 mm. Mononucleosides were purchased from Sigma Chemical Co. (St. Louis, MO) and used as standards for signal assignments without further purification.

Procedure for Acquisition of NMR Spectra. Carbon-13 nuclear magnetic resonance spectra were recorded with a Varian XL-100-15 NMR spectrometer (25.2 MHz). The field was locked to D_2O in the sample. Proton decoupling was used with all spectra. Fifty-degree radiofrequency pulses of about 30-μs duration were used in the time domain to obtain Fourier transform spectra of ±5000-Hz sweep widths with a Nicolet TT-100 data system. The calculation of chemical shifts was computer processed and determined with a resolution of ±0.05 ppm. Observed chemical shifts were related to internal dioxane, the signal of which was converted to the Me_4Si scale by using δ^D 67.4 (Stothers, 1972). Sample temperature was controlled to ±1.0 °C with a Varian temperature control unit. Difference spectra were determined by using Nicolet software which allowed one to subtract a stored spectrum from another stored spectrum on the basis of the resonance intensity and position of dioxane carbons as reference.

Results and Discussion

Chemical Shifts of [^{13}C]Methyl Groups of tRNA. The proton-decoupled ^{13}C NMR spectrum of unfractionated *E. coli* tRNA (1.52 mM in H_2O, pH 6.8) at 46 °C is shown in Figure 1. Efficient in vivo incorporation of [^{13}C]methyl groups into tRNA was evidenced in the higher chemical shift region where signals were now distinct in contrast to the previously observed, natural abundance spectra of tRNA (Komoroski & Allerhand, 1972, 1974; Agris et al., 1975b; Chang & Lee, 1976). Different preparations of the ^{13}C-methylated tRNA have given us a reproducible spectrum with respect to peak positions and peak intensities (Agris et al., 1975b; Fujiwara et al., 1978), although the signal to noise (S/N) ratios were, as expected, a function of averaging time. There has been no indication that any of the preparations were contaminated with [^{13}C]-methylmethionine. Consequently, an accurate quantitative analysis could be performed for the incorporation of [^{13}C]-methyl moieties into the *E. coli* tRNA molecules. Carbon-13

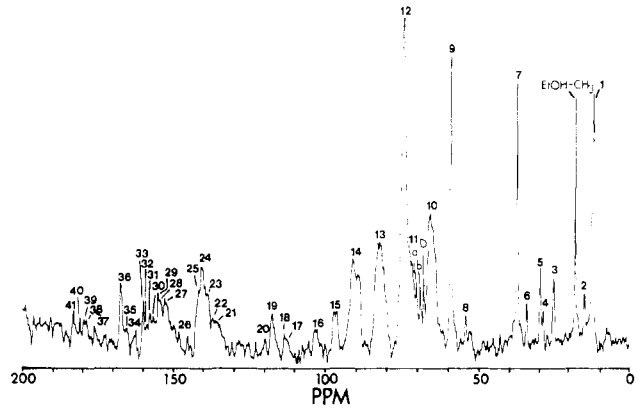

FIGURE 1: Proton-decoupled NMR spectrum of [^{13}C]methyl-enriched, unfractionated *E. coli* tRNA (1.52 mM in H_2O, 10% D_2O) at 46 °C. The spectrum was recorded at 25.2 MHz, with 8192 scans accumulated in each of four blocks. The horizontal scale is parts per million downfield from external Me_4Si. Resolved peaks are numbered. Signal assignments for methyl carbons in modified nucleosides (signals 1–9) are made in Table I, and those for natural abundance ribose and base ring carbons are given in Table II. Dioxane (peak D) and ethanol were utilized as internal standards for chemical shift measurements. The resonance of the 1-carbon of ethanol obscured the methyl resonances of Cm, Gm, and Um (signal 9). The methyl-carbon resonance of ethanol is denoted in the figure (EtOH-CH_3).

content of methyl groups was 56.7 atom % as determined by LC separation of bases from acid-hydrolyzed normal and ^{13}C-enriched tRNA, followed by mass spectroscopy of the bases (J. G. Tompson, K. C. Kuo, C. W. Gehrke, and P. F. Agris, unpublished experiments).

