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STUDIES ON THE EFFECT OF ZINC IONS ON tRNA STRUCTURE

A Dissertation

Presented for the

Doctor of Philosophy

Degree

The University of Tennessee, Knoxville

John Michael Flanagan

March 1986

TO MOM AND DAD
FOR THEIR LOVE AND SUPPORT

ACKNOWLEDGEMENTS

I express gratitude to all those people who have supported me during my tenure in the Biology Division. My sincerest gratitude goes to my advisor, Dr. K. Bruce Jacobson, for the time, thoughtful advice, and guidance he has given me over the years. Without his patience, goading and prodding none of this could have been possible. It has helped me to achieve things I did not think were possible.

I thank my dissertation committee, Drs. Larry Waters, Salil Niyogi, Gerard Bunick, and Don Olins. Their counsel, both individually and at the thesis meetings, was valued highly.

I thank the members of my lab group, past and present, including Dr. Eva Lee, Dr. Wendy Williams, Ms. Everline Wright, and Dr. Juan Ferre, who have helped me, technically and otherwise, over the years. I also thank Drs. Audrey Stevens and Robert K. Fujimura for their advice and encouragement.

A number of members of the Biology Division have helped me over the years. Dr. Waldo Cohn has reviewed manuscripts with insight and patience. Nette Crowe who prepared many of the figures appearing in this manuscript. Willena Carter and Dean Sheard who have provided valuable and essential assistance in the preparation of this and others manuscripts.

Finally, I especially thank my wife, Tammy Flanagan, for her understanding during the preparation of this manuscript.

This research was carried out in the Biology Division of Oak Ridge National Laboratory, and was supported by the Office of Health and Environmental Research, U.S. Department of Energy, under contract DE-AC05-84OR21400 with Martin Marietta Energy Systems, Inc.

ABSTRACT

The effects of divalent metal ions, particularly Mg^{2+} , Zn^{2+} and Cd^{2+} , on the structure of transfer ribonucleic acid (tRNA) in solution, were investigated using: chromatographic retention of tRNA on reverse-phase 5 (RPC-5) columns, ultraviolet difference spectroscopy, fluorescence intensity of the Wye (Y) base in yeast phenylalanine tRNA ($tRNA^{Phe}$), thermal denaturation, and imino-proton nuclear magnetic resonance (^1H -NMR) spectroscopy as probes.

Increased chromatographic retention of several tRNAs on RPC-5 was caused by Zn^{2+} but not by either Cd^{2+} or Mg^{2+} . Gradient and isocratic elution conditions were investigated. The chromatographic results were interpreted in terms of a model that evaluates the relative contributions of ionic and hydrophobic interactions. Of the two kinds, the hydrophobic forces increased markedly and the ionic to a less extent. Of four tRNAs examined, $tRNA^{Phe}$ (yeast) was unique in that the increase in retention was related biphasically to Zn^{2+} concentration whereas the other tRNAs showed a monophasic dependence. Both Cd^{2+} and Mg^{2+} eliminated the retention caused by 10 mM Zn^{2+} at 10 mM and 35 mM respectively. The biphasic nature of this reversal by Cd^{2+} and Mg^{2+} may indicate that the chromatographic effect is due to the binding of Zn^{2+} to two sites.

The Zn^{2+} binding sites in tRNA were also examined with the imino-proton spectra of yeast $tRNA^{Phe}$ and E. coli $tRNA^{Val}$. The largest effects of Zn^{2+} were on the imino-proton resonances of

(G-15)•(C-58), (G-19)•(C-56), (G-10)•(C-25), and (U-12)•(A-23).

Zn²⁺ appears to bind at both the Mg²⁺ sites in the loop formed by residues A-9, G-10, A-11, and U-12 (P-10 loop) and to the Mg²⁺ site in the "elbow" of the L structure near G-19, and G-20. Zn²⁺ may also bind in the terminal region of the extended D-helix, near G-15.

The increases in absorbance of tRNA at 294 nm, due to Zn²⁺, Cd²⁺ and Ni²⁺, suggest that these metals bind to sites on the bases, most probably the N-7 position of guanine; Mg²⁺ does not bind at this site. Since the increased absorbance of the 294-nm band saturates at micromolar metal ion concentrations these interactions are not responsible for the chromatographic effect that is not completed even at 20 mM.

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CHAPTER I

INTRODUCTION

Metal ions are essential for the normal function of almost all biological systems; they play important structural and functional roles in nearly every class of biological macromolecules. Though metal ions have long been recognized as integral components of the cellular machinery, the field of bioinorganic chemistry has, until recently, been neglected. Recent studies on interactions of metal ions with cellular components suggest that the modes of action of these molecules are extremely diverse [This subject is aptly reviewed in the series, Metal ions in Biological Systems, edited by Helmut Sigel (1973-1985)], with the physico-chemical properties of the specific ion involved being critical to its function. Because of the strict requirements placed on the chemistry of the metal ion involved, displacement of the ("correct") metal by another ("incorrect") metal can have disastrous consequences on macromolecular structure and function. This work is not meant as an exhaustive survey of the binding of divalent metal ions; in fact, I have limited the study to three: Mg^{2+} , Zn^{2+} , and Cd^{2+} . Both Mg^{2+} and Zn^{2+} are essential metal ions, while Cd^{2+} is one of the most toxic ones tested to date (Jacobson & Turner, 1981). These three ions also represent a broad range for the "hard-soft" classification scheme of metals (Pearson, 1963). A detailed discussion of the chemistry of these metals will be deferred until later in this chapter.

The macromolecule that has been chosen for study is tRNA, one of the most studied biologically relevant macromolecules; both in solution and in crystal form. The crystal structure of tRNA has been solved to a resolution of 2 Å (Hingerty et al., 1978; Quigley et al., 1979), while its solution structure has been investigated in detail by NMR spectroscopy (Patel, 1978; Reid, 1981), by x-ray and neutron small angle scattering (Li et al., 1985; Pilz et al., 1971), and by a number of other physical techniques. The primary cellular role of tRNA is as an "adapter" molecule in the flow of information from the genetic reservoir, DNA, to its ultimate destination, functional protein. Specifically, tRNA translates the information contained in the encrypted genetic code into the linear sequences of amino acids which define the three-dimensional, functional proteins. In fulfilling its role in translation tRNA must recognize and be recognized by a number of other protein and nucleic acid components, such as aminoacyl-tRNA synthetases, elongation factor Tu, numerous ribosomal proteins, possibly 5-S RNA, and mRNA. In almost all of these reactions, metal ions play an important role in not only the catalytic reactions of the proteins involved, but also the structural integrity of the tRNA (Goldberg, 1966; Lindahl et al., 1966; Karpel et al., 1975; Allende et al., 1965; Thompson et al., 1981; Svensson, 1967).

