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Nuclear Magnetic Resonance Studies on the Tertiary Folding of Transfer Ribonucleic Acid: Assignment of the 7-Methylguanosine Resonance†

Ralph E. Hurd and Brian R. Reid*

ABSTRACT: Analysis of the low-field nuclear magnetic resonance (NMR) spectra of several class 1 D4V5 transfer ribonucleic acid (tRNA) species containing 7-methylguanosine in their variable loops reveals a set of six to seven tertiary base pair resonances, one of which is always located at ca. −13.4 ppm. Other tRNA species which do not contain 7-methylguanosine do not contain the tertiary resonance at −13.4 ppm. Chemical removal of 7-methylguanosine from several tRNAs containing the same dihydrouridine (DHU) helix sequence as yeast tRNA^{Phe} results in the loss of the −13.4-ppm tertiary resonance. In the initiator methionine tRNA, which contains a different DHU helix sequence, the 7-methylguanosine hydrogen bond has been assigned at −14.55 ppm by chemical removal of this residue. In these experiments the aromatic C8H proton of 7-methylguanosine was also assigned (−9.1 ppm). The unexpectedly low-field position of the 7-methylguanosine resonance is explained by the deshielding effect of the delocalized positive charge in this nucleoside.

In a previous paper in this series (Reid et al., 1979) we have identified, by several methods, six or seven resonances in the low-field NMR[1] spectra of several class 1 tRNAs which are not derived from standard secondary base pairs. All of these transfer RNA species contain m^{7}G as the central nucleotide of a five-residue extra loop (Barrell & Clark, 1974). From the three-dimensional crystal structure of yeast tRNA^{Phe} (Kim et al., 1974a; Robertus et al., 1974; Sussman & Kim, 1976a,b; Quigley & Rich, 1976) it is evident that the ring nitrogen proton of m^{7}G46 is hydrogen bonded to N7 of guanosine-22 in the major groove of the DHU stem. The m^{7}G46–G22–C13 base triple (Figure 1) stabilizes the interaction of the DHU stem and the extra loop (G22–C13 is the terminal Watson–Crick pair in the DHU stem).

In this paper we establish that the crystallographically observed m^{7}G46–G22 tertiary interaction exists in solution for a series of class 1 (D4V5) tRNAs. Furthermore, we assign a specific resonance in the low-field (−15 to −11 ppm) proton NMR spectrum of each of these tRNAs to the hydrogen-bonded ring nitrogen proton of m^{7}G. Comparative studies on isoaccepting tRNA species which do not contain m^{7}G led to an initial tentative assignment of the m^{7}G hydrogen-bond resonance in those which do. This assignment was then confirmed by excision of m^{7}G from yeast tRNA^{Phe}, *Escherichia coli* tRNA$_1^{Val}$, *E. coli* tRNA$_m^{Met}$, and *E. coli* tRNA^{Lys}. These tRNA species have an identical DHU stem and hence should have a nearly identical environment surrounding the m^{7}G ring nitrogen proton. Further verification of our assignment was accomplished by removal of m^{7}G from *E. coli* tRNA$_f^{Met}$; the base pair sequence in the DHU stem of this tRNA differs from that of yeast tRNA^{Phe} and therefore changes the environment of the m^{7}G N1H hydrogen bond. The unexpectedly low-field position of the m^{7}G ring NH

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[1] Abbreviations used: NMR, nuclear magnetic resonance; tRNA, transfer ribonucleic acid; DHU, dihydrouridine; m^{7}G, N^7-methylguanosine; Ψ, pseudouridine.

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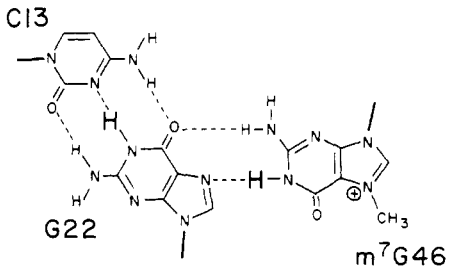

FIGURE 1: Geometry of the m^7G46–G22–C13 triple interaction in the crystal structure of yeast $tRNA^{Phe}$ showing the bonding of m^7G46 N1H to N7 of G22 [taken from Kim et al. (1974a)].

resonance is explained by NMR studies of monomeric m^7G in aprotic solvents.

Materials and Methods

Transfer RNA. *E. coli* $tRNA_m^{Met}$, *E. coli* $tRNA_1^{Val}$, and yeast $tRNA^{Phe}$ were purified from unfractionated tRNA as described previously [see Reid et al. (1979)]. *E. coli* $tRNA_1^{Gly}$ (1640 pmol/A_{260}), *E. coli* $tRNA_3^{Gly}$ (1700 pmol/A_{260}), and *E. coli* $tRNA^{Lys}$ (1500 pmol/A_{260}) were also purified to homogeneity by using benzoylated DEAE-cellulose (BD-cellulose) chromatography (Gillam et al., 1967), DEAE-Sephadex chromatography (Nishimura, 1971), and Sepharose 4B chromatography (Holmes et al., 1975). *E. coli* $tRNA_f^{Met}$, the first peak of methionine activity from BD-cellulose (Gillam et al., 1967), was chromatographed on DEAE-Sephadex as described by Nishimura (1971). A symmetrical peak of pure (1880 pmol/A_{260}) *E. coli* $tRNA_f^{Met}$ was obtained. Identification of these tRNA species was accomplished by RNase T1 fingerprints; the Barrell (1971) two-dimensional electrophoresis and/or the two-dimensional TLC system described by Schoemaker & Schimmel (1974) were used.

NMR Spectra. Six milligram samples of tRNA were dissolved in 10 mM sodium cacodylate, pH 7.0, containing 15 mM $MgCl_2$ and 100 mM NaCl to give a final volume of 0.18–0.19 mL. The sample was transferred to a 5 × 9 mm NMR microtube (Wilmad Glass Co.). Spectra were accumulated by correlation spectroscopy (Dadok & Sprecher, 1974) at 2500 Hz/s for 10 min on a Bruker HXS-360 spectrometer at the Stanford Magnetic Resonance Laboratory. The phased spectra were printed directly onto 8.5 × 11 in. sheets of plain white paper. Chemical shifts are reported as parts per million from the reference DSS (2,2-dimethylsilapentane-5-sulfonate); they were experimentally determined with respect to the H_2O peak to which was added the known chemical shift of water with respect to DSS at that temperature.

m^7G Removal. The chemical removal of m^7G from all tRNA species was carried out according to Simsek et al. (1973) with the exception that aniline was omitted from the reaction to prevent chain cleavage.