The first step in assigning the higher field shifted methyl-carbon resonances from tRNA was to classify their resonance positions into three categories: the carbons bonded directly to ring carbon or to sulfur (the highest upfield-shifted resonance groups, 10–20 ppm), the carbons bonded to nitrogen (20–40 ppm), and the carbons bonded to oxygen (the lower shifted resonance groups, 40–60 ppm). Knowledge of methylated nucleoside structures (Nishimura, 1972; Agris & Söll, 1977) and their content within *E. coli* tRNA (Hall, 1971), together with sequence data (Barrell & Clark, 1974; Sprinzl et al., 1978) and natural abundance spectral information of nucleosides and tRNA (Komoroski & Allerhand, 1972, 1974; Chang & Lee, 1976), provided the basis for unambiguous assignments of the [^{13}C]methyl-carbon resonances (Table I). Natural abundance spectra of commercially available methylated mononucleosides (or -nucleotides) found in *E. coli* tRNA were acquired at the same temperature as that of the tRNA. Chemical shifts for the methyl carbons of those nucleosides we investigated are listed in Table I along with literature values, the tRNA species in which each particular modified nucleoside is found, and its location in the cloverleaf secondary structure.

Signal 1 (Figure 1 and Table I) was assigned to the methyl carbon of ribothymidine (riboT) because the chemical shift of this methyl carbon is in close agreement with both that of the monomeric nucleoside and its previous assignment in the natural abundance spectra of tRNA. Furthermore, it is one of the most intense signals in the spectrum, which is in accordance with its presence in all tRNAs. A similar line of reasoning was used to assign resonances 3, 4, 5, and 7 to the methyl carbons of 2-methyladenosine (m^2A), N^6-methyladenosine (m^6A), 1-methylguanosine (m^1G), and 7-methylguanosine (m^7G), respectively. The signals 1, 7, and 4 have intensities in the approximate ratio of 8:5:1, showing close agreement with that of studies using radiochemical labeling in vivo and subsequent quantitation of the methylated nu-

Table I: Signal Assignments for Methylated Nucleosides of *E. coli* tRNA

signal[a]	chemical shift in tRNA[b]	chemical shift of nucleoside standard[b]	referenced chemical shift of standard[c]	assignment	rel amount in tRNA[d]	location in tRNA[e]	tRNA species[e]
1	11.9	11.0	12.0	m^5U (riboT)	1.00	TΨCG loop	all
2	14.7		14.3	ms^2i^6A	0.13	anticodon loop	Phe; Ser_1; Trp; Tyr
3	24.9	21.7		m^2A	0.25	anticodon loop	$Arg_{1,2}$; Asp_1; $Gln_{1,2}$; $Glu_{1,2}$; His
4	28.3	28.2	27.2, 27.6	m^6A	0.04	anticodon loop	Val_1
5	29.3	29.4	28.6	m^1G	0.14	anticodon loop	($Leu_{1,2}$)
6	33.7		33–36	mam^5s^2U	0.11	first base anticodon	$Glu_{1,2}$; Lys
7	36.8	36.3	35.8, 36.3	m^7G	0.50	extra loop	Ala_{1a}; $Arg_{1,2}$; Asn; Asp_1; Gly_3; His; Ile_1; Lys; fMet; Met; Phe; Thr; Trp; $Val_{1,2ab}$
8	53.6		51.0–51.3	mo^5U	0.10	first base anticodon	Ala_{1a}; $Ser_{1,2}$; Val_1
9	58.6	62.3	55.2, 57.9–60.4	Gm	0.10	h_2U loop	$Gln_{1,2}$; $Leu_{1,2}$; Met; Ser_1; Tyr
				Cm	0.06	anticodon loop	fMet; Ser_1; Tyr
			56.0	Um	0.06	anticodon loop	$Gln_{1,2}$

