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Effect of Cations on tRNA Structure†

P. H. Bolton and D. R. Kearns*

ABSTRACT: The structure of tRNA in the presence of certain cations has been investigated by high-resolution nuclear magnetic resonance (NMR). In the presence of 0.17 M Na at 22 °C the structure of tRNA is similar to, but not identical with, the native structure. At higher temperatures the difference between the native structure and the structure in the presence of 0.17 M Na increases. Magnesium sequentially stabilizes the tRNA structure in the presence of 0.17 M Na in the temperature range 22–44 °C, the number of magnesium required for the stabilization of the native structure increases from 2 ± 1 at 22 °C to 4 ± 1 at 44 °C, and the number of interactions with slowly exchanging hydrogen-bonded imino protons stabilized increases from 1 ± 1 at 22 °C to 4 ± 1 at 44 °C. The polyamines spermine and spermidine stabilize some, but not all, of the interactions stabilized by magnesium. For tRNA in the presence of 0.17 M Na at 44 °C, 3–4 spermine per tRNA stabilizes 2 ± 1 interactions with slowly exchanging hydrogen-bonded imino protons. The combination of spermine and magnesium was found to be more effective than either cation alone in stabilizing the native structure of tRNA. The NMR results offer no evidence that the structure of tRNA in the presence of both spermine and magnesium (at high levels of both cations) is different from the native structure. In the presence of tetraethylammonium or tetramethylammonium ions, the tRNA structure is extensively destabilized relative to the native structure. Addition of magnesium to such samples stabilizes the native structure in a nonsequential manner, and about eight magnesium per tRNA are needed for the stabilization. The NMR results are taken in conjunction with the results of other investigators to propose a comprehensive model for the interaction of tRNA with cations. The use of assignments of some resonances to specific tertiary interactions suggests that magnesium stabilizes the tertiary interactions of tRNA in the following order: $s^4U_8 \cdot A_{14}$, U_{33}, $A_{58} \cdot T_{54}$, and $G_{19} \cdot C_{56}$.

There has been considerable interest in determining the effect of various cations on the structure and stability of tRNA (Römer and Hach, 1975). Cations associated with tRNA in vivo are of special interest. A number of different experimental techniques have been used to investigate the interaction of polyvalent cations with tRNA and other polynucleotides (Römer and Hach, 1975; Sander and Ts'o, 1971; Danchin, 1972; Stein and Crothers, 1976a,b; Schrier and Schimmel, 1974, 1975; Pochon and Cohen, 1972; Kayne and Cohn, 1974; Wolfson and Kearns, 1975; Jones and Kearns, 1974; Lynch and Schimmel, 1974; Bina-Stein and Stein, 1976).

Studies of tRNA interaction with cations can be separated into two broad groups: those primarily concerned with the metal binding properties of tRNA (i.e., the number of binding sites, equilibrium constants) and those which have focused on the effect of cations on tRNA structure. It has been shown that tRNAs have about five strong binding sites for magnesium, with binding constants of about 10^5 M^{-1} in a wide variety of experimental conditions (Römer and Hach, 1975; Danchin, 1972; Schrier and Schimmel, 1974, 1975; Stein and Crothers, 1976a,b; Bina-Stein and Stein, 1976). From such studies it is known that tRNAs exhibit a set of strong polyvalent cation binding sites not found in either tRNA fragments or double helical or single-stranded RNA. Furthermore, there is considerable evidence that the strong binding sites are intimately associated with tRNA tertiary structure (Römer and Hach, 1975; Kayne and Cohn, 1974; Stein and Crothers, 1976b; Bina-Stein and Stein, 1976; Bolton and Kearns, 1977). However, recent reports have shown that in the presence of high levels of sodium, 0.17 M, tRNAs have only one or two strong binding sites at 4–22 °C (Stein and Crothers, 1976a,b; Bina-Stein, 1976; Bolton and Kearns, 1977). The manner in which magnesium and spermine bind to tRNA (cooperative, independent, or sequential) also appears to depend on the experi-

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mental conditions (Römer and Hach, 1975). In triethanolamine solutions the binding appears to be cooperative (Danchin, 1972; Schrier and Schimmel, 1974, 1975; Lynch and Schimmel, 1974), whereas in the presence of sodium it is not (Römer and Hach, 1975; Stein and Crothers, 1976a,b; Bina-Stein and Stein, 1976; Bolton and Kearns, 1977). While the binding of magnesium and polyamines to tRNA has been characterized in terms of binding constants and number of binding sites, comparatively little is known about the effects of cations on tRNA structure because it has been difficult to experimentally observe the effect of cations on tRNA structure and stability due to the lack of suitable methods for monitoring specific structural features. Although there are a number of reports which support the notion that magnesium is important in maintaining the native structure and biological function of tRNA, several investigators have reported that magnesium has little or no effect on tRNA structure in the presence of high levels of sodium, $\gtrsim$0.1 M (Reid and Robillard, 1975; Robillard et al., 1976; Goldstein et al., 1972; Yang and Crothers, 1972). Nevertheless, it is now generally accepted that polyvalent cations (magnesium and certain polyamines) are essential for the native structure of tRNA in conditions which closely parallel those found in vivo.

The effect of polyamines on tRNA structure is of special interest since certain polyamines can replace magnesium, at least partially, in stimulating the biological activity of tRNA in vitro (Santi and Webster, 1975; Cohen, 1971; Evans and Deutscher, 1976; Takeda and Igarashi, 1969; Igarashi et al., 1974; Sakai and Cohen, 1976). Furthermore, polyamines have been used in growing the crystals of tRNA used in x-ray diffraction studies (Sussman and Kim, 1976; Ladner et al., 1975; Quigley et al., 1975; Stout et al., 1976), and there is some evidence that tRNA structure in the presence of both polyamines and magnesium is not the same as in the presence of magnesium alone (Pochon and Cohen, 1972; Prinz et al., 1976). Since TMA and TEA[1] are reported to preferentially interact with A + T rich DNA (Melchior and von Hippel, 1973), it was of interest to determine if such an effect also occurs with tRNA and how the interaction of tRNA with magnesium is affected by the nature of the monovalent cation present.

In the experiments described in this paper, NMR is used to monitor the effect of cations on the structure of tRNA. Two kinds of structural change can be observed by NMR—stabilization of hydrogen bonding interactions which are manifested as an increase in the intensity in the low-field regions of the spectrum, and alterations in the stacking of residues which give rise to changes in the resonance positions of the hydrogen-bonded and carbon-bound protons. Both types of spectral change are observed in these experiments.

In this paper we are primarily interested in the stabilization of tRNA by magnesium and polyamines in different experimental conditions. By examination of tRNA spectra obtained in the presence of different levels of these cations, the number of cations which increase the intensity in the low-field region and/or induce changes in the resonance positions can be determined. These data allow the determination of the number of cations which affect tRNA structure. The spectra also allow the partial determination of the extent to which these cations stabilize the tRNA structure—as evidenced by increases in intensity in the low-field region (increase in the number of base pairs) and the shifting of resonance position. By simply comparing the spectra obtained in different conditions, the extent to which the structure of tRNA is identical with the native structure (the structure in the presence of high levels of sodium and magnesium) can be determined. This information about cation effects on tRNA structure can be obtained without recourse to assigning any resonances to specific protons of the molecule. If individual resonances in the tRNA spectra are assigned, additional information can be obtained from the spectra. At the present time, the assignments of most resonances from secondary structure interactions are not certain, so that any conclusions which are based on detailed assignments are less secure.

Materials and Methods

Unfractionated *Escherichia coli* and yeast tRNA were obtained from Plenum Scientific. RPC-5 was purchased from Miles Laboratories and Astro Enterprises, RPC-7 was prepared as described elsewhere (Shugart and Stulberg, 1974), and BD-cellulose was obtained from Plenum Scientific. TEA and TMA were obtained from J. T. Baker Chemical Corporation. Spermine, spermidine, putrescine, and cadaverine were products of calbiochem. All other chemicals were of reagent grade or higher, and doubly distilled water was used in all solutions.

E. coli $tRNA^{Met}_f$, $tRNA^{Val}_1$, and $tRNA^{Asp}$ and yeast $tRNA^{Phe}$ were purified through combining RPC-5 (Pearson et al., 1970), RPC-7 (Shugart and Stulberg, 1974), and BD-cellulose (Gilliam et al., 1967) chromatography. All tRNA preparations had activities of at least 1.5 nM/A_{260} unit. An A_{260} unit is the amount of material which when dissolved in 1 mL of distilled water has an absorbance of 1.0 at 260 nm when measured in a 1-cm light path. Amino acid acceptor assays were performed as described elsewhere (Weeren et al., 1970). The *E. coli* tRNA used in these experiments was less than 5% photo-cross-linked as determined by the method of Ofengand and Bierbaum (1973). After purification, the tRNA samples were stored as ethanol precipitates at −20 °C. The NMR samples were prepared by vacuum dialysis against a solution containing 0.2 M Na and 5 mM EDTA and then several times against the solution used in the NMR experiment. Trace levels of EDTA may lead to anomalous results in the titration of the tRNA samples with magnesium. In one sample, a trace level of ~0.5 mM EDTA gave rise to irreproducible results. The optical densities of the samples were measured after dialysis, the concentrations were computed assuming 1.6 nM/A_{260} unit, and the concentration of tRNA in the NMR experiments was about 1 mM.