The structure of tRNA. A limited discussion of the structure-function relationships of tRNA will be presented. For a more

thorough treatment the reader is referred to the large number of review articles (Rich, 1977; Soll et al., 1978; Altman, 1978; Goddard, 1977; Kim, 1976; Kim, 1981; Schimmel & Redfield, 1980; Patel, 1978). Summarized here are some of the important structural features of this molecule, which are essential to understanding its interaction with metal ions in solution.

Typically, tRNAs are polynucleotide chains 75 to 90 nucleotides long. They contain not only the four major nucleotides, (those of adenine, guanine, uracil and cytosine), but also approximately 10% "rare" or "minor" nucleotides. The primary sequences of nearly 200 tRNAs have been collected (Sprinzl et al., 1985), and all can be arranged in the "cloverleaf" secondary structure first proposed by Holley et al. (1965). This representation will be shown in Figure 16 for yeast tRNA^{Phe}. The basic feature of this representation is the folding back of a single polynucleotide chain upon itself, with the formation of double-helical stems and looped-out single stranded regions.

The four stems of the cloverleaf each contain four to seven "Watson-Crick" base-pairs organized into double helices. The "acceptor" stem usually contains seven base-pairs and is the locus for both the 3' and 5' termini of the polynucleotide chain. At one end of the acceptor stem is the single-stranded A-C-C-A sequence, which constitutes the 3' terminus of the molecule. The other end of the acceptor stem, its 5' terminus, is involved in the first base-pair. The terminal 3' adenosine of the tRNA contains a free hydroxyl group, which can be esterified to its

cognate amino acid in the "charging" reaction. The dihydrouridine arm ("D-arm"), so named because of the presence of one or more dihydrouridine (D) residues invariably found in the single-stranded loop region at the end of this arm. The "anticodon arm" contains the triplet of bases which can recognize a complementary sequence on the mRNA during translation of the latter on the ribosome and specify the amino acid to be added to the growing peptide chain. The bases that make up the anticodon are located in the single-stranded loop at the end of this arm. The T or TΨC arm, (ribothymidine-pseudouridine-cytidine), contains this highly conserved sequence in its loop. Between the TΨC and anticodon stems is a single stranded region called the "extra loop"; in some tRNAs (class II) this region is composed of 13-21 nucleotides which then form what is called the "extra" arm, i.e., a helical stem and loop structure.

All tRNA sequences can be aligned in register so that the same nucleotide always occurs in some positions; these are called invariant nucleotides. In other positions, only purines, pyrimidines, or hypermodified purines are found; these are the "semi-invariants". Most of the invariant and semi-invariant nucleotides are found in the loop regions (Fig. 16), and seem to be required for maintenance of tertiary folding.

Even before the first X-ray crystal structure analysis of tRNA in 1973 (Kim et al., 1973), chemical and physical evidence was used to predict a tertiary folding of tRNAs. Two such models, one from small angle x-ray scattering (Pilz et al., 1971) which

correctly predicted the L-shape of the molecule, and another, theoretical model (Levitt, 1969) which correctly placed most of the tertiary base-pairs, helped define the conformation of the molecule.

Crystals of tRNA have been obtained in several laboratories (Cramer, 1971), and their structures have been solved by three independent groups on the orthorhombic (Holbrook et al., 1978; Sussman et al., 1978) and monoclinic (Hingerty et al., 1978; Stout et al., 1978; Jack, 1976) forms of yeast tRNA^{Phe} crystals. More recently, a number of other tRNAs have been crystallized and their structures determined; among them are yeast tRNA^{Asp} (Moras et al., 1980), initiator tRNAs^{fMet} from yeast (Schevitz et al., 1979) and E. coli (Woo et al., 1980), and yeast tRNA^{Gly} (Wright et al., 1979).

The crystal structures analyzed to date have revealed that tRNAs all have more or less the same L-shaped configuration, with each arm being about 70 Å long and 20 Å across; the latter dimension being indicative of an A-RNA-type helix. The two forms of yeast tRNA^{Phe} (orthorhombic and monoclinic) are practically identical although their packing in the crystal is different. The other tRNAs that have been analyzed all show small but significant variations from the structure seen in tRNA^{Phe}.

The two ends of the L are formed by the acceptor CCA end and the anticodon loop which are about 80 Å apart. The outer rim of the L-corner is formed by the TΨC loop. The acceptor and TΨC stems are stacked to form a continuous 11-base-pair double-helix,

as are the D and anticodon stems, although the overall helix formed by these stems has a 26° kink.

The L-structure is maintained not only by base-pairs of the Watson-Crick type, which account for most of the horizontal base-base interactions (predominantly in the stem regions), but also nonstandard base-pairs and base-triplets, and the base "stacking" interactions. The nonstandard base-pairs and base-triplets are generally found in the outer corner and hinge regions of the L where the two helical domains join. The base stacking interactions are the most striking feature of tRNA architecture; these interactions serve generally to reinforce the structure. The existence of these interactions has also been confirmed in solution by ¹H-NMR for all of the tRNAs studied to date (Hare et al., 1985; Hare & Reid, 1982; Roy & Redfield, 1982; Heerschap et al., 1982, 1983a,b).