[a] Signal numbers refer to those of Figure 1. [b] Chemical shifts are in parts per million relative to Me_4Si. [c] Referenced chemical shifts were obtained from the following sources: for signal 1, Dorman & Roberts (1970) and Sugiyama et al. (1974); signal 2, Uesugi & Ikehara (1977); signal 4, Chang & Lee (1976), Komoroski & Allerhand (1974), and Thorpe et al. (1974); signal 5, Chang & Lee (1976) and Komoroski & Allerhand (1974); signal 6, Sarneski et al. (1975); signal 7, Chang & Lee (1976) and Komoroski & Allerhand (1974); signal 8, Stothers (1972); and signal 9, Komoroski & Allerhand (1974) and Stothers (1972). [d] Relative amount in tRNA from Agris et al. (1975b), Hall (1971), Munns et al. (1974), and Sprinzl et al. (1978). [e] Location in specific tRNA species from sequence data collected by Barrell & Clark (1974) and Sprinzl et al. (1978).

cleosides of *E. coli* tRNA (Munns et al., 1974; Agris et al., 1975b).

Signal 2 has been assigned to the methyl-carbon resonance of the methylthio moiety of 2-(methylthio)-N^6-(2-isopentenyl)adenosine (ms^2i^6A). The biosynthesis of this nucleoside in tRNA has been shown to involve the stepwise thiolation of the 2 position of i^6A, followed by the methionine-derived methylation of the thio group of s^2i^6A (Agris et al., 1975a). Resonance 2 was found to be of a size consistent with the knowledge that ms^2i^6A occurs in approximately 13% of all *E. coli* tRNA species (Table I; Sprinzl et al., 1978). In addition, the methyl carbon of 2-(methylthio)inosine has been found to give a resonance signal at 14.3 ppm (Uesugi & Ikehara, 1977), quite near to that of signal 2 from the tRNA (14.7 ppm).

5-[(Methylamino)methyl]-2-thiouridine (mam^5s^2U) occurs at the first position of the anticodon of approximately 10% of all sequenced tRNA species (Table I; Sprinzl et al., 1978). Resonance 6 at 33.7 ppm has been assigned to this methyl carbon through consideration of literature values for chemical shifts of similar methyl groups in the range of 33–36 ppm (Sarneski et al., 1975) and consideration of a signal intensity consistent with the occurrence of the nucleoside in tRNA (Table I). This methyl group has also been shown to be derived from methionine (Taya & Nishimura, 1973).

Signal 8 occurs at 53.6 ppm in the area of the most downfield resonances of methyl carbons. Those methyl groups possessing chemical shifts in this region are usually oxygen-bonded, methoxy carbons. We have therefore tentatively assigned signal 8 to the methyl carbon of the methyl ester of uridine-5-oxyacetic acid (mo^5U). The unesterified nucleoside has between shown to occur in approximately 10% of the published *E. coli* tRNA sequences (Table I). However, it is highly likely that esterification occurs in vivo with methionine as the methyl donor (Kwong & Lane, 1970) and can be lost during isolation of the tRNA as has been shown for a similar nucleoside in yeast tRNA (Kuntzel et al., 1975; Bronskill et al., 1972). Chemical shifts for similar methyl esters occur in the range of 51.0–51.3 ppm (Stothers, 1972). Carbon resonances of the oxygen-bonded methyl groups at the 2′ position of ribose (Gm, Um, and Cm) are responsible for signals near 59 ppm (Komoroski & Allerhand, 1974; Fujiwara et al., 1978). In Figure 1 these resonances are obscured by that of the 1-carbon (methylene) of ethanol (used as a standard in the "melting" experiments discussed later) but have been shown previously to be [^{13}C]methyl-enriched and to occur at this spectral location (Fujiwara et al., 1978).