The solutions used in the NMR experiments are: 0.1 M NaCl and 10 mM KH_2PO_4 (Na), 0.1 M NaCl, 10 mM $MgCl_2$, and 10 mM KH_2PO_4 (Mg), 0.1 M TMA and 10 mM KH_2PO_4 (TMA), and 0.1 M TEA and 10 mM KH_2PO_4 (TEA). All solutions were at pH 7.0. The total sodium concentration of the NMR samples was about 0.17 M (0.1 M free sodium and about 0.07 M bound to tRNA). Similarly, the concentration of TMA and TEA was about 0.17 M. A concentration of 0.17 M monovalent cation was chosen to minimize changes in ionic strength during the polyvalent cation titrations and to allow comparison of the results obtained here with those of other investigators.

The cation titrations were performed in situ by the addition of the proper volume of 10 mM cation, followed by addition of an equimolar amount of KH_2PO_4 at pH 7.0 to ensure that the pH would not change. After addition of the polyvalent cation and buffer, the samples were reduced to their original volume by a stream of dry, filtered nitrogen. In this manner reproducible spectral intensities (better than 5%) were ob-

[1] Abbreviations used are: TMA, tetramethylammonium; TEA, tetraethylammonium; BD-cellulose, benzoylated DEAE-cellulose; EDTA, ethylenediaminetetraacetic acid.

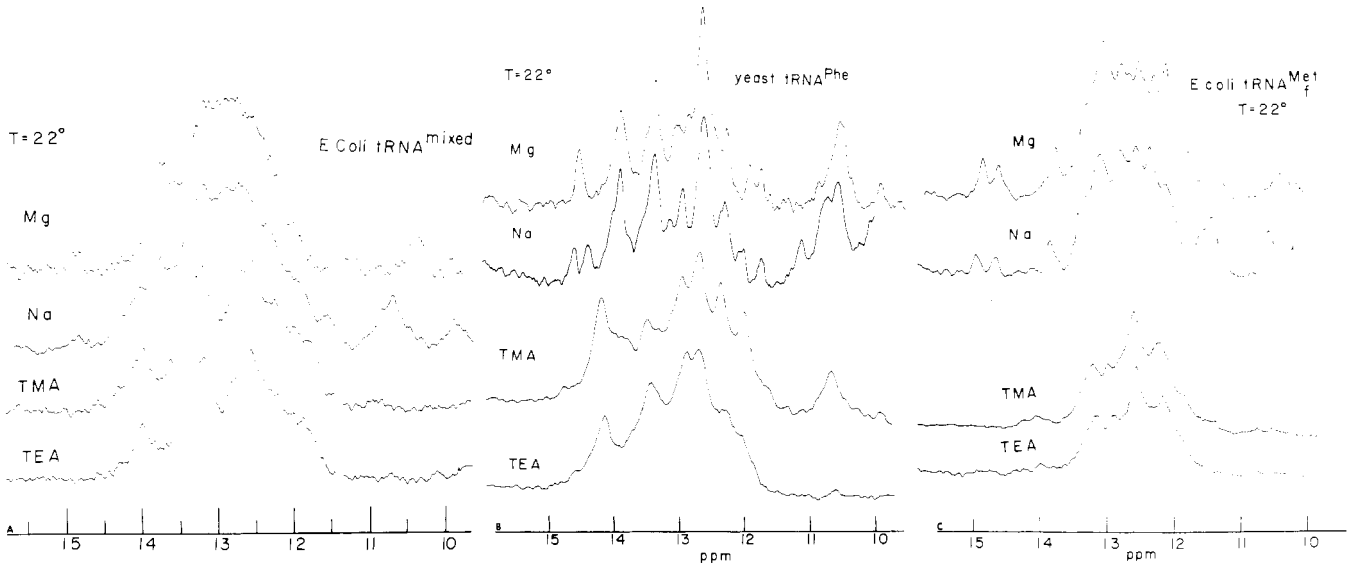

FIGURE 1: NMR spectra (300 MHz) of *E. coli* $tRNA^{mixed}$, *E. coli* $tRNA^{Met}$, and yeast $tRNA^{Phe}$ at 22 °C in the presence of TEA, TMA, Na, and Mg. See text for details.

tained. The error in producing the correct cation/tRNA ratio in this manner is estimated to be 10% and was reproducible to the accuracy of the NMR measurements.

NMR spectra were obtained with a Varian HR-300 spectrometer operated in the continuous wave field sweep mode. The spectra were averaged with a Nicolet 1020A computer to improve the signal-to-noise ratio. Spectra were integrated as described elsewhere (Bolton et al., 1976). In determining the integrated intensity the baseline must accurately be known, and because of possible uncertainty in this, the determination of the total intensity of a given spectrum is only accurate to about 10%. When two successive spectra are obtained on the same sample using identical instrument settings, the spectra are superimposable to within the noise level. Therefore, on comparing the spectra obtained on a sample before and after addition of polyvalent cations, differences in intensity can be determined with a much higher accuracy. When there are proportional changes in all parts of the spectrum, there are difficulties in accurately measuring small differences (5–10% of the total). However, most of the changes which were observed in this investigation experimentally are localized at just a few spectral positions.

Results

In this section we present the spectra obtained for samples of tRNA in the presence of different cations. The intensity of the low-field spectra gives the total number of secondary and tertiary interactions with slowly exchanging imino protons (extent of base pairing), and the resonance positions of the imino and methyl protons offer information about the tRNA conformation. Thus, the interaction of tRNA with magnesium and polyamines, as well as other cations, can be monitored by observing the spectral changes induced by the polyvalent cations (Bolton and Kearns, 1977).

In presenting the results we will use the spectrum of tRNA in solutions containing 0.17 M Na–10 mM Mg at pH 7 (referred to as native tRNA) as a reference. Native class I tRNAs exhibit 24 ± 2 resonances in the low-field region (Bolton et al., 1976; Bolton and Kearns, 1975, 1976; Daniel and Cohn, 1975, 1976; Geerdes and Hilbers, 1977; Reid and Robillard, 1975; Robillard et al., 1976; Kan and Ts'o, 1977; Reid et al., 1977; Hurd et al., 1977). This indicates that there are several tertiary interactions which have resonances in this region in addition to the resonances from secondary structure base pairs. In the methyl region, 1–3 ppm, the resonance of the T_{54} methyl protons is the principal feature of interest. The position of this resonance is primarily sensitive to the stacking of the bases adjacent to T_{54} (Kan and Ts'o, 1974; Kan et al., 1977; Kastrup and Schmidt, 1975). By using native tRNA as a reference state, we are also able to determine whether or not there are conditions in which tRNAs exhibit the native spectrum in the absence of magnesium.

The spectra of tRNA in the different experimental conditions will be analyzed as to the number of resonances in the low-field region, resonance positions compared with those of native tRNA, the number of polyvalent cations which affect the spectra, and the net gain in intensity in the low-field spectra induced by their presence. This analysis does not require the assignment of any of the low-field resonances to specific secondary or tertiary interactions. However, additional information can be inferred if the resonances in the spectra can be assigned to specific interactions and this will be presented in the Discussion section.

The spectra in Figure 1 show that tRNAs in the presence of TMA or TEA exhibit less overall intensity in the low-field region than does native tRNA. This loss in intensity, corresponding to about 10 ± 2 protons in TEA and 8 ± 2 protons in TMA, indicates that tRNAs are destabilized in TEA or TMA relative to native tRNA.

In contrast to the overall loss in intensity of the low-field spectra in TEA or TMA, there is a gain in intensity, relative to the spectrum of native tRNA, in the region between 14.0 and 14.5 ppm. This gain in intensity is observed for yeast $tRNA^{Phe}$ and *E. coli* $tRNA^{mixed}$ but not for *E. coli* $tRNA^{Met}_f$. Examination of the NMR spectra of mononucleotides in TEA and TMA gave no indication of an interaction which could cause such a shift in intensity.