Nucleoside Spectra. Guanosine and 7-methylguanosine samples were prepared in the following manner. A 0.05-mmol amount of each was weighed out as a dry powder. Two milliliters of double-distilled H_2O was added to each sample and the pH of the resulting solution or suspension adjusted to 4.0. These samples were then lyophilized and resuspended in 0.5 mL of dry Me_2SO-d_6. Monomer spectra were also obtained on the Bruker HXS-360 spectrometer at the Stanford Magnetic Resonance Laboratory. The average free induction decay (FID) collected from four quadrature mode pulses of 13.5-ms duration each, and separated by 20 s (over 5 times the longest T_1 observed), was Fourier transformed, phased, and printed directly on white paper for the figures presented. Chemical shifts for these monomer spectra are reported as parts per million from Me_4Si.

Results

Our initial conclusion concerning the spectral position of the m^7G46 ring NH proton was derived from comparative NMR studies on the low-field spectra of isoaccepting $tRNA^{Gly}$ species. The five definite ring NH tertiary interactions seen in the crystal structure of yeast $tRNA^{Phe}$ (8–14, 46–22, 19–56, 54–58, and 15–48) and a possible sixth ring NH interaction involving Ψ55 are occupied by identical nucleotides in the sequence of *E. coli* $tRNA_3^{Gly}$ (see Figure 2). Like yeast $tRNA^{Phe}$ (and *E. coli* $tRNA_1^{Val}$, $tRNA_m^{Met}$, and $tRNA^{Lys}$), *E. coli* $tRNA_3^{Gly}$ contains a similarly positioned set of extra, noncloverleaf resonances in its NMR spectrum at −14.8 (−14.3 with U8 instead of s^4U8), −14.2, −13.4, −12.9, ca. −12.5, −11.8, −11.5, and −11.4 ppm (see spectra in the first paper of this series). On the basis of previous work, the two very low-field tertiary resonances between −14 and −15 ppm can be assigned to the two tertiary AU-reversed Hoogsteen pairs, namely, s^4U8–A14 and T54–A58 [see Hurd & Reid (1979)]. Unlike $tRNA_3^{Gly}$, which is a standard class 1 D4V5 tRNA, *E. coli* $tRNA_1^{Gly}$ is a D4V4 tRNA, which does not contain m^7G in its variable loop (Hill et al., 1973). The spectrum of pure *E. coli* $tRNA_1^{Gly}$ is shown in Figure 3; it is a quite well-resolved spectrum containing 25 to 26 protons between −11 and −15 ppm. The secondary Watson–Crick base pairs are satisfactorily assigned on the basis of neighboring ring current shifts as shown in Figure 3, leaving five additional base pair resonances indicated by asterisks. The extra resonances at −14.9 and −14.2 ppm are precisely where we expected the s^4U–A and TA tertiary resonances, leaving additional resonances at −12.9, −11.7, and −11.5 ppm. Thus, the tertiary resonances observed at −13.4 and at ca. −12.5 ppm in $tRNA_3^{Gly}$, $tRNA_1^{Val}$, $tRNA_m^{Met}$, $tRNA^{Lys}$, and yeast $tRNA^{Phe}$ are absent in the spectrum of $tRNA_1^{Gly}$. The tertiary resonance observed at ca. −12.5 ± 0.25 ppm in D4V5 tRNAs has been assigned to the reverse Watson–Crick tertiary base pair G15–C48 on the basis of the effects of paramagnetic ions bound close to this site [see Hurd et al. (1979)]. *E. coli* $tRNA_1^{Gly}$ contains A15, and the last residue of the variable loop (analogous to position 48) is occupied by C; since neither A nor C contains a ring NH proton, the absence of the reverse Watson–Crick tertiary resonance (G15–C48) at ca. −12.5 ppm is to be expected in $tRNA_1^{Gly}$. This then leaves the tertiary resonance at −13.4 ppm, present in $tRNA_3^{Gly}$ but absent in $tRNA_1^{Gly}$, as the most likely candidate for the m^7G46–G22 resonance. Further support for this hypothesis came from an examination of the spectrum of *E. coli* $tRNA_2^{Gly}$ (not shown) which is also devoid of m^7G (Roberts & Carbon, 1974). The foregoing deductions depend upon the assumption that the isoaccepting *E. coli* $tRNA^{Gly}$ species have a three-dimensional structure similar to yeast $tRNA^{Phe}$. Analysis of coordinated base changes has suggested a general folding scheme involving the same tertiary base pairs for such related tRNAs (Klug et al., 1974; Kim et al., 1974b).

We next attempted to corroborate this hypothesis by chemical removal of m^7G46 from those species which do contain this residue. The 360-MHz low-field NMR spectrum of yeast $tRNA^{Phe}$ and the spectrum of yeast $tRNA^{Phe}$ with m^7G46 removed (−m^7G) are presented in Figure 4. The extent of removal of m^7G was estimated to be ~70% by hydrolysis and quantitative TLC analysis (Nishimura, 1972) of the mononucleotides. Furthermore, incubation of an aliquot of the sample with aniline according to Simsek et al. (1973) followed by fragment separation on hot Sephadex G-100