Assignment of Natural Abundance Carbon-13 Signals from tRNA. We have recorded and assigned the natural abundance ^{13}C NMR spectral signals of ribose and base ring carbons of unfractionated *E. coli* tRNA (Figure 1 and Table II). Numerous resonances of the tRNA molecule can be assigned by comparison with the ^{13}C chemical shifts of mononucleosides. In producing Table II, we have taken advantage of those assignments made by investigators of tRNA natural abundance spectroscopy (Komoroski & Allerhand, 1972, 1974; Chang & Lee, 1976), mononucleosides or nucleotides (Dorman & Roberts, 1970; Jones et al., 1970), and ^{13}C enrichment of tRNA base ring carbons (Hamill et al., 1976; J. G. Tompson, and P. F. Agris, unpublished experiments). Even though the observed tRNA molecules were not purified to amino acid specificity or to a single species, closely spaced resonances of individual ring carbons of major and minor bases have been assigned (Table II). The most readily assigned natural abundance signals are the relatively intense resonances provided by the ribose carbons, 5′ (signal 10, 65.2 ppm), 3′ and 2′ (signal 12, 74.0 ppm), 4′ (signal 13, 82.0 ppm), and 1′ (signal 14, 89.5 ppm), each occurring approximately 76 times per tRNA molecule. Base ring carbons assigned to signals listed in Table II occur to a lesser degree and most likely in more varied environments from consideration of the yeast $tRNA^{Phe}$ crystal structure (Kim et al., 1974).

Since many of the signals denoted in Table II have been previously assigned and since this report pays particular attention to the modified nucleosides of tRNA, we will limit discussion to those ribose and base ring carbons, the resonances of which are affected by nucleoside modifications. The 2′-O-methylation of nucleosides induces an upfield shift of the 3′-carbon resonances and a downfield shift of the 2′-carbon signal relative to that of the parent ribose (Aldefer & Ts'o,

Table II: Chemical Shifts and Assignments of the Natural Abundance Carbon-13 Resonances of Unfractionated *E. coli* tRNA

signal[a]	chemical shift[b]	assignment[c]		chemical shift of nucleoside or nucleotide carbon[d]
		carbon	moiety	
10	65.2	5′	ribose	ribose (5′) of nucleotides
11a,b	71.4–72.0	3′	ribose	ribose (3′) of residues with 2′-*O*-methyl groups of mononucleotides
12	74.0	2′, 3′	ribose	ribose (2′, 3′) of mononucleotides
13	82.0	4′	ribose	ribose (4′) of mononucleotides
14	89.5	1′	ribose	ribose (1′) of mononucleotides
15	96.8	5	cytosine	CMP (5) 97.4
16	102.7	5	uracil	UMP (5) 103.5; poly(U) (5) 103.9
17	111.7	5	riboT	TMP (5) 112.3
18	113.0	5	s^4U	s^4U (5) 113.7
19	117.0	5	guanine	GMP (5) 116.6; m^1G (5) 116.3
20	119.1	5	adenine	AMP (5) 118.9; poly(A) (5) 119.2
21	135.3	8	m^7G	m^7G (8) 135.4
22	136.4	6	riboT; s^4U	TMP (6) 137.3; s^4U (6) 136.9
23	138.1	8	guanine	GMP (8) 138.2; m^1G (8) 137.9
24	140.1	8	adenine	AMP (8) 140.8; poly(A) (8) 140.4
25	141.5	6	cytosine; uracil	CMP (6) 142.7; UMP (6) 142.2; Ψ (6) 141.6; poly(U) (6) 142.5
26	144.6–147.5	4	m^2A; m^1G	m^1G (4) 147.0; m^2A (4) 147.5
27	151.5	4	adenine	AMP (4) 149.3; poly(A) (4) 149.1
		4	guanine	GMP (4) 151.9
		2	uracil	UMP (2) 152.7; poly(U) (2) 152.8
28	153.5	2	adenine	AMP (2) 153.3; poly(A) (2) 153.6
29	154.2	2	guanine	GMP (2) 154.5; m^1G (2) 154.6
		2	h_2U	h_2U (2) 154.5
30	155.8	6	adenine	AMP (6) 155.9; poly(A) (6) 156.0
31	156.8	2	cytosine	CMP (2) 157.0
32	157.8	6	m^1G	m^1G (6) 158.3
33	159.1	6	guanine	GMP (6) 159.2
34	161.3	6, 2	m^7G	m^7G (6) 162.0; m^7G (2) 161.1
35	164.0	4	uracil	UMP (4) 164.7; TMP (4) 164.9; Ψ (4) 165.3; poly(U) (4) 164.9
36	166.5	4	cytosine	CMP (4) 166.7
37	174.9	4	h_2U	h_2U (4) 173.8
38–41	177.4–181.7	*e*		