The addition of magnesium to tRNA in TMA induced the gain of intensity in the low-field spectra (8 ± 2 protons) shown in Figure 2. The addition of up to 8 Mg/tRNA increases the intensity of the low-field region and shifts the positions of some resonances, but further addition of magnesium induced little or no additional spectral change. The spectrum in the presence of TMA and 8 Mg/tRNA is almost the same as that of native tRNA. The spectral changes induced by the addition of magnesium are seen to be nonsequential in that each addition

TABLE I: Summary of NMR Results on Cation Binding to tRNA.

tRNA species	Exptl conditions: Temp (°C)	Exptl conditions: Cations	No. of ions which stabilize structure	No. of interactions stabilized	Is stabilization sequential?	Is stabilized structure native?
Yeast $tRNA^{Phe}$	22	0.17 M Na	2 ± 1 (Mg)	1 ± 1	Yes	Yes
Yeast $tRNA^{Phe}$	37	0.17 M Na	2 ± 1 (Mg)	2 ± 1	Yes	Yes
Yeast $tRNA^{Phe}$	44	0.17 M Na	4 ± 1 (Mg)	4 ± 1	Yes	Yes
E. coli $tRNA^{Val}_1$	44	0.17 M Na	4 ± 1 (Mg)	4 ± 1	Yes	Yes
E. coli $tRNA^{Met}_f$	44	0.17 M Na	4 ± 1 (Mg)	4 ± 1	Yes	Yes
E. coli $tRNA^{mixed}$	44	0.17 M Na	4 ± 1 (Mg)	4 ± 1	Yes	Yes
Yeast $tRNA^{Phe}$	44	0.17 M Na	3 ± 1 (Sp)	2 ± 1	Yes	No
E. coli $tRNA^{Val}_1$	44	0.17 M Na	3 ± 1 (Sp)	2 ± 1	Yes	No
E. coli $tRNA^{mixed}$	44	0.17 M Na	3 ± 1 (Sp)	2 ± 1	Yes	No
Yeast $tRNA^{Phe}$	44	0.17 M Na, 3 Sp	2 ± 1 (Mg)	2 ± 1	Yes	Yes
E. coli $tRNA^{Val}_1$	44	0.17 M Na, 3 Sp	2 ± 1 (Mg)	2 ± 1	Yes	Yes
E. coli $tRNA^{mixed}$	44	0.17 M Na, 3 Sp	2 ± 1 (Mg)	2 ± 1	Yes	Yes
Yeast $tRNA^{Phe}$	22	0.17 M TMA	8 ± 2 (Mg)	8 ± 2	No	Yes
E. coli $tRNA^{Phe}$	22	0.17 M TMA	8 ± 2 (Mg)	8 ± 2	No	Yes

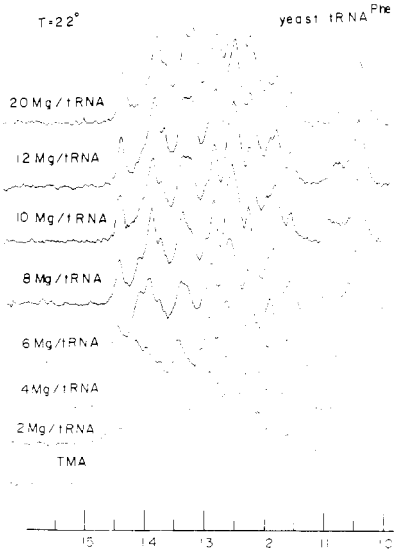

FIGURE 2: NMR spectrum (300 MHz) of yeast $tRNA^{Phe}$ in TMA in the presence of increasing amounts of magnesium. The spectra were obtained at 22 °C.

of magnesium induces about the same proportion of the total spectral change in the different spectral features affected. Similar results were obtained for *E. coli* $tRNA^{Met}_f$.

These NMR results on tRNA indicate that tRNAs are destabilized in the presence of alkylammonium ions, that these ions induce a gain in intensity between 14.0 and 14.5 ppm, that about 8 Mg/tRNA are required to obtain a spectrum similar to that of native tRNA, and that magnesium stabilizes tRNA in TMA in a nonsequential manner. These results are summarized in Table I.

Spectra of tRNA in the presence of 0.17 M Na are shown in Figures 3–7. The results in Figure 6 show the effect of temperature on the spectrum of yeast $tRNA^{Phe}$ in the range 22–44 °C. The spectra show that there is 1 ± 1 resonance less in the spectrum at 22 °C than in the spectrum of native tRNA. At 37 °C there are 2 ± 1 less and at 44 °C 4 ± 1 less. The spectra also show that the losses in intensity with increasing temperature occur at specific spectral locations and that some of the losses occur at lower temperature than do others. In addition to the observation that there are fewer low-field resonances in the presence of 0.17 M Na than for native $tRNA^{Phe}$, the spectra show that the positions of some resonances are not the same as in the native $tRNA^{Phe}$ spectrum. The spectral region between 14.0 and 14.5 ppm shows the most striking difference at 22 °C (Bolton and Kearns, 1977; Hilbers et al., 1973). In the native $tRNA^{Phe}$ spectrum there is a single peak in this region corresponding to two protons, whereas in the spectrum of $tRNA^{Phe}$ in the presence of 0.17 M Na there are two peaks, each corresponding to one proton. This result indicates that the structure of $tRNA^{Phe}$ is not the same as the native conformation in 0.17 M Na even at 22 °C, even though most of the resonances are present and the overall spectrum is quite similar to that of native tRNA. The results below on the methyl protons support the notion that tRNA is close to the native conformation in 0.17 M Na at 22 °C (Goldstein et al., 1972).

The spectra of the methyl region of several tRNA are shown in Figure 4. The spectra show that the resonance position of the methyl protons of T_{54} is about the same in 0.17 M Na at 22 °C as in the native tRNA spectrum (~1.1 ppm). From this it may be inferred that the stacking of the TΨC loop is essentially the same in the presence of 0.17 M Na as in native tRNA. However, at 44 °C the spectra of tRNA in the presence of 0.17 M Na show that the resonance position of the T_{54} methyl shifts to lower field than in the native tRNA spectrum. Since the resonance position of the resonance of T_{54} in completely melted tRNA is about 1.8 ppm (Kan and Ts'o, 1974; Kastrup and Schmidt, 1975; Kan et al., 1977), this result indicates that the stacking of residues adjacent to T_{54}, in 0.17 M Na at 44 °C, is somewhat less than in native tRNA but still more than in completely melted tRNA.

These results on tRNA in the presence of 0.17 M Na show that $tRNA^{Phe}$ does not assume the native conformation in this condition even at 22 °C, but the differences are small. At higher temperatures the differences between the spectra of tRNA in 0.17 M Na and native tRNA increase such that at 44 °C there are substantial differences between them. These results are summarized in Table I and discussed further below.

Since the structure of tRNA in the presence of 0.17 M Na is not the native conformation, it was of interest to examine the manner in which magnesium induces formation of the native

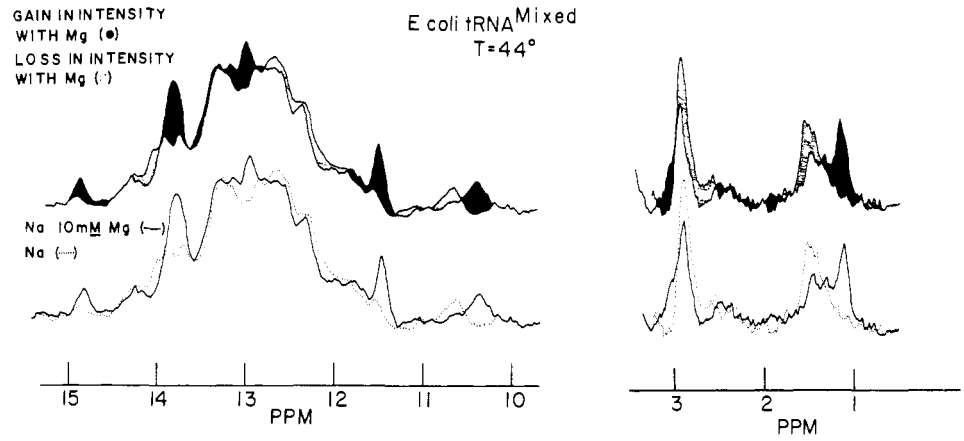

FIGURE 3: NMR spectrum (300 MHz) of *E. coli* $tRNA^{mixed}$ in the presence and absence of magnesium. The bottom spectra are the superposition of the spectrum in the presence of magnesium (solid line) and the spectrum in the absence of magnesium (dashed line). The top spectra are the superposition of the spectra in the presence and absence of magnesium, and the darkened areas are those in which there is a gain of intensity with magnesium and the shaded areas are those in which there is a loss of intensity with magnesium. The spectra were obtained at 44 °C.