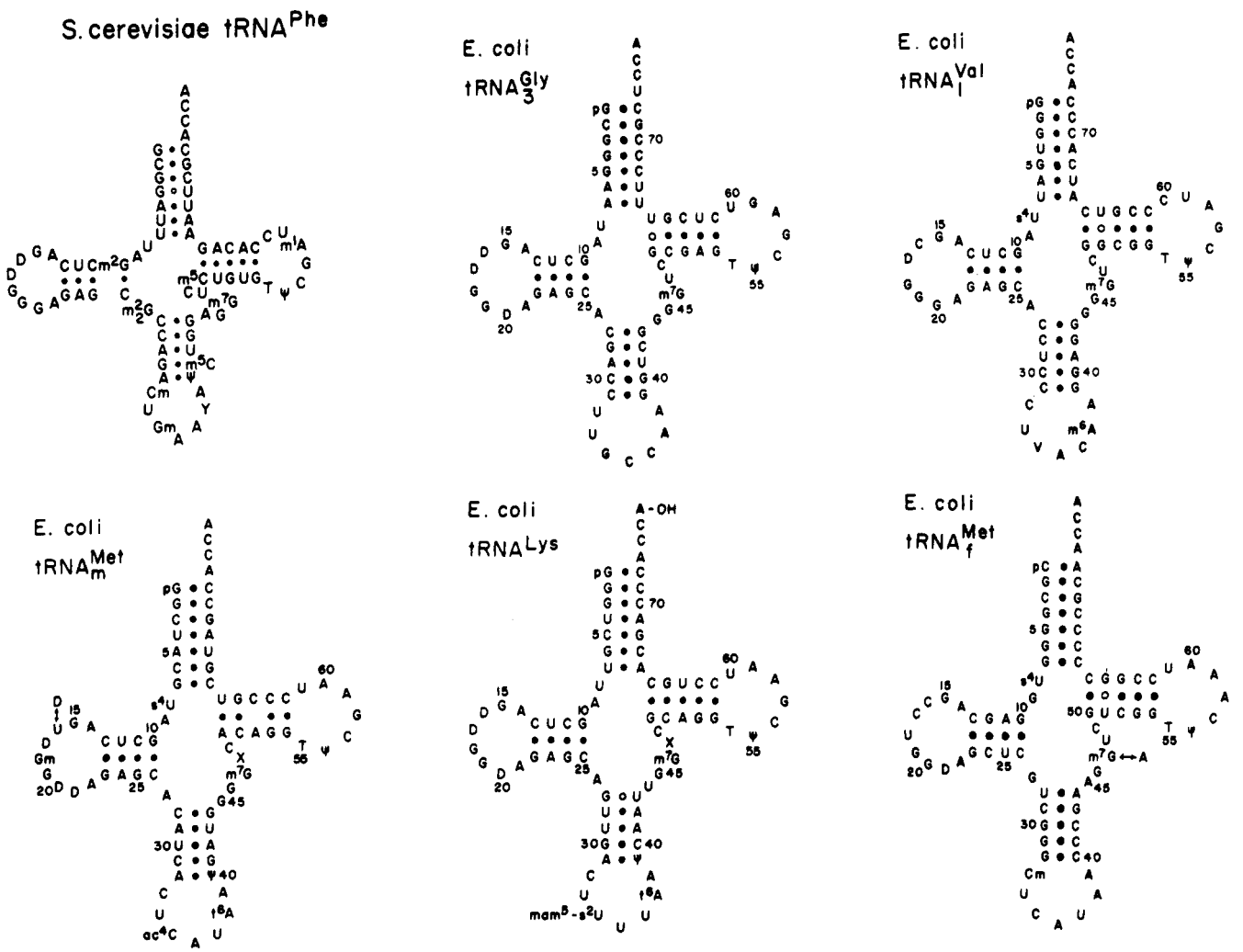

FIGURE 2: Cloverleaf sequences of the tRNA species used in this study [taken from Barrell & Clark (1974)].

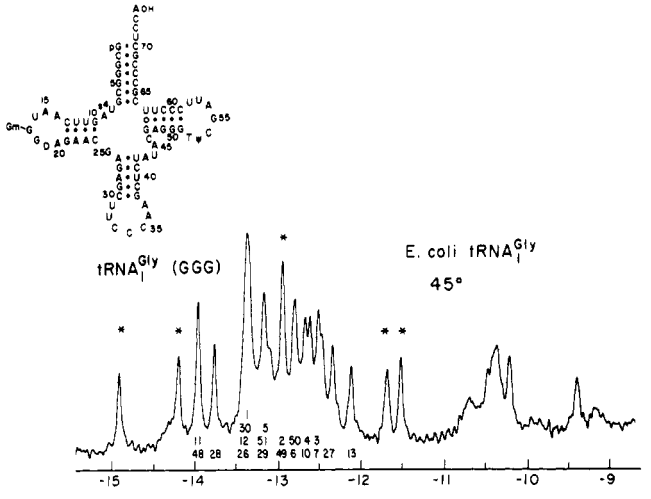

FIGURE 3: The 360-MHz low-field NMR spectrum of *E. coli* tRNA$_1^{Gly}$ in the presence of magnesium. Secondary base pair assignments from sequence-dependent ring currents shifts are designated by their respective base number; additional resonances are denoted by asterisks.

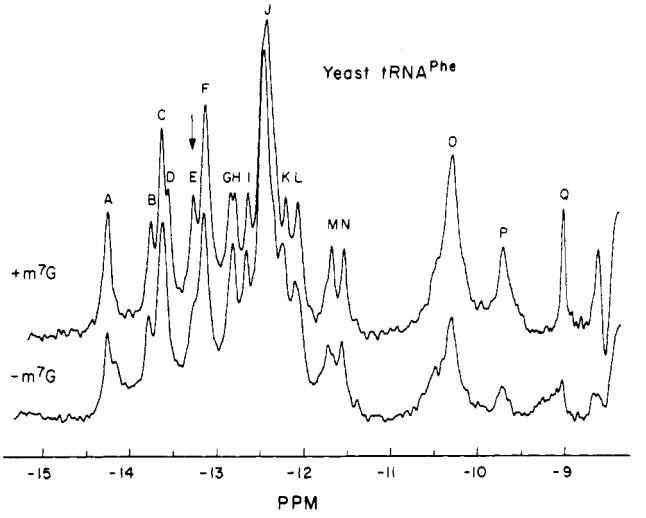

FIGURE 4: NMR spectra of yeast tRNA^{Phe} before and after removal of m^{7}G46 as described in the text. Both spectra were taken at 45 °C in 10 mM sodium cacodylate, 100 mM NaCl, and 15 mM $MgCl_2$, pH 7.0.

revealed 30% intact tRNA, and 70% of the material chromatographed as two resolved fragments of chain lengths 46 and 30. In the NMR spectrum, removal of m^{7}G46 results in the loss of a low-field resonance at −13.4 ppm (peak E, indicated by an arrow). A further interesting point is a quantitatively similar loss of intensity in peak Q at −9.1 ppm. This latter peak was found to be the only peak remaining in the low-field spectrum when the tRNA was dissolved in D_2O solvents, thus establishing that it is not a rapidly exchanging NH proton and suggesting that it may be a deshielded aromatic proton. The loss of this resonance upon m^{7}G removal has led us to assign peak Q at −9.1 ppm to the aromatic C8H proton of m^{7}G46. The reason for the anomalous downfield shift of this aromatic proton will be discussed later.