[a] Signal numbers refer to those of Figure 1. [b] Chemical shifts are in parts per million downfield from Me_4Si. [c] Assignments were made for those signals numbered 10–16, 18–20, 22, 24, 26, 27, 29–31, and 33–37 in Figure 1. Signals numbered 17, 21, 23, 25, 28, and 32 only designate the presumed locations of resonances within the spectrum of Figure 1 for the corresponding ring carbons listed in the table. [d] Chemical shifts of nucleotide, nucleoside, and polymer carbons were taken from literature values cited by Dorman & Roberts (1970), Hamill et al. (1976), Komoroski & Allerhand (1974), Jones et al. (1970), Mantsch & Smith (1972), and Miwa et al. (1977). [e] Presently not assigned.

1977). The two separated signals 11a and 11b between signals 10 and 12 for ribose 5′ and ribose 2′ and 3′ carbons (Figure 1) probably arise from different 3′ carbons rather than 2′ carbons of methylated ribose moieties.

Methylation of the C_5 position of uridine in the synthesis of riboT produces a change in the chemical shift for this carbon and C_6 (Jones et al., 1970). Signal 17 at 111.7 ppm and signal 22 at 136.4 ppm are assigned to C_5 and C_6 of riboT, respectively. The prevalent occurrence of m^7G in tRNA probably gives rise to the signals at 135.3 ppm (signal 21) and at 161.3 ppm (signal 34), which are considered to be due to the C_8 of m^7G and the combined resonances of C_2 and C_6 of m^7G, respectively (Table II; Chang & Lee, 1976). On the basis of the chemical shifts of modified nucleosides, signals 18, 26, and 32 of the tRNA spectrum (Figure 1) were assigned to the C_5 of s^4U, C_8 of m^1A, and C_6 of m^1G, respectively. Signals observed at the lowest field, signals 38–41 (177.4–181.7 ppm) do not correspond to any known carbon signals for the four major bases.

Temperature Transitions of [^{13}C]Methyl Signals of tRNA. The ^{13}C NMR spectra of [^{13}C]methyl-enriched tRNA at 8.9, 30.0, and 46.0 °C are shown in Figure 2. Narrowing of signal lines at the higher temperature is quite evident for some of the methyl carbons (signals 1–8) but most pronounced for riboT (signal 1) and m^7G (signal 7). Resonance lines for the ribose carbons (signals 10–14) also narrow between 8.9 and 46.0 °C. However, narrowing of the signals representative of the base ring carbons (signals above 90 ppm) is not as evident.

Changes in chemical shifts were determined with the aid of difference spectroscopy (Figure 2). The presence of dioxane and ethanol signals established reference points for examination of chemical shift changes. Furthermore, with difference spectroscopy the resonance fraction that remains unchanged in each residue of the tRNA is canceled by comparison of the spectra at two temperatures. The resulting spectrum, therefore, emphasizes differences in structural conformations yielded by the thermal perturbation of the tRNA molecule. Under experimental melting conditions, two separate thermal transitions were investigated, transition I between 8.9 and 30.0 °C and transition II between 30.0 and 46.0 °C. Transition I melting probably disrupts much of the tertiary structure, whereas transition II melting completes denaturation of tertiary structure and begins some secondary-structure unfolding (Kearns, 1976).