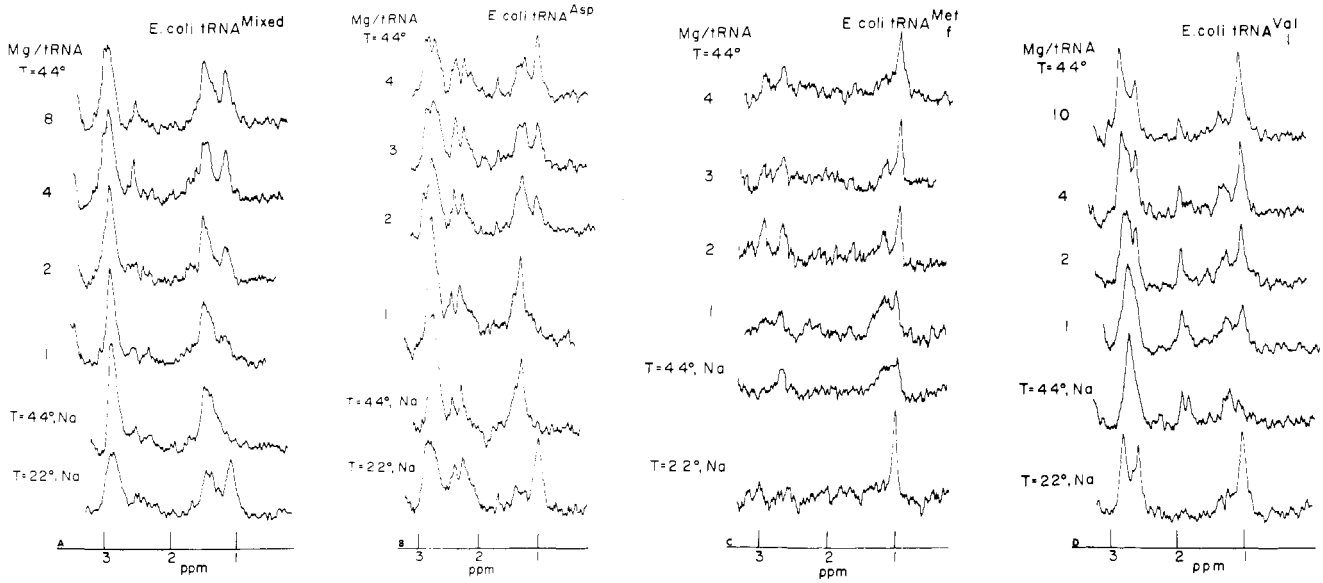

FIGURE 4: NMR spectra (300 MHz) of *E. coli* $tRNA^{mixed}$, *E. coli* $tRNA^{Asp}$, *E. coli* $tRNA^{Met}_f$, and *E. coli* $tRNA^{Val}_1$. The methyl region spectra are shown at 22 and 44 °C in the absence of magnesium and at 44 °C in the presence of different concentrations of magnesium.

conformation. The results in Figure 7 show the effect of magnesium on the low-field spectrum of yeast $tRNA^{Phe}$ at 22 °C in 0.17 M Na. The addition of 1 Mg/tRNA induces the coalescence of the two peaks between 14.0 and 14.5 ppm. The addition of a second magnesium induces a gain in intensity near 13.1 ppm, but the further addition of magnesium induces little or no change in the spectrum. These results show that magnesium increases the number of resonances in the low-field region by 1 ± 1, that 2 ± 1 magnesium are required to obtain a spectrum comparable to that of native tRNA, and that the spectral changes induced by magnesium are sequential.

At 37 °C, similar spectral changes are observed upon the addition of magnesium to yeast $tRNA^{Phe}$ (Figure 7). The addition of 1 Mg/tRNA primarily affects the spectral region between 14.0 and 14.5 ppm, and the addition of up to 2 ± 1 magnesium per tRNA increases the intensity of the low-field region. At 2 Mg/tRNA the spectrum is comparable to that of native trna, and the further addition of magnesium induces little or no change in the spectrum. At 44 °C the addition of magnesium to yeast $tRNA^{Phe}$, *E. coli* $tRNA^{Val}_1$, and *E. coli* $tRNA^{Met}_f$ induces the gain of 4 ± 1 resonances in the low-field region. The gain in intensity is complete at 4 Mg/tRNA, and the spectral changes induced by magnesium are seen to be sequential (see Figure 5). At 4 Mg/tRNA, the spectra are comparable to those of native tRNA, and the further addition of magnesium induces little change in the spectra. The effect of magnesium on the methyl region spectra of tRNA in 0.17 M Na at 44 °C was also investigated and the spectra are shown in Figure 4. The addition of magnesium to tRNA induces the shift of the resonance from the T_{54} methyl protons to 1.1 ppm from about 1.3 ppm. In all cases examined no more than 4 Mg/tRNA are required to shift the resonance to the position observed in native tRNA, and almost all of the change occurs at 2 ± 1 Mg/tRNA, indicating that the effect of magnesium is sequential since 4 Mg/tRNA are required for the native low-field spectrum. It is also of interest to note that the T_{54} resonance is observed either at the position of the partially denatured tRNA or that of native tRNA (the resonance position does not shift continuously between the two positions with the addition of magnesium), indicating that the two conformations do not rapidly equilibrate on the NMR time scale.

These results on the interaction of tRNA in 0.17 M Na with magnesium indicate that the number of magnesiums which

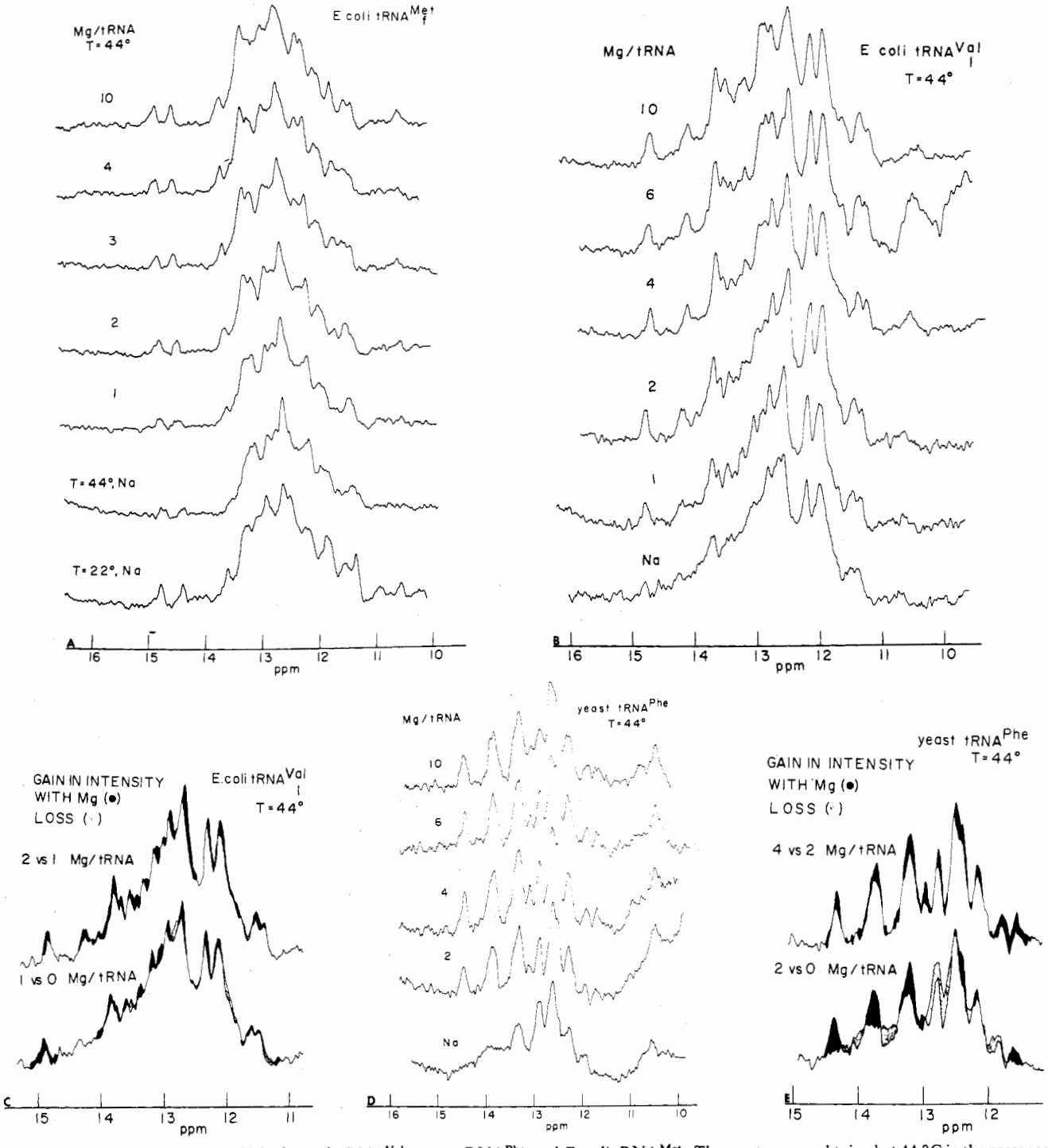

FIGURE 5: NMR spectra (300 MHz) of *E. coli* $tRNA^{Val}_1$, yeast $tRNA^{Phe}$, and *E. coli* $tRNA^{Met}_f$. The spectra were obtained at 44 °C in the presence of the indicated levels of magnesium. Also shown is the superposition of the spectra in the presence of various levels of magnesium for $tRNA^{Val}_1$ and $tRNA^{Phe}$ (see Figure 1).

stabilize tRNA structure (the number required for observation of the native tRNA spectrum) and the number of interactions stabilized increase with temperature from 22 to 44 °C. In all cases the stabilization is found to be sequential. These results on the interaction of tRNA with magnesium are given in Table I.