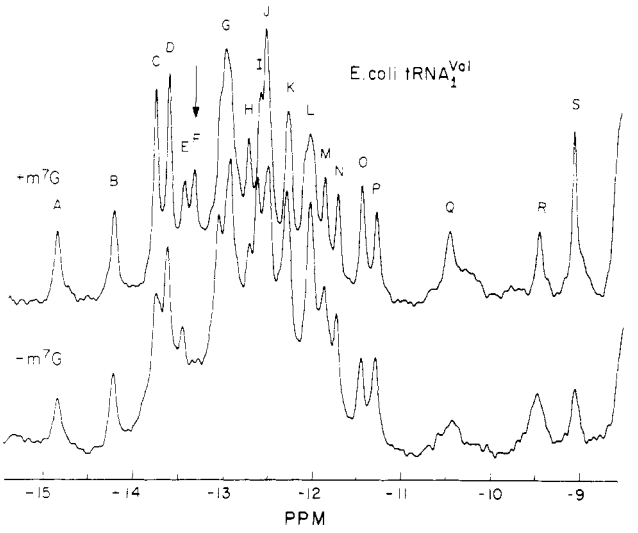

FIGURE 5: NMR spectra of *E. coli* tRNA^{Val} before and after m^{7}G46 removal: 45 °C in 10 mM sodium cacodylate, 100 mM NaCl, and 15 mM $MgCl_2$, pH 7.0.

In addition to the loss of the m^{7}G resonance, several small shifts and perturbations are also evident; e.g., peak D moves slightly downfield and one of the two protons in peak A broadens and moves upfield slightly. One or more of the constituent protons in peak J experience slight shifts toward the downfield side of this complex, approximately seven-proton peak. It is possible that some of these changes are due to the loss of the ring current effect of m^{7}G on neighboring base pairs. However, it is also eminently reasonable to expect that removal of one of the V loop–D helix interactions will lead to a general destabilization of tertiary structure compared to the control tRNA at a given temperature; this is especially evident in the case of the tertiary resonances between −9.5 and −10.5 ppm. In Figure 5 the low-field NMR spectra of *E. coli* tRNA$_1^{Val}$ with and without m^{7}G are presented. Loss of ~70% of the intensity of peak S at −9.1 ppm (the aromatic C8H) again reflects the loss of m^{7}G. In the extreme low-field spectrum the loss of peak F assigns the m^{7}G46–G22 hydrogen bond in tRNA^{Val} at −13.4 ppm and agrees well with our comparative assignment of this resonance in *E. coli* tRNA$_3^{Gly}$ and our chemical assignment in yeast tRNA^{Phe}. We expect to find the m^{7}G46–G22 resonance at a similar chemical shift since the bases occupying positions proximal to m^{7}G46 in the crystal structure of yeast tRNA^{Phe} are virtually identical in all of these tRNA species (see Figures 8 and 2). Additional effects resulting from removal of the m^{7}G in tRNA$_1^{Val}$ include broadening of peak A and an upfield shift of one of the two protons in peak C; also, there are shifts (but no intensity loss) in peaks G, I–J, and L and broadening of peak Q—these effects will be discussed later.

E. coli tRNA$_m^{Met}$ and *E. coli* tRNA^{Lys} are class 1 (D4V5) species which, like tRNA$_1^{Val}$, contain the same residues as yeast tRNA^{Phe} in the three-dimensional environment of the m^{7}G. The low-field NMR spectra of tRNA$_m^{Met}$ and tRNA^{Lys}, as well as the corresponding m^{7}G-deficient spectra, are shown in Figures 6 and 7, respectively. Loss of the −9.1-ppm resonance in the m^{7}G-deficient tRNA$_m^{Met}$ spectrum and in the m^{7}G-deficient tRNA^{Lys} spectrum corroborates our assignment of the m^{7}G C8H proton. Assignment of the hydrogen-bonded ring NH proton of the m^{7}G tertiary interaction in these two species is somewhat more ambiguous than in yeast tRNA^{Phe} and *E. coli* tRNA$_1^{Val}$. However, considering these tRNAs as a homologous series with yeast tRNA^{Phe}, the loss of intensity in peak F (−13.5 ppm) in tRNA$_m^{Met}$ (Figure 6) is the most

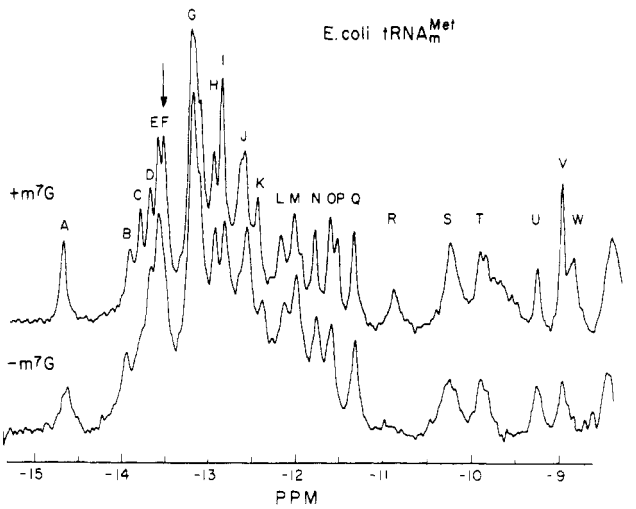

FIGURE 6: NMR spectra of *E. coli* tRNA$_m^{Met}$ before and after removal of m^{7}G. Conditions are as in Figure 4.

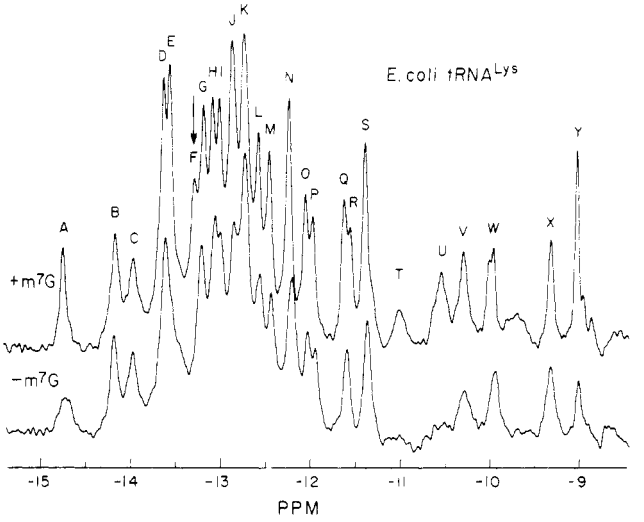

FIGURE 7: NMR spectra of *E. coli* tRNA^{Lys} before and after removal of m^{7}G. Conditions are as in Figure 4.