As shown in Table III, it is important to note that the resonances of ribose carbons 2′, 3′, and 4′ of tRNA shifted downfield, predominantly in the temperature range of 30–46 °C and not in the range of 8.9–30 °C. The significant change in their chemical shift values at higher temperature may be best interpreted as indicating the occurrence of environmental alterations in the phosphate-ribose backbone after the breakage

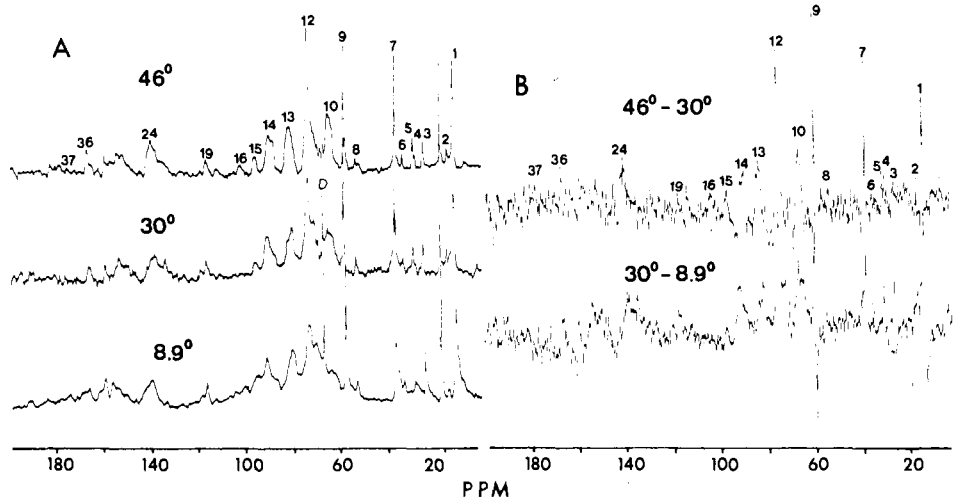

FIGURE 2: (A) Temperature dependence of the proton-decoupled ^{13}C-enriched NMR spectra of unfractionated *E. coli* tRNA (1.52 mM in H_2O, 10% D_2O). Spectra were recorded at 25.2 MHz with 8192 scans accumulated in each of four blocks. Chemical shifts are referenced to external Me_4Si with dioxane (D) and ethanol used as internal standards. Signal assignments are made in Tables I and II. (B) ^{13}C NMR difference spectra of [^{13}C]methyl-enriched tRNA. The two spectra were derived from the three spectra shown in Figure 2A.

Table III: Temperature-Dependent Carbon-13 Chemical Shift Changes of the Ribose Carbons of Unfractionated *E. coli* tRNA[a]

carbon	8.9 °C	30 °C	46 °C
$C_{1'}$	91.5	90.8	90.4
$C_{2'}$	73.7	73.7	74.0
$C_{3'}$	71.1	71.1	74.0
$C_{4'}$	80.6	80.6	81.7
$C_{5'}$	65.3	65.2	65.2

[a] Chemical shifts, given in parts per million downfield from Me_4Si, were measured relative to the chemical shift of the internal standard, dioxane (δ^D 67.4).

of tertiary interactions (Hayashi et al., 1977; Miwa et al., 1977). However, the upfield shifts of the ribose carbon 1′ signal and the insensitivity of the carbon 5′ resonance to increased temperatures are not understood at present but have been noted by other investigators (Miwa et al., 1977).