The interaction of tRNA in 0.17 M Na with spermine was also investigated. The spectra of yeast $tRNA^{Phe}$ and *E. coli* $tRNA^{Val}_1$ in Figure 8 show that spermine induces a gain in intensity of the low-field spectra of these tRNA by about 2 ± 1 resonances. Addition of more than three spermine per tRNA has little or no additional effect on the spectra. The spectra also show that spermine does not induce the native conformation of tRNA as monitored by NMR. The combination of spermine and magnesium was therefore investigated with the results shown in Figures 9 and 10. The spectra show that the addition of up to 2 Mg/tRNA in the presence of 0.17 M Na and 3–4 spermine/tRNA increases the intensity of the low-field spectra by 2 ± 1 resonances, and that the spectral changes induced by magnesium are sequential. The spectrum of tRNA in the presence of magnesium and spermine is that of native tRNA, and the combination of magnesium and spermine, at low levels of each cation, is found to be more effective than either cation alone in forming the native structure of tRNA.

TABLE II: Comparison of Results on the Binding of Polyvalent Cations to tRNA.

Exptl condition	Poly-valent cation	No. of strong binding sites	Bonding constant (M^{-1})	Type of binding	Structural features stabilized
Triethanolamine (4 °C)[a-c]	Mn	5 ± 1	2.7×10^5	Cooperative	
	Sp	4 ± 1	3.7 ± 10^6	Cooperative	
	Mg	5 ± 1	1×10^5	Cooperative	
TMA (22 °C)[d]	Mg	(8)[l]		Sequential	Secondary and tertiary
Na (4 °C)[e-g]	Mg	1	3×10^4	Independent	D stem, D loop
Na (10-45 °C)[h]	Mg	5 ± 1	9×10^4	Independent	Tertiary structure
Na (44 °C)[d]	Mg	(4)[l]		Sequential	$(s^4)U_8$·A_{14} U_{33}, A_{58}·T_{54} G_{19}·C_{56}
Na (22 °C)[d]	Mg	(2)[l]		Sequential	U_8·A_{14} G_{19}·C_{56}
Na (37 °C)[d]	Mg	(2)[l]		Sequential	U_8·A_{14} G_{19}·C_{56}
Na (44 °C([d]	Sp	(3-4)[l]		Sequential	$(s^4)U_8$·A_{14} U_{33},A_{58}·T_{54} (partial)
Sp (44 °C)[d]	Mg	(2-3)[l]		Sequential	A_{58}·T_{54} G_{19}·C_{56}
Na (25 °C)[i-k]	Eu	3	10^8	Sequential	
	Eu	4	1.5×10^5	Independent	

[a] Danchin (1972). [b] Schrier and Schimmel (1974). [c] Schrier and Schimmel (1975). [d] This paper. [e] Stein and Crothers (1976a). [f] Stein and Crothers (1976b). [g] Bina-Stein and Stein (1976). [h] Römer and Hach (1975). [i] Jones and Kearns (1974). [j] Kayne and Cohn (1974). [k] Wolfson and Kearns (1975). [l] Number of polyvalent cations which stabilize tRNA structure.

Taken together, the above results show that the stabilization of tRNA with polyvalent cations is strongly dependent on the experimental conditions since the NMR results show that the number of cations needed to form the native conformation, the number of interactions stabilized, and whether or not the stabilization is sequential depend on the number and type of cation present and the temperature. These results and those of other investigators can be used to arrive at a comprehensive picture for the interaction of tRNA with cations. The results of this section are summarized in Table I.

Discussion

In comparing the above results with those obtained by other investigators, it is important to consider the exact experimental conditions in which the effect of polyvalent cations on the conformation of tRNA was observed. Table II contains a partial list of the results obtained here and elsewhere on the interaction of tRNA with polyvalent cations. The features of particular interest are: (i) the number of strong binding sites, (ii) the number of polyvalent cations which stabilize tRNA structure, (iii) whether the binding of the polyvalent cations is sequential or not, (iv) whether the polyvalent cations stabilize the native conformation of tRNA, and (v) the specific interactions stabilized by the polyvalent cations. Since the first four points can be discussed without recourse to any assignment of the NMR resonances, and hence are the most secure results, they will be discussed first.

Interaction of tRNA with Magnesium. Römer and Hach (1975) reviewed the literature on the effect of magnesium on the structure of tRNA and concluded that magnesium binds sequentially to tRNA when the tertiary structure, but not the secondary structure, is disrupted. They also pointed out that the number of magnesium ions required to stabilize the native conformation of tRNA may depend on the type and concentration of monovalent ions present and the temperature at which the experiments are performed.

The number of strong binding sites for magnesium has been reported to be as many as five and as few as one. While the NMR results offer no information about the binding constants, the results do allow the determination of the number of magnesiums which stabilize the structure of tRNA. The results in Table II show that the NMR (in 0.17 M Na) results are compatible with the equilibrium binding studies, if it is assumed that the number of strongly bound magnesiums is the same as the number of magnesiums which stabilize tRNA structure. In other conditions (TMA), the number determined by NMR is eight, which is somewhat higher than the number determined in any other condition. This may be due to the extreme destabilization of tRNA in the presence of this cation or to the fact that several magnesiums in addition to the strongly bound ones are required to stabilize the structure.

The NMR results also correlate well with the results of other investigators with regard to whether the binding of magnesium to tRNA is sequential or not. These results and others show that the binding of magnesium to tRNA in the presence of 0.17 to 0.032 M Na in the temperature range from 4 to 45 °C is sequential (see Table II). However, when tRNA is in the presence of destabilizing monovalent cations (TMA or triethanolamine), the binding is nonsequential (Table II).

In developing a picture for the interaction of magnesium with tRNA, it is important to consider the conformational changes that occur in the different experimental conditions. The NMR results presented here and elsewhere and the results of other investigators indicate that in the presence of 0.17 M Na at 22-37 °C the tRNA is in a conformation similar to, but not identical with, the native conformation. For tRNA in the presence of 0.17 M Na at 44 °C there is evidence that the tRNAs exhibit most, if not all, of the secondary structure, but the tertiary structure is disrupted. At a lower Na concentration, 0.032 M, it is thought that the secondary structure is present between 10 and 45 °C, but the tertiary structure is disrupted. In the presence of large monovalent cations such as triethanolamine and TMA, the NMR results presented here show that at least some of the secondary structure is destabilized in

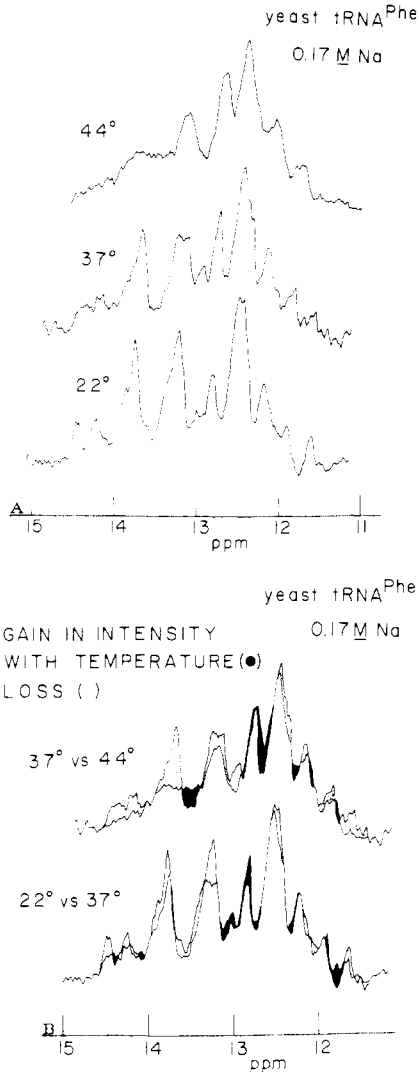

FIGURE 6: NMR spectra (300 MHz) of yeast tRNA^{Phe}. The spectra were obtained at 22, 37, and 44 °C in the absence of magnesium. Also shown is the superposition of the spectra at 22 and 37 °C as well as at 37 and 44 °C; the dark areas are those at which there is a gain of intensity as the temperature is increased and the shaded areas the regions in which there is loss of intensity with an increase in temperature.

the presence of this cation, and evidence has been presented that the secondary structure is destabilized in the presence of triethanolamine. This view of tRNA conformation in different experimental conditions suggests the following picture for the interaction of tRNA with magnesium. When the tertiary structure as well as some of the secondary structure of a tRNA is destabilized, the binding of magnesium is cooperative, and there are about five strong magnesium binding sites and the magnesium binds nonsequentially. In addition, the NMR results suggest that there may be several magnesiums in addition to the strongly bound magnesium which participate in stabilizing the tRNA conformation. When tRNA tertiary structure is preferentially disrupted but secondary structure is intact, the binding of magnesium is sequential, and about 5 ± 1 magnesiums bind strongly as well as stabilize the structure of tRNA. When most of the tertiary structure is present, the number of strong magnesium binding sites is small, 1–2, and the magnesium binds sequentially. One implication of this picture is that strong magnesium binding sites are associated with tRNA tertiary structure as discussed below.