likely correlation with the removal of m^{7}G; peaks C and D appear to have merely coalesced into the downfield side of peak E. Similarly, the clear loss of a proton in the F–G region (−13.35 ppm) in tRNA^{Lys} (Figure 7) makes this the most reasonable assignment for the m^{7}G tertiary hydrogen bond. A common feature in all four of these homologous tRNA species is that m^{7}G removal causes broadening of peak A. In a parallel paper, we have assigned peak A to the reversed Hoogsteen tertiary base pair between residues 8 and 14 (−14.8 ± 0.1 ppm in bacterial species containing s^{4}U8 and −14.3 ppm in species containing U8). Additional perturbations resulting from m^{7}G removal are observed in peaks E, J, and Q in tRNA^{Lys} (Figure 7) and in the equivalent peaks C, I, and P in *E. coli* tRNA$_m^{Met}$ (Figure 6).

The environment surrounding the m^{7}G tertiary interaction in *E. coli* tRNA$_f^{Met}$ differs from that of the homologous series of tRNAs already presented. In the crystal structure of yeast tRNA^{Phe} the m^{7}G residue at position 46 is stacked with A9 and with the A of UA12 (Sussman & Kim, 1976a,b) (see Figure 8). In tRNA$_f^{Met}$ the analogous environment of m^{7}G would be G9 and the C of GC12 (see Figure 2). Based on the weaker ring current of guanine and cytosine compared to adenine (Geissner-Prettre & Pullman, 1970), the neighboring upfield shift on the m^{7}G ring NH proton in tRNA$_f^{Met}$ is expected to be much less than that in yeast tRNA^{Phe}, *E. coli*

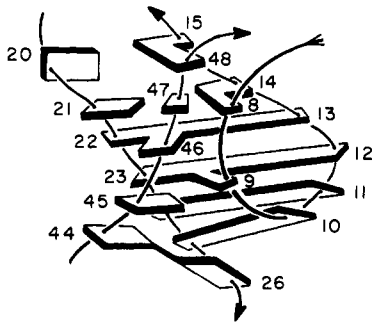

FIGURE 8: Diagrammatic representation of the base stacking environment surrounding m^{7}G46 in the crystal structure of yeast tRNA^{Phe} [redrawn from Sussman & Kim (1976a)].

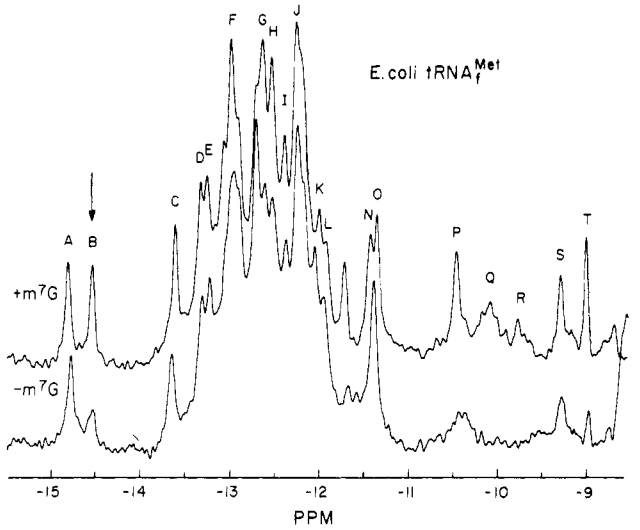

FIGURE 9: NMR spectra of *E. coli* tRNA^{fMet} before and after m^{7}G removal. Both spectra were taken at 45 °C in 10 mM sodium cacodylate, 100 mM NaCl, and 15 mM $MgCl_2$, pH 7.0.

tRNA^{Val}, etc. Therefore, we should find the m^{7}G ring NH proton of *E. coli* tRNA$_f^{Met}$ at lower field than the corresponding resonance in yeast tRNA^{Phe}. In Figure 9, the 360-MHz low-field NMR spectrum of *E. coli* tRNA$_f^{Met}$ with and without m^{7}G is shown. Peak B at −14.55 ppm is clearly assignable to the m^{7}G46 ring NH proton. This assignment is consistent with the differences in environment of the m^{7}G in *E. coli* tRNA$_f^{Met}$ compared to its environment in yeast tRNA^{Phe}. The loss of peak T (−9.0 ppm) on m^{7}G removal in this species assigns the aromatic C8H proton; hence, it appears that the ring current effects on the m^{7}G C8H proton out in the groove are similar in tRNA$_f^{Met}$ and in the other species of tRNA studied. Other perturbations in the tRNA$_f^{Met}$ spectrum include shifts, but no loss, in peaks G and H as well as peaks N and O which coalesce. Peak M at −11.7 ppm splits and loses intensity, and there is a general destabilization of the tertiary resonances in the −9.5- to −10.5-ppm region.

An interesting result of our assignment of the m^{7}G46–G22 interaction to a −13.4-ppm resonance in yeast tRNA^{Phe} is that, when combined with environmental upfield ring current shifts, one is forced to use a −15.1-ppm inherent unshifted position for the m^{7}G N1H proton in the m^{7}G46–G22–C13 triple interaction [using the environmental upfield ring current shifts estimated by Robillard et al. (1976)]. To investigate a possible basis for this abnormally low-field starting position, we compared the low-field NMR spectra of the monomers, 7-methylguanosine and guanosine, in dry Me_2SO-d_6. These spectra are shown in Figure 10.

Methylation of guanosine at N7 leads, via delocalization

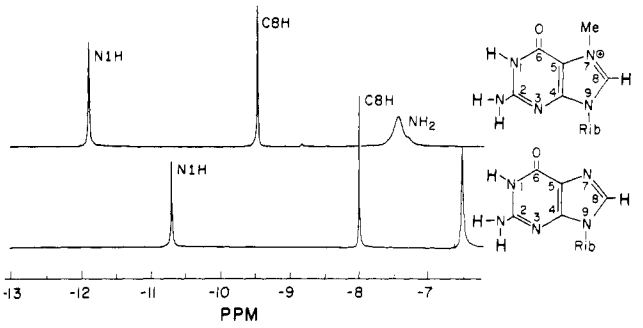

FIGURE 10: Low-field portion of the NMR spectra of 7-methylguanosine and guanosine in dry Me_2SO-d_6.

of the positive charge, to a large downfield shift of not only C8H, but also of the ring nitrogen (N1H) and the amino protons. N1H of 7-methylguanosine is over 1.2 ppm further downfield than its counterpart in guanosine. Thus, considering that the inherent, unshifted position of a guanosine ring nitrogen proton in a Watson–Crick GC base pair is ca. −13.4 ppm, the positive charge effect in the m^{7}G–GC interaction alone can account for 1.2 ppm of the 1.7-ppm discrepancy. The remaining 0.5 ppm probably comes from the in-plane downfield shift from the ring current of G22 acting on the m^{7}G ring NH which would not occur in a m^{7}G–C pair. These results strongly support our assignment of the m^{7}G46–G22 resonance. The C8H proton of m^{7}G is at −9.5 ppm in Me_2SO and appears to be minimally shifted in the tRNA molecules studied.