In contrast to the ribose carbon signals, chemical shifts of some of the modified nucleosides were changed by the first temperature transition (Figure 3). We can clearly separate the observed methyl resonances into four classes with respect to their chemical shift changes during thermal denaturation of the tRNA. Signals 1, 3, and 6 exhibited chemical shift changes only during transition I (8.9–30.0 °C), signals 4 and 7 exhibited changes only during transition II (30.0–46.0 °C), signal 5 had chemical shift changes during both transitions, and signals 2 and 8 had no perceptible chemical shift changes. Because the observed methyl signals are assigned with a high degree of certainty and correspond to individual methylated residues, we may extend our discussion of their chemical shift changes to actual site-specific perturbations in tRNA structure.

Signal 1 is assigned to riboT which is located in the TΨCG loop of every *E. coli* tRNA species. This resonance exhibits the largest change in chemical shift, 15 Hz (or 0.6 ppm), of all the observed methyl resonances (Figure 3). The riboT chemical shift change occurs during the initial thermal transition only, indicating disruption of tertiary structures in which riboT residues are normally restricted. Narrowing of the riboT methyl carbon and its chemical shift change correspond with that found for the methyl protons of riboT in yeast $tRNA^{Phe}$ (Kan et al., 1977) and *E. coli* $tRNA_1^{Val}$ (Kastrup & Schmidt, 1975). The TΨCG loop is covered by the h_2U loop as shown in the yeast $tRNA^{Phe}$ crystal structure

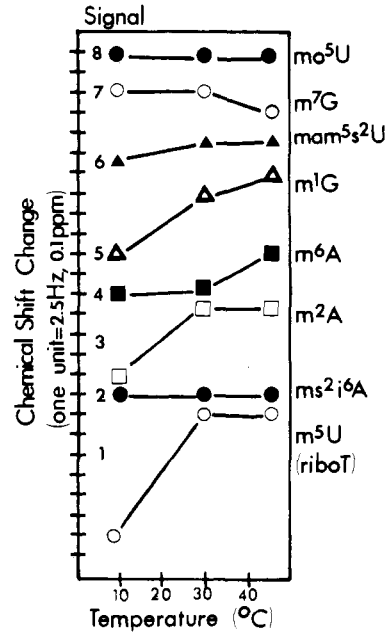

FIGURE 3: Changes in chemical shifts of methyl resonances as a function of temperature. Each unit along the vertical axis represents a shift of 2.5 Hz (0.1 ppm). All resonances experienced downfield shifts except for m^7G (upfield shift) and mo^5U and ms^2i^6A (no change). These changes were derived from analysis of the spectra taken at three temperatures as well as the difference spectra shown in Figure 2. Signal numbers at the left-hand side of the figure correspond to those in Figures 1 and 2.

(Ladner et al., 1975) and in most if not all tRNA species is inaccessible to chemical modification (Shulman & Pelka, 1976), ribonuclease activity (Penswick & Holley, 1965), isotope labeling (Gamble et al., 1976), and oligonucleotide binding (Uhlenbeck, 1972). Additional information about the interaction of the TΨCG loop and the h_2U loop could possibly be obtained by analysis of a resonance specific to a residue in the h_2U loop. The resonance signal 37 was assigned to C_4 of h_2U. Signal 37 also exhibited a downfield shift (12 Hz) between 8.9 and 30 °C. The changes in chemical shifts and therefore environments for carbons of both h_2U and riboT are in close agreement with the observations made with optical and proton NMR methods (Rich & RajBhandary, 1976) and could imply a disengagement of the two loop regions in which

the nucleosides reside. TΨCG becomes available for binding to the complementary oligonucleotide (CGAA) in 5S rRNA when the tRNA interacts with the elongation factor, mRNA, and the ribosome during protein synthesis (Erdmann et al., 1973; Schwarz et al., 1974; Sprinzl et al., 1976). We believe that the low-temperature structural transition exhibited through changes in the riboT resonance is analogous to the natural dynamics of the tRNA molecule while it functions in protein synthesis.