The majority of the tertiary interactions appear to be between invariant or semi-invariant nucleotides. One example of a non-standard base-pair is that of G•U, which is found in the acceptor stem of tRNA^{Phe} (G-4)•(U-69) and in the TΨC stem of E. coli tRNA^{Val} (G-50)•(U-64), and elsewhere. Because of its overall structural similarity to a Watson-Crick base-pair, it has little effect on the double-helical structure of these regions. The (G-15)•(C-48) base-pair is a "reverse" Watson-Crick base-pair, with both chains running parallel. Antiparallel orientation is observed in the "reverse-Hoogsteen" base-pair (m¹A-58)•(T-54). In this base-pair, the Watson-Crick site on the adenine (N-1) is methylated so that

Hoogsteen pairing interaction is possible. Methylation of the N-1 position gives this residue a positive charge, which probably helps to favor this type of interaction. The purine-purine base-pair (m^2G-26)•(A-44) is longer than a normal Watson-Crick pair and is also nonplanar due to the steric repulsion of the dimethylamino group of (G-26) and the C2-H of (A-44). This base-pair is at the junction of the anticodon and D-stems and since it is stacked on both it gives rise to the 26° kink.

There are also several base-triplets consisting of a Watson-Crick base-pair to which, in the major groove side, another base is associated via one hydrogen bond in (m^2G-10)•(C-25)•(G-45) or via two bonds in (G-22)•(C-13)•(m^7G-46) and in (U-12)•(A-23)•(A-9), the latter being similar to the A•A base-pair found in the poly(AH⁺)•poly(AH⁺) duplex (Rich et al., 1961). In all of these interactions the polynucleotide chains are of necessity simultaneously parallel and antiparallel.

In yeast tRNA^{Phe}, 42 bases are involved in helical structures of the A-RNA type and thus involved in base-interactions. Of the remaining 34 bases, 29 more, which are not involved in traditional helical structures, are also stacked. Out of the 76 nucleotides in this molecule, a total of 71 are involved in stacking interactions with only G-20, U-47, the terminal A-76, and the two dihydrouridines (D-16 and D-17) not being involved in any stacking. The dihydrouricil residues are non-planar and non-aromatic; thus they are inherently incapable of stacking.

Of the 29 bases that are stacked, but not in an A form RNA helix, a number are involved in interesting stacking patterns such as: the stacking of the Watson-Crick base-pair (m^2G-10)•(C-25) over the (m^2_2G-26) of the long purine•purine pair mentioned above. Further in the TΨC loop, both the reversed Hoogsteen pair (m^1A-58)•(T-54) and the constant (G-18)•(C-61) pair, which contains only one hydrogen bond, are stacked on the Watson-Crick pair (G-53)•(C-61); the latter is also invariant. The two guanines of in this region (G-18 and G-53) are also involved in an interaction with G-57 where the latter residue is intercalated between G-18 and G-53, forming a "guanosine sandwich". Also involved in intercalation interactions are: G-45 and m^7G-46 which have A-9 between them; C-13 between U-8 and A-9; and G18 between m^1A-58 and G-57. Because base insertion requires a larger spacing between adjacent residues, the ribose-phosphate backbone must readjust; this is done by switching one of the ribose puckerings from the preferred C3'-endo to C2'-endo.

At all physiological pH's, tRNA is polyanionic, and therefore must be surrounded by numerous cations. Due to its polyanionic nature, the water associated with this molecule is electroconstricted and therefore has a greater density than bulk water (Li et al., 1983). What is suprising, however, is that certain cations must be present to stabilize the native structure of tRNA (Fresco et al., 1966; Soll et al., 1980; Schimmel & Redfield, 1981). Moreover, for divalent cations, binding under certain conditions can be cooperative and having both strong and weak

sites. This has been shown by numerous experimental techniques: equilibrium dialysis, NMR spectroscopy, fluorescence quenching, and calorimetry (Stein & Crothers, 1976; Bina-Stein & Stein, 1976; Scheier & Schimmel, 1974; Dachin & Gueron, 1970; Karpel et al., 1975; Jones & Kearns, 1974; Lynch & Schimmel, 1974).

The strong binding sites are characterized by association constants of about 10^5 liters/mole (Bina-Stein & Stein, 1976), and appear to be cooperative at low monovalent ion concentrations. The time constants for exchange of Mg^{2+} are on the order of seconds due to the high activation energy, around 30 kcal/mol, involved in displacing tightly bound Mg^{2+} (Lynch & Schimmel, 1974). Weak binding sites have association constants on the order of 10^3 liter/mole (Bina-Stein & Stein, 1976) and do not display cooperativity.

Transfer RNA, like other nucleic acids, contains four different types of sites that may bind metal ions: the negatively charge phosphate oxygen atoms, the ribose hydroxyls, the base ring nitrogens, and the exocyclic amino and keto groups of the bases. These sites have varying affinities for Mg^{2+} , with the negatively charged phosphates having the highest affinities and the ribose hydroxyls the lowest. Mg^{2+} appears to be essential for the maintenance of the native tRNA structure because it can cross-link phosphate groups (Holbrook et al., 1977). This can clearly be seen in all of the crystal structures to date; most of the strong Mg^{2+} binding sites are in regions of sharp bends in the polynucleotide backbone where anionic phosphates oxygens are brought

close together (Holbrook et al., 1977; Hingerty et al., 1978; Quigley et al., 1978).

The chemistry of Mg^{2+} , Zn^{2+} and Cd^{2+} . A complete characterization of these three metals and their associated interactions would be impossible in the present framework; a more complete description can be found in the book Inorganic Biochemistry, edited by Eichhorn, (1975). Both Cd^{2+} and Zn^{2+} are transition metals while Mg^{2+} is an alkaline earth metal. The electronic configuration of Mg^{2+} is the same as that of Ar or, $1s^2 2s^2 p^6$, while for Zn^{2+} it is $[Ar]3d^{10}$, and Cd^{2+} , $[Kr]4d^{10}$.

All alkaline earth metals are very electropositive; therefore most of the compounds formed by alkaline earth metals are ionic in character. Due to its small size, Mg^{2+} can approach a ligand closely and have a polarizing influence on it, giving these complexes covalent character. To a large extent, the attached ligands of alkaline earth metal complexes assume a regular stereochemistry dictated only by intra-ligand repulsion i.e. there are no steric requirements imposed upon the ligands by the metal ions themselves. Therefore, complexes involving alkaline earth metals assume coordination geometries with high symmetries (i.e. octahedral). In aqueous solution, Mg^{2+} forms the octahedral complex, $Mg[H_2O]_6^{2+}$.