In the case of the *E. coli* tRNA$_1^{Val}$ spectrum, we have already assigned the tertiary resonances at −14.9, −14.3, and −12.25 ppm, which left the extra resonances at −13.4, −12.9, −11.95, and −11.35 ppm as potential candidates for the m^{7}G46 hydrogen bond. Although the effects between −11 and −13 ppm upon removal of m^{7}G from tRNA$_1^{Val}$ and yeast tRNA^{Phe} appear to be shifts rather than losses, we sought alternative methods of further narrowing down which of these remaining additional peaks might be due to m^{7}G46. Johnston & Redfield (1979) have developed an elegant method of identifying hydrogen-bonded GU pairs in tRNA spectra. Using preirradiation FT NMR methods, one can detect negative nuclear Overhauser effects (NOEs) between protons which must, because of the marked distance dependence of the NOE, be located very close together (~3 Å). The only situation in which two low-field (ring NH) protons can approach this distance is when they are in the same base pair, i.e., G N1H and U N3H in a GU pair. *E. coli* tRNA$_1^{Val}$ contains a GU pair at position 50, and so we used the Johnston–Redfield technique to try to find the GU resonances among the remaining unassigned extra resonances. The results are shown in Figure 11. When the resonance at −11.35 ppm is irradiated and the resulting spectrum subtracted from a control irradiated in the valley at −11.6 ppm, distinct NOE cross-relaxation to the peak at −11.95 ppm is observed; conversely, when the −11.95-ppm peak is preirradiated, the NOE at −11.35 ppm is observed. This clearly identifies peak M and peak P in Figure 5 as being the GU pair at position 50 and further limits the choice for the m^{7}G6 resonance to the extra resonances at −12.9 or −13.4 ppm. The monomer spectra, the presence of a tertiary resonance at −12.9 ppm in tRNA$_1^{Gly}$, and the chemical removal studies all indicate that the more deshielded resonance at −13.4 ppm is the m^{7}G46–G22 hydrogen bond.

Discussion

Our initial comparative studies on three isoaccepting tRNA^{Gly} species from *E. coli*, two of which contain no m^{7}G

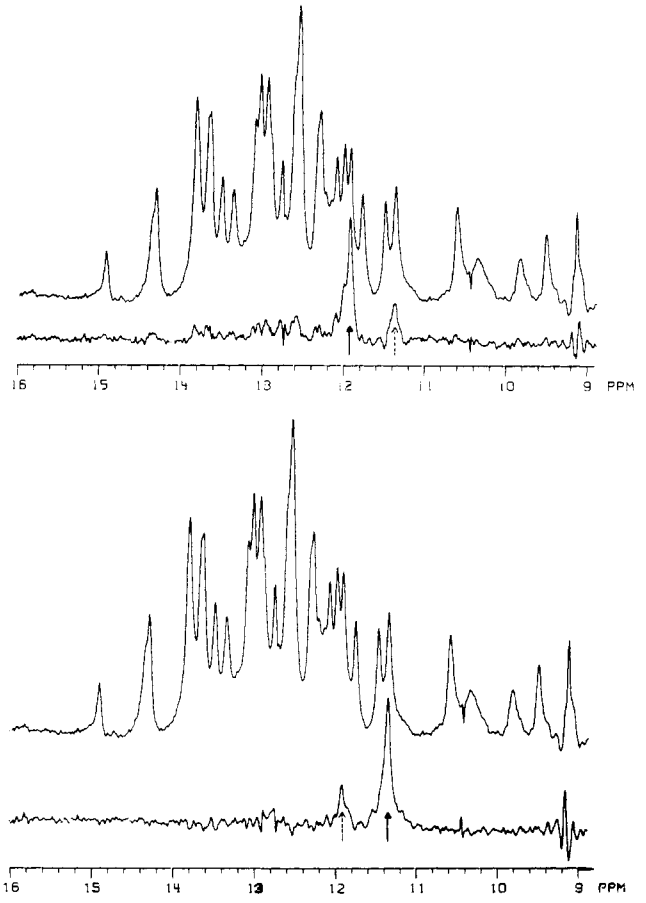

FIGURE 11: Identification of the GU base pair resonances in *E. coli* $tRNA^{Val}$ by means of the nuclear Overhauser effect. Preirradiation was carried out for 100 ms with an f2 frequency centered at −11.95 ppm, and the spectrum was acquired 1 ms later with a 0.37-ms Redfield 214 detection pulse. The resulting spectrum was subtracted from a control spectrum preirradiated at −11.6 ppm, and the difference spectrum is plotted below the control spectrum. In the lower spectrum the f2 preirradiation was centered at −11.35 ppm. None of the other peaks between −11 and −13 ppm exhibited any NOE. This experiment was carried out with a sample containing 50% s^4U8.