Interaction of tRNA with Polyamines. Certain polyamines which may be bound to tRNA in vivo have been implicated in a number of biochemical processes. In particular, polyamines stimulate the aminoacylation of tRNA (Santi and Webster, 1975; Takeda and Igarashi, 1969; Sakai and Cohen, 1976), the addition of nucleotides to tRNA by nucleotidyltransferase (Evans and Deutscher, 1976), mRNA-mediated ribosomal binding of tRNA (Igarashi et al., 1974), as well as other cellular processes (Cohen, 1971). In addition to the biochemical studies, physical chemical studies have indicated that the conformation of tRNA in the presence of polyamines and magnesium may be different from that obtained in the presence of just magnesium, but with no polyamine. It is therefore of interest to compare the effect that polyamines and magnesium have on the structure of tRNA (Pochon and Cohen, 1972; Evans and Deutscher, 1976; Prinz et al., 1976).

The NMR results presented above indicate that spermine stabilizes the structure of tRNA, but not nearly as effectively as does magnesium at 44 °C in the presence of 0.17 M Na. Spermine sequentially stabilizes the structure of tRNA, and about 3–4 spermine per tRNA increase the intensity of the low-field spectra. These results are consistent with those of other investigators, considering the differences in experimental conditions (see discussion above). The combination of spermine and magnesium was found to be more effective than either cation alone, at low levels, in stabilizing the native conformation of tRNA. Spermidine was found to have essentially the same effect on the spectra of tRNA as spermine, but cadaverine and putrescine were found to have little or no effect on the spectra. This result suggests that it is the asymmetric triamine unit which is required.

Prinz et al. (1976) recently reported that the fluorescence enhancement of ethidium bromide and the circular dichroic (CD) spectrum and the T_m of yeast tRNA^{Phe} are affected by the addition of spermine to solutions of tRNA containing low levels of magnesium (1 mM) at low ionic strength, but none of these changes was observed for tRNA in the presence of 10 mM Mg. Similarly, Evans and Deutscher (1976) have shown that spermine stimulates the activity of tRNA nucleotidyltransferase, with the increase in activity being greatest at the lowest levels of magnesium investigated, 0.1 mM, and decreasing as the magnesium concentration is increased. All of these results are consistent with the proposal that spermine affects the conformation of tRNA in the presence of low levels of magnesium by stabilizing the native structure and that the tRNA structure formed in the presence of excess magnesium and spermine is the same as the structure formed in the presence of excess magnesium alone.

The above result is important in the interpretation of x-ray diffraction studies on yeast tRNA^{Phe} (Sussman and Kim, 1976; Ladner et al., 1975; Quigley et al., 1975; Stout et al., 1976). Due to the structural differences between magnesium and spermine, it is plausible, without considering the contrary evidence mentioned above, that spermine would stabilize a nonnative conformation of tRNA. However, the NMR results demonstrate that the combination of magnesium and spermine stabilizes the *same* native structure found when just magnesium is present. A similar conclusion was reached by Chen et al., who examined the Raman spectrum of yeast tRNA^{Phe} under various experimental conditions (Thomas et al., 1973; Chen et al., 1975). However, they did not examine the effect of spermine, separately, on the Raman spectrum of tRNA.

Structural Features Stabilized by Magnesium and Polyamines. To use the NMR data to determine which structural features of tRNA are stabilized by magnesium and polyamines, the resonances must first be assigned to specific interactions. For our purposes here we will only be concerned with the assignment of certain resonances to common tertiary

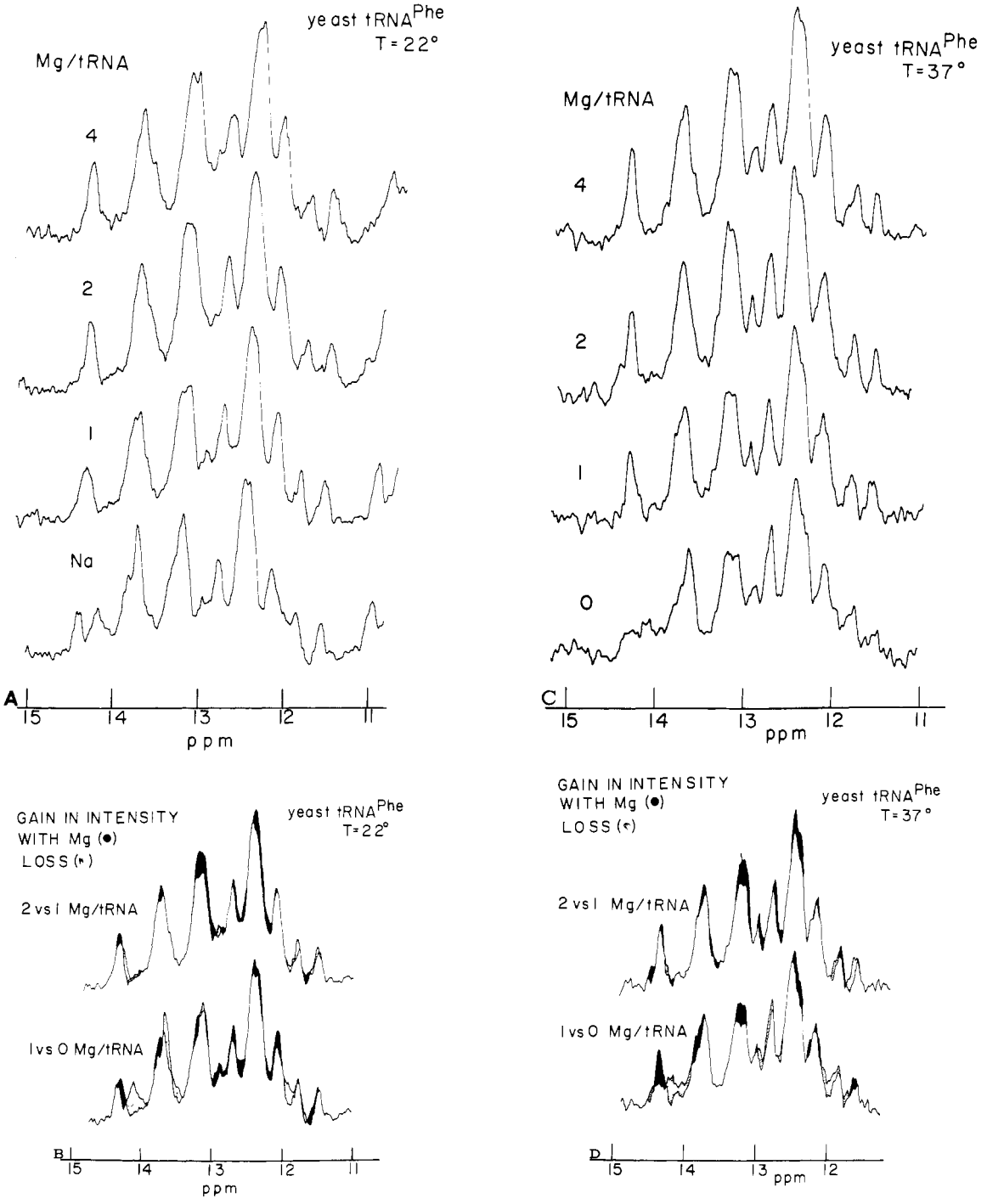

FIGURE 7: NMR spectra (300 MHz) of yeast $tRNA^{Phe}$ in the presence of different levels of magnesium at 22 and 37 °C. Also shown is the superposition of spectra in the presence of different levels of magnesium. The dark areas are those in which there is a gain of intensity when the magnesium concentration is increased and the shaded areas are those at which there is a loss of intensity.

interactions in order to show that magnesium and polyamines specifically stabilize the tertiary structure of tRNA. Additional assignment of the common resonances to *specific* tertiary interactions will only be considered in order to determine the order in which the tertiary interactions are stabilized.

The first low-field resonance to be unequivocally assigned to a tertiary interaction was the one from the $s^4U_8 \cdot A_{14}$ tertiary structure base pair which gives rise to a resolved resonance at 14.9 ppm in the spectra of both pure and mixed *E. coli* tRNA (Bolton and Kearns, 1975; Wong and Kearns, 1974; Wong et al., 1975). This assignment was subsequently confirmed by others (Daniel and Cohn, 1975; Reid et al., 1975). The fact that a resolved resonance from a tertiary structure base pair could be observed in mixed *E. coli* tRNA suggested that it would be possible to observe resolved resonances from other common tertiary interactions in the mixed tRNA spectra. In other experiments, we identified such resonances at 13.8, 13.0, and 11.5 ppm in both yeast and *E. coli* tRNA (see Figure 3) (Bolton and Kearns, 1975). The experimental evidence for assigning these resonances to common tertiary interactions is based on the following: (i) Integration of the low-field NMR spectra of tRNA containing adequate levels of magnesium indicated that there are at least 3–4 more resonances per molecule than can be accounted for in terms of cloverleaf secondary structure base pairs (Reid and Robillard, 1975; Bolton and Kearns, 1975; Bolton et al., 1976; Daniel and Cohn,