Zn^{2+} and Cd^{2+} are both transition metals, and each has full outer d orbitals. The stability of complexes formed by the first

row transition elements with a given ligand follows the general order



the Irving-Williams order of stability (Irving & Williams, 1948). The decrease in stability of Zn^{2+} complexes as compared to Cu^{2+} can be explained by the lack of crystal field stabilization energy (CFSE) for Zn^{2+} , which has a full d shell. This also explains why, in the series from Ca^{2+} to Zn^{2+} , the stability plots have a double hump, with nodes at Ca^{2+} (d^0), Mn^{2+} (d^5) and Zn^{2+} (d^{10}). Correcting for CFSE gives a linearly increasing stability curve for the elements from Ca^{2+} to Zn^{2+} . This would be the expected result in the absence of crystal field stabilization energy since the ligands' attraction for the metal ion will be greater as the effective charge on the metal increases.

The $2+$ species is the only aquated ion known for Zn, and all complexes observed to date are with this species. Due to the lack of crystal field stabilization forces, the stereochemistry of its complexes are determined entirely by electrostatic and covalent bonding forces, and by ligand size. Hence, a large number of geometries have been observed for Zn^{2+} complexes such as: tetrahedral, octahedral, trigonal bipyramidal, and square pyramidal, the most prevalent geometry being the tetrahedral, with trigonal and square pyramidal being the least prevalent.

Like Zn^{2+} , complexes involving Cd^{2+} will not have any CFSE since it too has a full d orbital. Because both cadmium and zinc

are in the same group of the Periodic Table we would expect their chemistry to be similar. This, however, is an oversimplification; since cadmium is a larger ion and thus has a lower charge density, it is more polarizable than Zn^{2+} ; therefore, its complexes will have greater covalent character than those of Zn^{2+} . Like Zn^{2+} , Cd^{2+} tends to form tetrahedral complexes in solution, although its coordination complexes are not restricted to this geometry. This in fact is one of the characteristics of Cd^{2+} ; its coordination complexes can assume widely different geometries. In this respect, Cd^{2+} more closely resembles the third member of the group, Hg^{2+} , than it does Zn^{2+} .

It would seem that correlations of stability constant values with the charge and size of the metal ion should occur and indeed a few have been observed. For a broad range of metal ions and ligands, it is clear that few correlations do occur and more fundamental properties of metals are needed to explain their reactive properties. In the case of Fe^{3+} and Hg^{2+} the stability constants for halide complexation are exactly reversed from each other. For Fe^{3+} they decrease in the order $\text{F}^- > \text{Cl}^- > \text{Br}^- > \text{I}^-$, while for Hg^{2+} , they increase in that order. This has led a number of authors (Schwarzenbach, 1956; Ahrlund et al., 1958) to conclude that there are two classes of metal ions: those that behave like Fe^{3+} , and those that behave like Hg^{2+} . Pearson (1963) formulated these tendencies into a quantitative scale based on the polarizability of these metal ions and a ρ value was calculated for each ion from thermodynamic properties. Ions like Fe^{2+} were

called "hard" (large value of σ_p) since they can not be easily polarized and they like to complex with ions that are also "hard". Ions like Hg^{2+} were designated as "soft" (smaller values of σ_p) since they can be more easily polarized. As a general rule, hard metals prefer donor atoms such as nitrogen, oxygen or fluorine, while soft metals prefer iodine, sulfur, or phosphates.

The σ_p values for Mg^{2+} , Zn^{2+} , and Cd^{2+} are 0.167, 0.115, and 0.081, respectively. Mg^{2+} is considered a hard metal, Cd^{2+} a soft metal and Zn^{2+} is a borderline metal in this classification scheme.

Complexes of chelating ligands (polydentate), are more stable than those of comparable monodentate ligands. For example, the stability constants for the complexation of Ni^{2+} by ethylenediamine (en) are larger than those for complexation by two NH_3 molecules. This is, in part, due to the increased entropy that results from formation of three moles of products as compared to two of reactants when en displaces NH_3 . It must also be noted that ΔH is also negative for this reaction; this is generally believed to be due to repulsion between the two NH_3 groups in the transition complex for the formation of the new Ni(en)^{2+} complex (Martell, 1976).

Complexes of Mg^{2+} , Zn^{2+} and Cd^{2+} with nucleotides. To provide some insight on metal-nucleic acid complexes some known complexes of these three and other related metals and nucleotides are discussed as a first approximation of their interactions in nucleic acids. This topic has been reviewed extensively in the

book, Nucleic Acid-Metal Ion Interaction edited by Spiro (1980). The crystal structures of a large number of complexes of metals with nucleic acid components have also been compiled and reviewed previously (Marzelli, 1977; Hodgson, 1977; Gellert & Bau, 1979; Swaminathan & Sundaralingam, 1981). In this discussion we exclude interactions with the ribose portions of these molecules since, as a general rule, sugar molecules complex poorly with alkaline earth and transition metals; the lack of complex formation with these metal ions, is in part, due to the low polarizability of these sites. In fact, while the purine and pyrimidines serve as excellent ligands for metal coordination, the stability constants of metal-nucleotide complexes have a large component of electrostatic attraction between the electrophilic metal center and the anionic phosphate groups. This is reflected in the increasing stability constants for metal ion complex formation in the series $\text{Ado} < \text{AMP} < \text{ADP} < \text{ATP}$; the stability of the complex increases with increasing phosphate content. The stabilities of these complexes are also affected by the nature of the metal ion involved. For the first row transition metals the order of stability of binding to ATP^{-4} follows the Irving-Williams order: $\text{Ba}^{2+} < \text{Sr}^{2+} < \text{Mg}^{2+} < \text{Co}^{2+} < \text{Mn}^{2+} < \text{Zn}^{2+} < \text{Ni}^{2+} < \text{Cu}^{2+}$ [Khan & Martell, 1966]. This order has been shown to be independent of the base in the nucleotide. Though the stability constants follow the predicted order, the differences in log K values was very small. In this study there was no evidence for any of these metals, except for Cu^{2+} , binding to the bases.