in the variable loop, suggested that the m^7G46–G22 interaction in $tRNA_3^{Gly}$ was responsible for the hydrogen-bonded ring NH resonance at ca. −13.4 ppm in the NMR spectrum of this tRNA. This was corroborated by chemical removal of m^7G in yeast $tRNA^{Phe}$, *E. coli* $tRNA_1^{Val}$, *E. coli* $tRNA_m^{Met}$, and *E. coli* $tRNA^{Lys}$; in all cases a resonance at −13.4 ± 0.1 ppm was lost from the spectrum. The observed chemical shift of a given proton in tRNA is largely determined by the net ring current shift contributed by the surrounding nucleotides in the immediate three-dimensional environment of that proton. As shown in Figure 8, in the crystal structure of yeast $tRNA^{Phe}$ the environment of m^7G46 is defined by A21, U47, U8, A14, G22, C13, A23, U12, A9 and G45. Thus, the net ring current shift suffered by the N1H of m^7G46 is determined by the DHU helix nucleotides and the adjacent residues 45 and 47. In the tRNAs studied in this series we have chosen species with the same nucleotide sequence as yeast $tRNA^{Phe}$ in the DHU helix and DHU loop–helix junction so that A21, A14, G22, C13, A23, U12, and A9 remain constant (see Figure 2). Furthermore, G45 is constant in all of these species and the residue following m^7G is either U or a modified U. Seen in this light, it is now apparent why the observed low-field resonance from the m^7G–CG13 interaction is always found close to −13.4 ppm. The substitution of C for A23 and of G for A9, which occurs in $tRNA_f^{Met}$, is expected to result in a much lower upfield shift on the m^7G ring NH proton (Geissner-Prettre & Pullman, 1970). In agreement with this, the m^7G N1H proton is found at −14.55 ppm in $tRNA_f^{Met}$. The surprisingly deshielded low-field position of the hydrogen-bonded N1H proton of m^7G in tRNA is explained in the m^7G monomer spectrum; the positive charge at N7 is apparently delocalized throughout the purine ring system and produces downfield shifts of over 1 ppm on C8H, N1H, and the amino protons compared to their positions in guanosine.

Chemical removal of m^7G by cleavage of the glycosidic bond might be considered a somewhat harsh procedure, and certainly there is more than a single effect on the spectrum when compared with the control sample. Ring currents from proximal bases, especially purines, are extraordinarily sensitive monitors of small structural changes with shifts of up to 0.1 ppm resulting from movements of far less than 1 Å when favorably oriented. Thus, relatively minor reorientation of bases in the modified tRNAs combined with removal of the ring current effect of the m^7G itself is expected to produce more than just one simple change in the spectrum. When one takes these effects into consideration, the modified and control spectra are quite comparable and the assignment of the m^7G interaction to the indicated proton loss in each case is the most reasonable interpretation of the data. This is especially true in the case of $tRNA_f^{Met}$, where there are virtually no changes between −12.8 and −15 ppm (apart from the loss at −14.55 ppm) upon m^7G removal. When combined with the extraordinary deshielding of m^7G N1H and the extra upfield shift from A9 and A23, the $tRNA_f^{Met}$ data alone point to a chemical shift of ca. −13.5 ppm for the m^7G hydrogen bond in $tRNA_1^{Val}$ and yeast $tRNA^{Phe}$, thus corroborating the experimental result with these two tRNAs. One can also take a different, systematic elimination approach to assigning the m^7G hydrogen bond. Of the seven to eight resonances not derived from secondary Watson–Crick pairs in the $tRNA_1^{Val}$ spectrum, the peaks at −14.9, −14.3, and −12.25 ppm have already been assigned to the 8–14, 54–58, and 15–48 interactions, respectively, thus leaving unassigned resonances at −13.4, −12.9, −11.95, −11.5, and −11.35 ppm. The NOE results assign the −11.95- and −11.35-ppm resonances to the secondary GU pair at position 50, leaving resonances at −13.4, −12.9, and −11.5 ppm. The presence of a −12.9-ppm tertiary resonance in $tRNA_1^{Gly}$, which does not contain the m^7G46 tertiary interaction, argues against this resonance being m^7G (it is probably G19–56 and will be the subject of a future report). The extremely deshielded chemical shift of m^7G N1H makes the −11.5-ppm resonance an unlikely candidate and thus subtractively leads to the −13.4-ppm assignment for the 46–22 tertiary interaction.

The m^7G46–G22 resonance in yeast $tRNA^{Phe}$ has been tentatively assigned at −12.47 ppm by Robillard et al. (1976) based on their calculations of the net ring current shift experienced by the m^7G N1H. Although there is an extra, noncloverleaf, resonance at approximately this position, they did not then realize the extremely deshielding effect of the delocalized positive charge in estimating the unshifted starting position of the m^7G resonance; it is obvious that this parameter has a direct effect on the final position at which this resonance will be observed. Using a very similar approach, Geerdes & Hilbers (1977) subsequently also misassigned the m^7G46 N1H hydrogen bond at −12.5 ppm. They report that the upfield shift on m^7G N1H is predicted to be 0.57 ppm greater when using the Quigley et al. (1975) coordinates compared to the Sussman & Kim (1976b) coordinates; yet they predict the m^7G resonance at −12.5 ppm in both cases. Kan & Ts'o (1977) have also attempted to calculate the "theoretical NMR

spectrum of yeast tRNA^{Phe}" from published crystal coordinates; they report an upfield shift of 1.76 ppm on the m^{7}G N1H proton, although they did not attempt to predict the expected position of this resonance due to uncertainties in the inherent unshifted starting position. Kearns & colleagues do not acknowledge the existence of the hydrogen-bonded m^{7}G N1H resonance in tRNA low-field NMR spectra (Bolton et al., 1976; Kearns, 1976). Romer & Varadi (1977) have also misassigned the m^{7}G resonance in yeast tRNA^{Phe} at −12.5 ppm; their reasons appear to be based on the fact that they "found only one tertiary resonance at −12.4 ppm" and the fact that the m^{7}G resonance in tRNA$_f^{Met}$ was reported to be at −13.3 ppm by Daniel & Cohn (1976). The latter assignment of the tRNA$_f^{Met}$ m^{7}G resonance was based partly on the effect of attaching a spin-label to s^{4}U8; the structural perturbations caused by the alkylation of residue 8 with a moderately bulky alkyl nitroxide are not known, and furthermore the region around −14.55 ppm, where we have assigned the m^{7}G resonance in tRNA$_f^{Met}$, was broadened beyond recognition in the s^{4}U8 spin-labeled tRNA.

A more important result by Daniel & Cohn (1976) was the NMR spectra of native tRNA$_{f1}^{Met}$ and tRNA$_{f3}^{Met}$. The latter tRNA differs by a substitution of A for m^{7}G; on the basis of the geometry surrounding the m^{7}G–CG13 triple, this substitution should cause an increased upfield ring current shift on A14–s^{4}U8 (which we have already assigned at −14.8 ppm) and of course a loss of the m^{7}G resonance from the spectrum since A does not contain a ring NH proton. Hence, we would reinterpret the changes which they observed at the extreme low-field end of the tRNA$_{f3}^{Met}$ spectrum (Daniel & Cohn, 1976) as an upfield shift of the 8–14 resonance from −14.8 to −14.4 ppm and the loss of the m^{7}G resonance at −14.55 ppm.