Signals 3 and 5 are assigned to the methyl carbons of m^2A and m^1G, respectively. These modified nucleosides are consistently located adjacent to the third nucleoside (3′ end) of the anticodon and together are found in one-third of the *E. coli* tRNA species (Table I). The methyl resonance of m^2A exhibited a downfield shift of 8 Hz during the 8.9–30 °C transition and no change between 30–46 °C, whereas that of the m^1G shifted downfield 10 Hz during the first thermal transition and an additional 2 Hz during the second (Figure 3). Anticodon bases are believed to be in a stacked conformation (Quigley & Rich, 1976). The modified base adjacent to the 3′ end of the anticodon participates in this base stacking with the character of the modification demonstrably important to the strength of the interaction of anticodon with its complementary oligonucleotide (Vögeli et al., 1977). Therefore, we believe the downfield shifts exhibited by the methyl carbons of m^2A and m^1G are indicative of the disruption of the tRNA anticodon base stacking that includes these adjacent modified nucleosides.

Two other signals are attributed to modified nucleosides adjacent to the 3′ end of anticodons. Resonances 2 and 4 are assigned to the methyl groups of ms^2i^6A and m^6A, respectively. These nucleosides, consistently located adjacent to anticodons, are collectively found in some 15% of *E. coli* tRNA species (Table I). In contrast to chemical shifts of signals for m^2A and m^1G, the chemical shift of ms^2i^6A did not change during thermal transition and that of m^6A shifted downfield 5 Hz only during the second transition, 30–46 °C (Figure 3). The methyl of ms^2i^6A may have lacked responsiveness to the presumed thermal disturbance of anticodon stacking due to its bond to sulfur and further distance from the aromatic ring than that of m^2A and m^1G. The methyl of m^6A is also distant from the aromatic ring but may have responded because it is an amino methyl. Its resonance shifted only at the higher temperature change because of the nature of the base stacking in those tRNA species carrying the modification. Temperature-dependent changes in the proton chemical shift of m^6A methyl protons of *E. coli* $tRNA_1^{Val}$ have also been described (Kastrup & Schmidt, 1975).

Resonances 6 and 8 are assigned to the methyl carbons of mam^5s^2U [5-[(methylamino)methyl]-2-thiouridine] and mo^5U (methyl ester of 5-oxyacetic acid of uridine), respectively. Each of these modified nucleosides is found at the first position of anticodons occurring in some 10% of all tRNA species (Table I). There was a slight chemical change (2.5 Hz) observed for the methyl carbon of mam^5s^2U between 8.9 and 30 °C and no change at all for that of mo^5U (Figure 3). Inability to detect a change in chemical shift comparable to that of m^2A or m^1G may be due to incomplete base stacking at the 5′ end of the anticodon (the "Wobble" base position) and the larger distance of the observed methyl from the aromatic ring.

Signal 7, assigned to m^7G, had the rather notable behavior of a small upfield shift of 3.5 Hz between 30 and 46 °C. This thermal-induced behavior of the methyl carbon of m^7G is analogous to the slight shielding at higher temperatures of the corresponding methyl protons observed by proton NMR spectroscopy of yeast $tRNA^{Phe}$ (Kan & Ts'o, 1974; Kan et al., 1977). Half of all *E. coli* tRNA species contain m^7G, and the nucleoside is consistently located in the "extra" or variable loop. If the m^7G residues in *E. coli* tRNA species are presumed to be hydrogen-bonded to the 22nd nucleosides within the tertiary structures as an extension of crystal structure information from yeast $tRNA^{Phe}$ (Quigley & Rich, 1976), then the m^7G would possibly remain in a stacked-based conformation of the variable loop. Additional interaction would be electrostatic in nature and involve the charged quarternary nitrogen with the closely neighboring phosphate of the ninth nucleoside (Quigley & Rich, 1976).

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