Other studies, using ^{15}N -NMR as a probe, suggest that Ni^{2+} , Zn^{2+} and Mn^{2+} bind at the N-7 position of the adenine in AMP as well as the phosphates (Cohen & Hughes, 1962), whereas Ca^{2+} and Mg^{2+} , the alkaline earth metals, bind exclusively to phosphate oxygens. The stability of alkaline earth metal ion complexes decreases with increasing atomic number. This can be explained by the fact that for a group in the Periodic Table, the charge-to-radius ratio decreases with increasing atomic number. Since Mg^{2+} has the smallest charge-to-radius ratio in the alkaline earth series its complexes with nucleotides will be the strongest.

Binding of metals at the amino group of the bases has been ruled out because, a) on theoretical grounds the would-be free electron pair on the nitrogen is required to stabilize an already electron-deficient aromatic ring system (Schneider et al., 1964), and b) there is little or no experimental evidence for this mode of binding from NMR experiments, (Eichhorn et al., 1966; Happe & Morales, 1966), and x-ray crystallographic studies (Carrabine & Sundaralingam, 1971). These studies show that, for the 5' nucleotides of adenine, the N-7 position is the preferred site for interaction. The predominance of metal interaction at the N-7 position is probably due to its proximity to the phosphate groups when the nucleotide is in the preferred anti-conformation.

Other non-phosphate sites for metal binding on nucleic acid components are at the O^2 and N^3 site on cytosine, and the N-7 and O^6 sites on guanosine and inosine. Base interaction for nucleotides of uridine and thymidine are not normally observed, although

they are possible; binding of the metal could occur at the N-3 (deprotonated), O², or O⁴ positions. A number of possible sites which may be seen for the free base, nucleoside or nucleotide, (N-1 of A, N-3 and O⁴ of T, O⁶, and N-1 of G, and O², N-3 of C) are highly unlikely in double stranded polymers, where Watson-Crick base-pairing exists, because these sites are involved in the interbase interactions. Binding of metal ions at the N-3 position of purines is usually not observed because of steric interference due to the pentose moiety at the N-9 position in nucleosides and nucleotides.

Binding of metal ions to these "soft" sites increases with the increasing softness of the metal, which occurs as we progress across and down the Periodic Table. An example of this is thought to be the increased stability, 40 times greater, for the $[\text{Mn} \cdot (5'\text{-GMP})]^{2+}$ complex as compared to $[\text{Mg} \cdot (5'\text{-GMP})]^{2+}$ (Podder, 1974), which has been attributed to the ability of Mn^{2+} to bind to the N-7 position of 5'-GMP. This does not mean, however, that "soft" metals do not interact with the phosphates; in fact, metals at the far right of the Periodic Table, such as Co^{2+} and Cu^{2+} , can form covalent complexes with phosphates. Prime examples of this behavior are $[\text{Cu}^{2+}(3'\text{-GMP})(\text{phen})(\text{H}_2\text{O})]$ (Wei et al., 1978), and $[\text{Co}_2(5'\text{-UMP})_2\text{-H}_2\text{O}]_n$ (Cartwright et al., 1977).

The observed principles of metal ion binding to nucleotides, as discussed above, serve as the basis for these studies on metal ion interaction with nucleic acids. In these studies the observed metal-ion nucleic acid interactions are qualitatively similar to

those seen at the nucleotide level; however, the complex stereochemical requirements of metal-binding sites on nucleic acids add additional constraints.

CHAPTER II

EFFECT OF ZINC IONS ON tRNA STRUCTURE:

REVERSED PHASE CHROMATOGRAPHY

Introduction

Divalent metal ions affect both the structure and biochemical activity of nucleic acids and considerable effort has been expended to determine the nature of these metal ion interactions (Mildvan & Loeb, 1979; Spiro, 1980). For both RNA and DNA there seem to be four generally accepted modes of interaction: direct (inner-sphere) coordination of the metal ion to phosphate groups; second (outer) sphere coordination through water bridges to the phosphates; diffuse atmospheric binding; and binding of the metal ions at electron-donor sites on the heterocyclic bases. These interactions are thought to neutralize the negatively charged phosphate groups, which helps to stabilize the secondary and tertiary structure of the nucleic acid.

Evidence for all four modes of interaction comes from X-ray structural analysis of yeast tRNA^{Phe} in the presence of various divalent metal ions (Teeter et al., 1980; Rubin et al., 1983; Brown et al., 1983). Rubin et al. (1983) found five strong binding sites for Zn²⁺ ion in their crystals; two of which had displaced previously bound Mg²⁺. These two sites represent the most tightly bound of the five Zn²⁺ ions.

The effects of ions on the overall structure of tRNA in solution has also been investigated using fluorescence (Ehrenberg et al., 1979; Ehrlich et al., 1980), ESR (Nöstnig-Laslo et al., 1985) neutron and X-ray scattering techniques (Li et al., 1983; Pilz et al., 1971), light scattering (Olson et al., 1976; Fritzinger & Fournier, 1982), and thermal denaturation (Eichhorn & Shin, 1968). These studies showed that a complete description of nucleic acids in solution must include not only the secondary and tertiary structure of the nucleic acid but also the tightly associated ions and the tightly bound water molecules.

The physicochemical properties of metal ions and their relevance to biomolecular interactions have been reviewed recently (Jacobson & Turner, 1981). These authors emphasized that the "hard-soft" characterization of metal ions provides a means of comparison for the potential of various metal ions to bind to charged and uncharged sites on nucleic acids. In the present study, the effects of Mg^{2+} (hard), Cd^{2+} (soft), and Zn^{2+} (intermediate) will be compared for their effect on the chromatographic behavior of tRNA. The σ_p [softness parameter defined by Pearson (1963)] for these divalent ions are 0.167, 0.115, and 0.081 for Mg^{2+} , Zn^{2+} and Cd^{2+} respectively.

With respect to the RPC-5 column, Hiatt and Jacobson (1980) showed that Zn^{2+} causes a marked increase in the concentration of salt required for tRNA elution while Mg^{2+} and Cd^{2+} have small specific effects. Cd^{2+} prevented the resolution of isoacceptors

of tyrosyl-tRNA that differ by the presence of guanine or queuine in the "wobble" position of the anticodon (Jacobson & Lee, 1984).