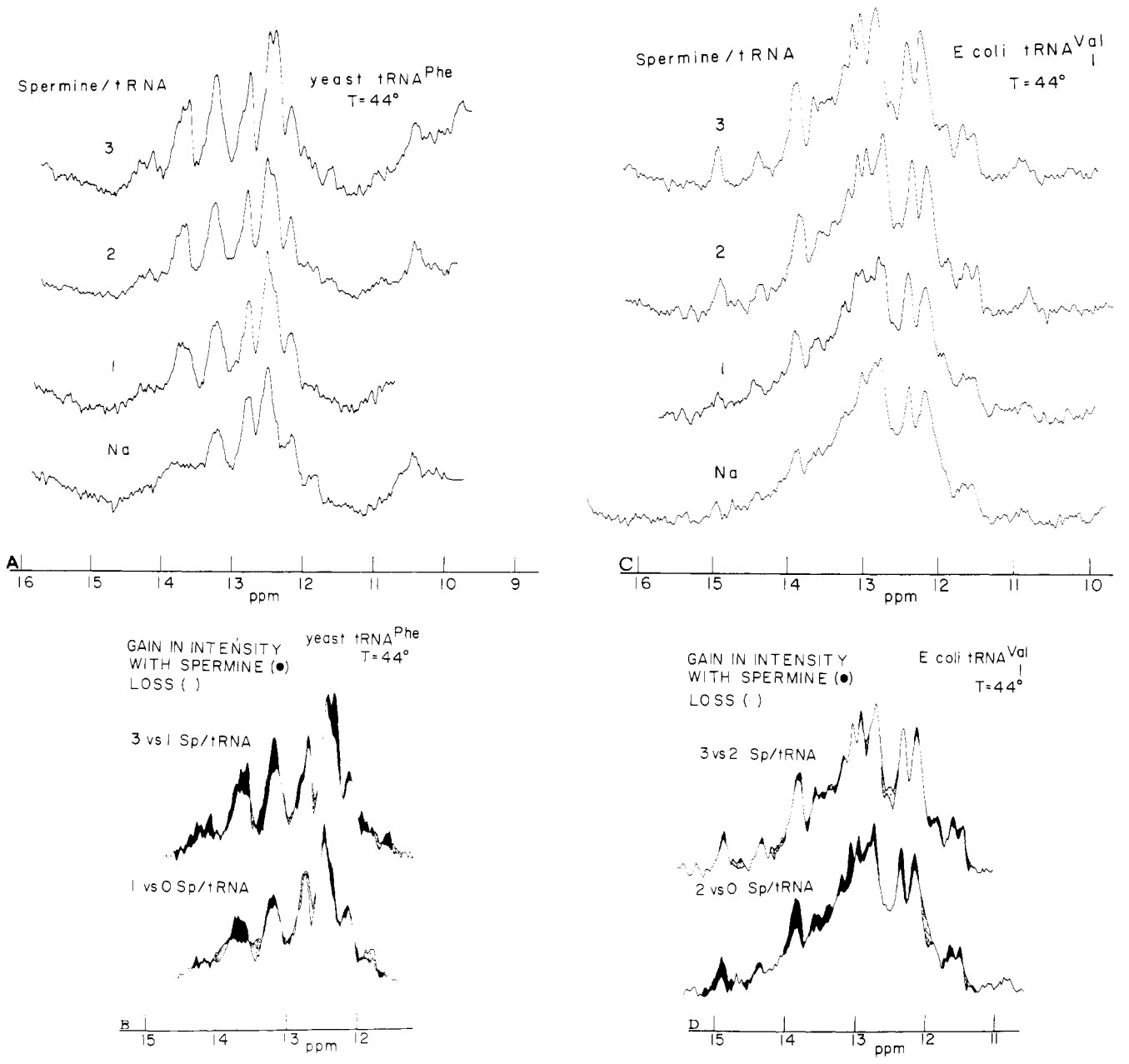

FIGURE 8: NMR spectra (300 MHz) of yeast $tRNA^{Phe}$ and *E. coli* $tRNA^{Val}$ at 44 °C in the presence of different concentrations of spermine. Also shown is the superposition of the spectra in the presence of different levels of spermine. The dark areas are those which gain intensity with an increase in spermine and the shaded areas are those which lose intensity.

1975; Geerdes and Hilbers, 1977; Kan and Ts'o, 1977; Bolton, 1976). (ii) The fact that *resolved* resonances appear in the spectrum of *E. coli* $tRNA^{mixed}$ (at 14.9, 13.8, 13.0, and 11.5 ppm) indicates they arise from base pairs or tertiary interactions which are common to most, if not all, tRNA. (iii) The resolved resonances exhibit early melting behavior in magnesium-deficient samples (Bolton and Kearns, 1975, 1977). (iv) The resolved resonances are specifically and sequentially broadened by paramagnetic metals in conditions such that most of the other resonances in the spectrum are not affected (Chao and Kearns, 1977; Kearns, 1976). (v) The methyl resonance from the common T_{54} residue in the TΨC loop is observed as a resolved peak in the spectra of both pure and mixed tRNA at 1.1 ppm, as shown in Figure 3 (Chao and Kearns, 1977; Kearns, 1976; Bolton, 1976). This also demonstrates that resolved resonances can be observed in the spectra of mixed tRNA from bases which are common to most tRNA even though differences in local environment might have given rise to a wide range of possible chemical shifts. (vi) Of the many tertiary structure interactions which have been proposed on the basis of the crystal-structure studies of yeast $tRNA^{Phe}$, only four (six, at most) are expected to give rise to resonances in the low-field spectrum (Quigley and Rich, 1976; Ladner et al., 1975; Sussman and Kim, 1976).

The assignment of the common resonances to tertiary interactions in conjunction with the results in Figure 3 implies that it is the tertiary structure which is stabilized by magnesium. Similarly, since results for pure species of tRNA show that magnesium induces gains of intensity at the positions of the common low-field resonances in pure species of tRNA and shifts the position of which methyl resonances can be assigned to tertiary interactions, it can be concluded that the tertiary structure is the structural feature stabilized by magnesium.

The results for spermine presented above (see Table I) show that spermine stabilizes some, but not all, of the interactions stabilized by magnesium. This is strong evidence that spermine

TABLE III: Effect of Magnesium on the Spectra of tRNA in 0.17 M Na.

tRNA species	Resonance positions affected by N Mg/tRNA (ppm) N = 1	N = 2	N = 4	Assignments of resonance
E. coli $tRNA^{mixed}$	14.9	14.9		s^4U_8·A_{14}
(44 °C)[a]	11.5	11.5		U_{33}
	1.1	1.1	1.1	T_{54}
		13.8	13.8	A_{58}·T_{54}
			13.0	G_{19}·C_{56}
E. coli $tRNA^{Val}_1$	14.9	14.9		s^4U_8·A_{14}
(44 °C)	1.1	1.1		T_{54}
		11.5		U_{33}
		14.3		AU_6
		13.8		A_{58}·T_{54}
			13.0	G_{19}·C_{56}
Yeast $tRNA^{Phe}$		14.2 (2)		U_8·A_{14}, AU_6
(44 °C)		13.8	13.8	A_{58}·T_{54}
		11.5	11.5	U_{33}
		13.2	13.2	G_{19}·C_{56}
		12.4	12.4	(?)
			13.0	(?)
E. coli $tRNA^{Met}_f$	14.8	14.8		s^4U_8·A_{14}
(44 °C)	14.6	14.6		AU_{11} (?)
	11.5	11.5		U_{33}
	1.1	1.1	1.1	T_{54}
		13.8	13.8	A_{58}·T_{54}
			12.5	(?)
			13.0	G_{19}·C_{56}
Yeast $tRNA^{Phe}$	14.4			U_8·A_{14}
(22 °C)		13.2		G_{19}·C_{56}
Yeast $tRNA^{Phe}$	14.4			U_8·A_{14}
(37 °C)	13.2	13.2		G_{19}·C_{56}
		12.4		(?)

[a] Bolton (1976).

is not as effective as magnesium in stabilizing the native structure of tRNA. However, the combination of spermine and magnesium was found to be more effective than either cation alone in stabilizing the native structure.

To use the NMR results to determine the order in which the tertiary interactions are stabilized by magnesium, it is necessary to have secure assignments for the resonances from tertiary interactions. As mentioned above, the assignments of the s^4U_8·A_{14} and T_{54} resonances are quite secure. The assignments of the other tertiary interaction resonances are less secure and have in part been based on the following observations. The 13.8-ppm resonance is assigned to the A_{58}·T_{54} tertiary structure base pair as it is the only common A·U(T) pair besides U_8·A_{14} (Bolton and Kearns, 1975) and on the basis of the examination of model compounds (Geerdes and Hilbers, 1977). The 13.0-ppm resonance is assigned to the G_{19}·C_{56} base pair as it is the only common tertiary base pair expected to have a resonance near 13.0 ppm and is common to all tRNAs (Bolton and Kearns, 1975). Finally, the 11.5-ppm resonance, originally attributed to G·C_{13} (Bolton and Kearns, 1975), is assigned to the ring nitrogen proton of U_{33} hydrogen bonded to the phosphate 36 (Bolton et al., 1976) on the basis of spectra of fragments of tRNA containing the anticodon loop (Wong and Kearns, 1974; Rordorf, 1975) and the crystal structure of yeast $tRNA^{Phe}$ (Quigley et al., 1975; Ladner et al., 1975; Sussman and Kim, 1976).