Finally, we should mention the perturbations observed in the rest of the spectrum upon removal of m^{7}G from tRNA. Several of these spectra were carried out at 45 °C to narrow the line widths. In the tRNA$_1^{Val}$ spectrum one of the two protons at −13.75 ppm shifts slightly, a resonance at −12.95 ppm shifts slightly, a proton at −12.55 ppm shifts downfield, and there is a slight shift in at least one proton in the complex peak at −12.05 ppm. Similar shifts are observed in the yeast tRNA^{Phe} spectrum. Our most reasonable explanation and assignment of these shifts is to tentatively attribute them to secondary base pairs 12, 11, 10, and the terminal CG13, respectively. These effects could be due to a relaxation of the distorted pitch of the DHU helix upon removing the m^{7}G–CG13 interaction or simply to the elimination of the ring current effect of m^{7}G on these proximal base pairs. In addition, there is a broadening and slight shifting in peak A of yeast tRNA^{Phe} (the U8–A14 resonance) and in peak A of tRNA$_1^{Val}$ (the s^{4}U8–A14 resonance); this is not unexpected in view of the close proximity of m^{7}G46 to U8. In addition to these shifts, in the case of tRNA$_m^{Met}$ and tRNA^{Lys} there appear to be possible intensity losses at −11.6, −12.8, −13.75, and −14.8 ppm. These are positions at which extra resonances from tertiary base pairs are expected in these two tRNAs [see Hurd et al. (1979)]. We suggest that the removal of the m^{7}G–CG13 triple interaction between the variable loop and the DHU stem might lead to a general destabilization of tertiary folding in tRNA$_m^{Met}$ and tRNA^{Lys}.

In an earlier collaborative investigation with Robillard et al. (1977), we studied the thermal unfolding sequence of yeast tRNA^{Phe} by using both high-field (aliphatic) and low-field NMR spectroscopy. The low-field tertiary ring NH assignments used in this study were derived by using the X-ray crystallographic coordinates to calculate the net through-space ring current shifts of all bases on every hydrogen-bonded ring NH proton in the molecule (Robillard et al., 1976). This approach led to assignments which agree quite well with our experimental assignments of tertiary resonances at −14.3 (8–14), −14.3 (54–58), −12.9 (19–56), and −11.6 ppm (15–48 or 18–55). The theoretical approach and our current experimental approach both reveal tertiary resonances at ca. −12.4 ppm. However, in yeast tRNA^{Phe}, we assign UA52 at −13.7 ppm with a tertiary resonance at −13.4 ppm, whereas Robillard et al. assign UA52 at −13.4 ppm with a tertiary resonance at −13.7 ppm. Despite this discrepancy there is obviously a tertiary resonance at −13.5 ± 0.2 ppm in addition to an ambiguously assigned tertiary resonance at −12.4 ppm. These two resonances are very probably derived from m^{7}G46–G22 and G15–C48, although it is difficult to state from the data alone which is which. The Robillard approach revealed that the m^{7}G46 N1H experienced a large upfield ring current shift. On the basis of this observation, and in the absence of any information concerning the inherent starting resonance position of a m^{7}G–G resonance, the m^{7}G46–G22 proton was guessed to be at −12.4 ppm with the G26–A44 resonance attributed to the tertiary resonance at lower field (Robillard et al., 1976, 1977). It now appears that these two assignments are incorrect. The hitherto unsuspected fact is that the m^{7}G46 N1H is highly deshielded into the extreme low-field end of the spectrum (below −15 ppm); its observed position at −13.4 ppm is still completely consistent with the large upfield ring current shift on this proton calculated by Robillard et al.

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Deoxyribonucleic Acid Binding Studies on Several New Anthracycline Antitumor Antibiotics. Sequence Preference and Structure–Activity Relationships of Marcellomycin and Its Analogues as Compared to Adriamycin†

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ABSTRACT: The deoxyribonucleic acid (DNA) binding characteristics of adriamycin and several new anthracycline glycosides, including marcellomycin, aclacinomycin, rudolfomycin, musettamycin, and pyrromycin, have been studied. The fluorescence spectra were determined for all six anthracyclines, and the fluorescence quenching effects caused by interactions with the natural DNAs poly(dAdT)–poly(dAdT) and poly(dGdC)–poly(dGdC) were characterized. Binding parameters were determined by Scatchard analyses of results obtained by spectrofluorometric titrations of anthracyclines with DNA. Consistent with earlier structure–activity relationship studies of nucleic acid synthesis inhibitory effects, the results demonstrate a correlation between the length of the glycosidic side chain and DNA binding affinity. In addition, the sugar residue 2-deoxyfucose appears to confer greater DNA binding ability than do the sugars rednosamine and cinerulose when present in the terminal position of the glycosidic side chain, also in agreement with earlier studies. The sequence preference of anthracycline–DNA interaction has been examined by using DNAs of varying GC content, including the naturally occurring calf thymus DNA (43% GC), *Clostridium perfringens* DNA (28% GC), and *Micrococcus luteus* DNA (72% GC) and the synthetic double-stranded copolymers poly(dGdC)–poly(dGdC) and poly(dAdT)–poly(dAdT). The results demonstrate that although adriamycin shows an absolute requirement for GC sequences for DNA binding, marcellomycin and its analogues showed no such sequence requirement. Furthermore, an AT preference for DNA binding was demonstrated with marcellomycin and its analogues.

The specificity of interaction of anthracycline antitumor antibiotics with DNA has been the subject of many studies using a variety of techniques, including equilibrium dialysis (Zunino et al., 1972; Arlandini et al., 1977), spectrophotometric methods (Calendi et al., 1965; Zunino et al., 1972; Gabbay et al., 1976), viscometric methods (Arlandini et al., 1977; Zunino et al., 1977), and fluorescence methods (Tsou & Yip, 1976; Zunino et al., 1976; DiMarco et al., 1977; Plumbridge & Brown, 1977; Zunino et al., 1977). As a result it is generally accepted that a major portion of the anthracycline–DNA interaction involves the insertion of the anthracycline ring between adjacent nucleotide bases of the DNA duplex by an intercalation mechanism (Lerman, 1961). This argument has been further substantiated by the studies of Waring (1970) and others [e.g., DiMarco et al. (1975), Gabbay et al. (1976), and Zunino et al. (1977)], who demonstrated that upon intercalation of anthracyclines between

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