RPC-5 chromatography has long been used to separate nucleic acids (Kelmers et al., 1971; Wells et al., 1980; Larson et al., 1979), and is sensitive to the molecular weight (Kelmers et al., 1971; Wells et al., 1980), base composition (Patient et al., 1979), and secondary and tertiary structure (Best et al., 1981; Wells et al., 1980) of the nucleic acids. The resolution of nucleic acids on RPC-5 is such that tRNAs that differ only by a methyl group (Bell et al., 1978; Shugart et al., 1968) or the presence or absence of dihydrouridine (Lo et al., 1981; Waters et al., 1973) are separable. Also tRNA^{Trp} is readily separated from Trp-tRNA^{Trp}, presumably because of the hydrophobic amino acid (Waters et al., 1975a and b). RPC-5 may be a powerful tool in distinguishing small changes in tRNA conformation. The support material for RPC-5 consists of a polymer of dichlorodifluoroethylene in bead form coated with tetraalkyl quaternary ammonium ions (Pearson et al., 1971). Chromatography on this matrix is thought to occur through mixed-mode interactions, where both ion-exchange and hydrophobic mechanisms play important roles in retention.

For this study the interactions between the RPC-5 chromatographic matrix and tRNA^{Phe} (yeast) and selected tRNAs from E. coli were examined in relation to three divalent cations, temperature, and pH.

Materials and Methods

Materials. Plaskon CTFE 2300 was purchased from Allied Chemical Co. (Morristown, NJ; no longer commercially available); Voltalef 300LD-micropowder from Produits Chimiques Ugina-Kuhlman (Cedex 21, 92087 Paris La-Defense, France; the American distributor is Atochem Polymers, 139 Harristown Road, Glen Rock, NJ); Adogen 464 from Ashland Chemical Co. (Columbus, OH); Aminex A28 from Bio-Rad Co. (Richmond, CA). The yeast tRNA^{Phe} was purchased from Boehringer Mannheim Biochemical Co. (7941 Castleway Drive, Indianapolis, IN 46250); E. coli tRNAs from Subriden RNA (P. O. Box 121, Rolling Bay, WA 98061). All other chemicals were of reagent grade.

Preparation of RPC-5 matrix and columns. The RPC-5 column matrix was prepared by adsorption of Adogen 464 to Plaskon beads, and the columns were slurry-packed with equipment and by procedures described previously (Flanagan et al., 1986). Chromatography was performed at 37°C at pH 4.6 in 10 mM NaOAc buffer except where stated otherwise. The elution was accomplished either by a linear gradient of NaCl (0.5-1.10 M) or by isocratic elution with the NaCl concentration fixed. The divalent ions were added as either chloride or acetate salts, usually the latter since the chloride forms are more acidic. For each experimental condition the column was equilibrated with 30 mL of solution before sample addition; the sample was dissolved in the same experimental solution. Elution was monitored at 260 nm. Salt concentration was

determined by refractive index measurement. Routine conditions for RPC-5 chromatography are described by Flanagan et al. (1986).

Chromatographic parameters. The fundamental parameter in liquid chromatography is the chromatographic capacity ratio k' , which is equal to n_s/n_m where n_s is the total number of moles of solute in the stationary phase, and n_m the total number of moles of solute in the mobile phase (Snyder & Kirkland, 1979). The capacity ratio is determined under isocratic conditions and can be related to the measurable quantities V_r , the elution volume of the solute, and V_o , the elution volume of an unretained solute, e.g., uridine, by the equation:

$$k' = \frac{V_r - V_o}{V_o} \quad [1]$$

The capacity ratio can also be expressed in terms of the equilibrium distribution constant:

$$k' = \frac{K_{eq}V_s}{V_m} \quad [2]$$

where V_s is the volume of the stationary phase and V_m the volume of the mobile phase.

Gradient elution of tRNA is accomplished by a progressive decrease in the relative affinities of the anionic sites on the tRNA for the column's quaternary ammonium ions, in response to the increasing concentration of counter ion in the eluent. As a

result, k' cannot be determined directly. For gradient elution we shall refer to the relative k' values of the solute tRNA which are large at the beginning of the chromatographic separation, and small at the point of elution. Relative k' values during migration down the column for each solute will be in the range $1 \leq k' \leq 5$ allowing the optimal resolution of all solute components (Snyder & Kirkland, 1979).

A second feature of gradient chromatography is that in the absence of multiple types of solute interactions with the column (i.e., ion-exchange and hydrophobic interactions), solutes leave the column with relative k' of about 1. Since all solutes have about the same relative k' when exiting the column, they should all have nearly the same peak width at half-height. This is not always the case in practice due to the existence of more than one mechanism for chromatography. Gradient chromatography also reduces peak tailing because the trailing edge of the peak will always be in a region of lower relative k' than the leading edge; thus solute molecules in the band tail will migrate at a faster rate relative to the band center, eliminating tailing. Gradient separations will be only weakly dependent upon column length because solvent composition is the primary basis of separation. In these experiments, the ionic strength was measured at the point of maximum peak height and elution conditions are compared on this basis.

Results

Gradient elution of tRNA^{Phe} in the presence or absence of Zn²⁺, Mg²⁺, or Cd²⁺. Chromatography of yeast tRNA^{Phe} in the presence of Mg²⁺, Cd²⁺, Zn²⁺ or in the absence of divalent metal ions is depicted in Figure 1. The presence of 10 mM Mg²⁺ or Cd²⁺ had little effect on the retention of yeast tRNA^{Phe}.

Ten mM Zn²⁺, however, produced marked changes in chromatographic behavior. Zn²⁺ caused the tRNA to elute as a broad, tailing band which emerged very late in the gradient. The broadened and asymmetric nature of the elution band in the presence of Zn²⁺ is quite unexpected if ion-exchange was the only mechanism for retention since, as discussed above, even late-eluting peaks should still be sharp and symmetrical. Peak tailing has been shown, in some cases (Sokolowski & Wahlund, 1980), to be due to the presence of multiple chromatographic mechanisms during the separation; this would also account for the increased band width observed in the presence of Zn²⁺. Recovery of tRNA in all cases exceeded 85% in the presence or absence of the divalent cations.