Some of the NMR results are presented in Table III. This table lists the positions of those resonances affected by the addition of a given amount of magnesium or spermine. The assignments listed are as given above. The table shows that for

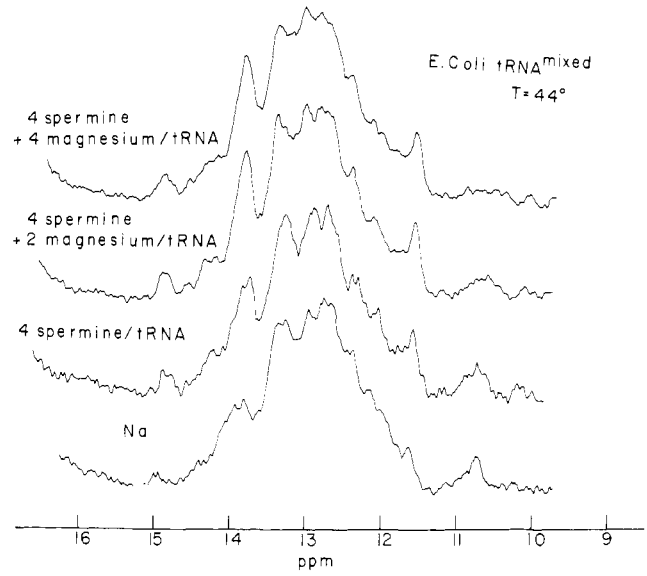

FIGURE 9: NMR spectra (300 MHz) of *E. coli* $tRNA^{mixed}$ at 44 °C in the absence of polyvalent cation (0.17 M NaCl) and in the presence of 4 spermine/tRNA and spermine plus different levels of magnesium.

tRNA in the presence of 0.17 M Na at 44 °C the s^4U_8·A_{14} interaction is the first one to be stabilized by magnesium. This is in agreement with the results of Stein and Crothers (1976a,b), who suggested that the most strongly bound magnesium to tRNA in the presence of 0.17 M Na at 4 °C is near the s^4U_8·A_{14} interaction. Using the assignments given here, the order in which the tertiary interactions are stabilized by magnesium is (s^4)U_8·A_{14}, U_{33}, A_{58}·T_{54}, and G_{19}·C_{56}.

Interaction of tRNA with Tetraalkylammonium Ions. In the presence of tetraalkylammonium ions, tRNAs exhibit an overall loss in intensity in the low-field region, but there is a net gain in intensity in the spectra of *E. coli* $tRNA^{mixed}$ and yeast $tRNA^{Phe}$, but not *E. coli* $tRNA^{Met}_f$, in the region between 14.0 and 14.5 ppm. Upon addition of magnesium to yeast $tRNA^{Phe}$ in the presence of TMA the resonances between 14.0 and 14.5 ppm shift to higher field. This phenomenon can be attributed to a specific interaction between A·U base pairs and the tetraalkylammonium ions. Since *E. coli* $tRNA^{Met}_f$ has only two A·U base pairs, both of which may be destabilized in the presence of TMA and TEA, the effect is not observable for this tRNA. The suggestion that TEA and TMA somehow specifically interact with A·U base pairs is consistent with the results of Melchior and von Hippel (1973), who showed that these ions, at the proper concentration, eliminated the base composition dependence of the melting of DNA. Investigation of the interaction of mononucleotides with TEA and TMA gave no evidence of a special interaction with either A or U. This result indicates that the interaction of TMA and TEA with poly(A)–poly(U) would be an interesting investigation. Such studies may give information about the nature of the recognition of specific nucleotide sequences in polynucleotides.

Concluding Remarks

The polyamines spermine and spermidine have been shown to stimulate the biological activity of tRNA, and at one time it was thought that tRNA in the absence of magnesium is biologically active in the presence of polyamines (Igarashi et al., 1971). It is now believed that the samples were contaminated with magnesium (Chakrabartty et al., 1975). The NMR re-

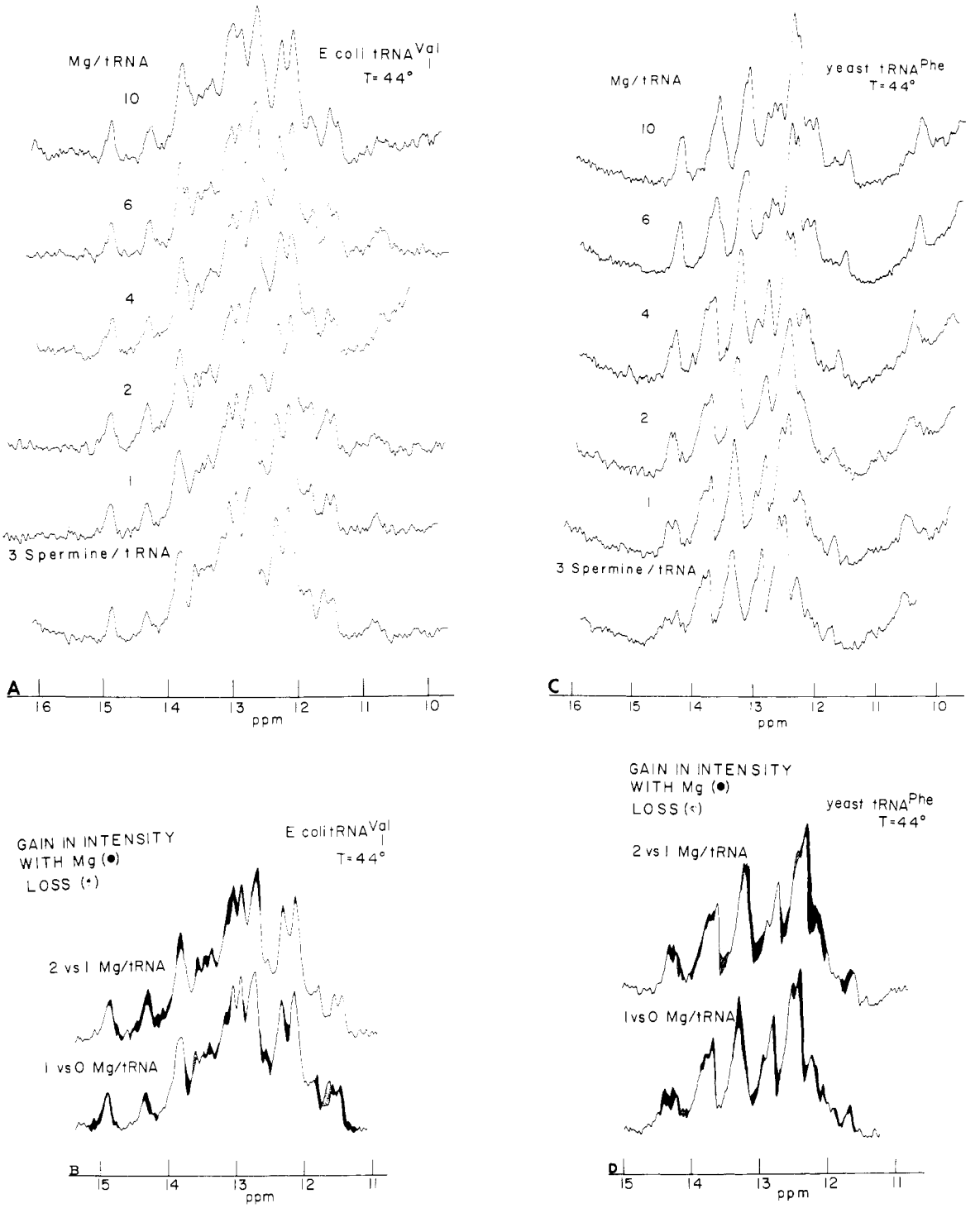

FIGURE 10: NMR spectra (300 MHz) of yeast tRNA^{Phe} and *E. coli* tRNA^{Val} in the presence of 3 spermine/tRNA and different levels of magnesium plus spermine. Also shown is the superposition of spectra in the presence of different levels of magnesium (spermine level constant). The dark areas are those which gain intensity upon the increase in magnesium concentration, and the shaded areas are those in which there is loss of intensity.

sults presented here show that these polyamines are not as effective as magnesium in stabilizing the native structure of tRNA and that the presence of polyamines, alone, does not induce the formation of the native structure in the absence of magnesium. However, our results show that spermine and spermidine induce the formation of the native structure in samples containing low levels of magnesium.

It has been reported that magnesium has no effect on the intensity in the spectrum of yeast tRNA^{Phe} in the temperature range considered here. Since the results of Hilbers et al. (1973) and those presented here and elsewhere (Bolton and Kearns, 1977) clearly demonstrate a magnesium effect on the spectrum of this tRNA, it is concluded that the samples for which no magnesium effect was observed may have been contaminated with magnesium.

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