The relationship between tRNA^{Phe} retention on RPC-5 and Zn²⁺ concentration is shown in Figure 2. At concentrations up to 0.5 mM, Zn²⁺ had no effect on yeast tRNA^{Phe} retention. Above 0.5 mM Zn²⁺, the retention was increased in a biphasic manner. The effect of Zn²⁺ was also investigated with RPC-5 in which Voltalef 300LD-micropowder replaced the Plaskon as the solid

Figure 1: RPC-5 chromatography of tRNA^{Phe} in the absence of metals and in the presence of Mg²⁺, Cd²⁺, and Zn²⁺. In the presence of Mg²⁺ or Cd²⁺, tRNA^{Phe} eluted at the same ionic strength as for no metal. Yeast tRNA^{Phe} (100 µg) was chromatographed on RPC-5 (1 × 10 cm) column at 35°C using a 100 mL linear gradient from 0.5 to 1.1 M NaCl that included 10 mM acetate pH 4.6. The flow rate was 1 mL/min, and 1 mL fractions were collected. The concentration of NaCl was determined by refractive index. The divalent cation concentration was 10 mM when present.

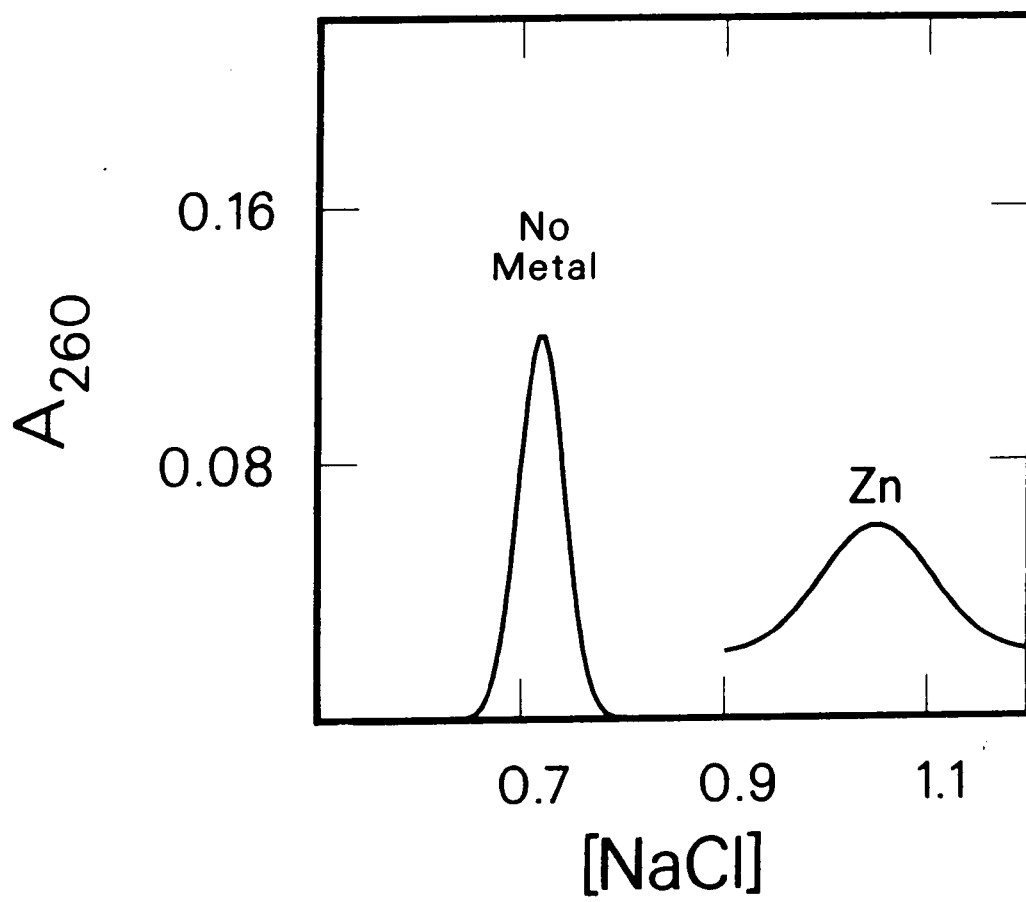


Figure 1

Figure 2: The dependence of tRNA^{Phe} retention on Zn²⁺ concentration. Two RPC-5 columns were prepared, one with Plaskon (- □ -) and the other with Voltalef (- ■ -) powder as the solid support material. Yeast tRNA^{Phe} (50-100 µg) was chromatographed at various concentrations of Zn²⁺ under the conditions described in Figure 1. The ionic strength of the peak fraction was determined from the refractive index and corrected for the contribution of Zn(OAc)₂. The ionic strength of the peak fraction is plotted relative to the Zn²⁺ concentration in the elution buffer.

Ionic Strength of Peak (M)

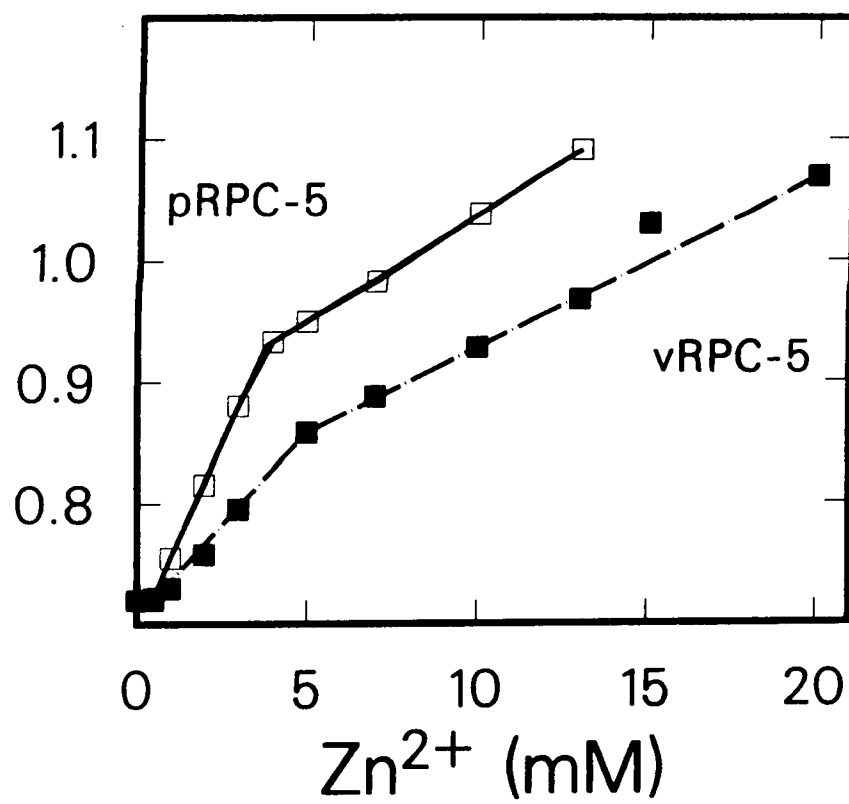


Figure 2

support material (vRPC-5) (Flanagan et al., 1986). Columns prepared with this material demonstrated a Zn^{2+} effect, however the tRNA eluted at a somewhat lower ionic strength at all Zn^{2+} concentrations above 0.5 mM Zn^{2+} .

Zn^{2+} ions also affected the chromatography of three closely related E. coli tRNAs (for valine, lysine and isoleucine) (Figure 3). These tRNAs co-chromatographed on RPC-5 under the conditions chosen, and were affected similarly by Zn^{2+} . As with tRNA^{Phe} , Zn^{2+} increased the retention of all three tRNAs, though the functional dependence of this increase was not biphasic. In contrast to tRNA^{Phe} (Figure 2), there was a linear relationship between the retention on RPC-5 and concentration of Zn^{2+} .

The quaternary ammonium ion used in RPC-5 chromatography is composed of a methyl group and three hydrocarbon chains of 8-10 methylene groups, which are generally thought to be able to interact with the tRNA and affect retention. Aminex A-28 is also effective for separating tRNA isoacceptors though its quaternary ammonium ion contains only methyl groups and is covalently attached to a styrene divinylbenzene copolymer. The effect of Zn^{2+} on yeast tRNA^{Phe} chromatography on Aminex A-28 was evaluated for a wide range of Zn^{2+} concentrations (Figure 4). No increased retention due to Zn^{2+} was observed at concentrations up to 30 mM.

Isocratic chromatography on RPC-5. The mechanism of chromatography on RPC-5 includes ion-exchange and hydrophobic

Figure 3: Effect of Zn^{2+} concentration on ionic strength for elution of three tRNAs. *E. coli* tRNAs valine ($\square$), leucine ($\triangle$), or isoleucine ($\circ$) (50-100 μg) were chromatographed in a 100 mL linear gradient from 0.4 to 1.0 M NaCl, and increasing fixed concentrations of $\text{Zn}(\text{OAc})_2$ (all other conditions same as Figure 1).

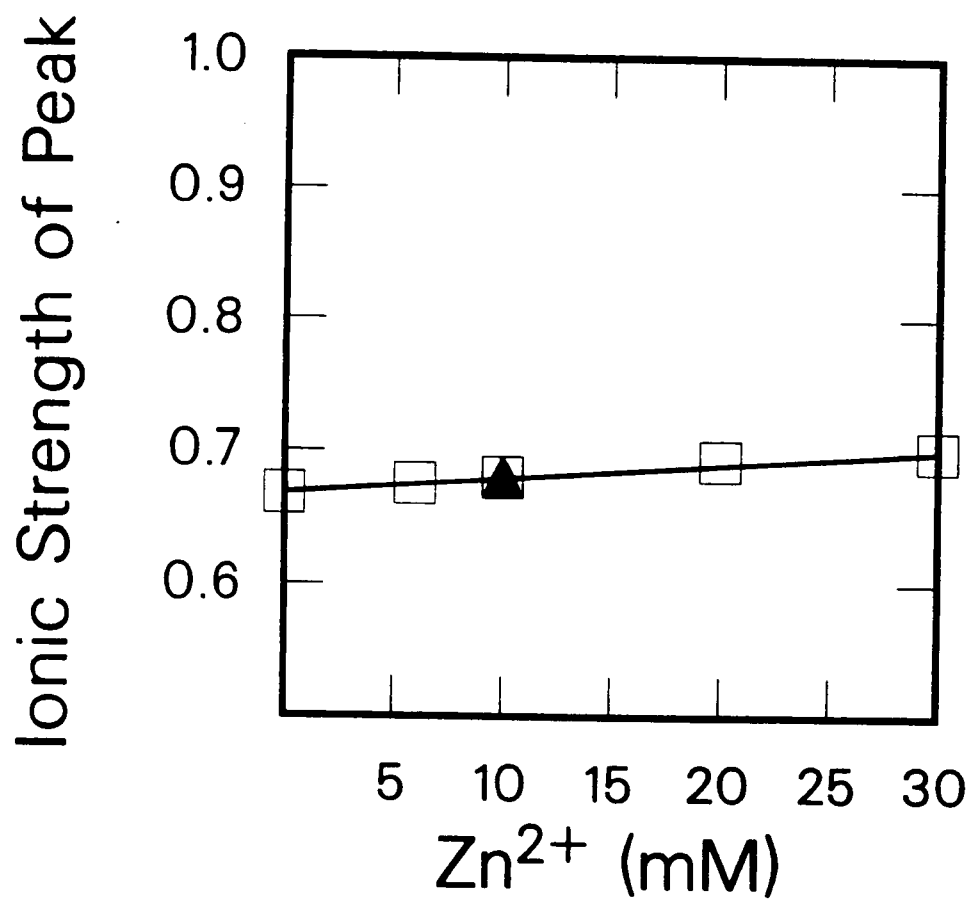


Figure 3

Figure 4: Chromatography of tRNA^{Phe} at various divalent cation concentration on Aminex A28. Yeast tRNA^{Phe} was chromatographed on Aminex A28 (1 × 20 cm), using the chromatographic conditions in Figure 1, with various fixed concentrations of Zn(OAc)₂ (○) or 10 mM Mg(OAc)₂ (□).

Ionic Strength of Peak (M)

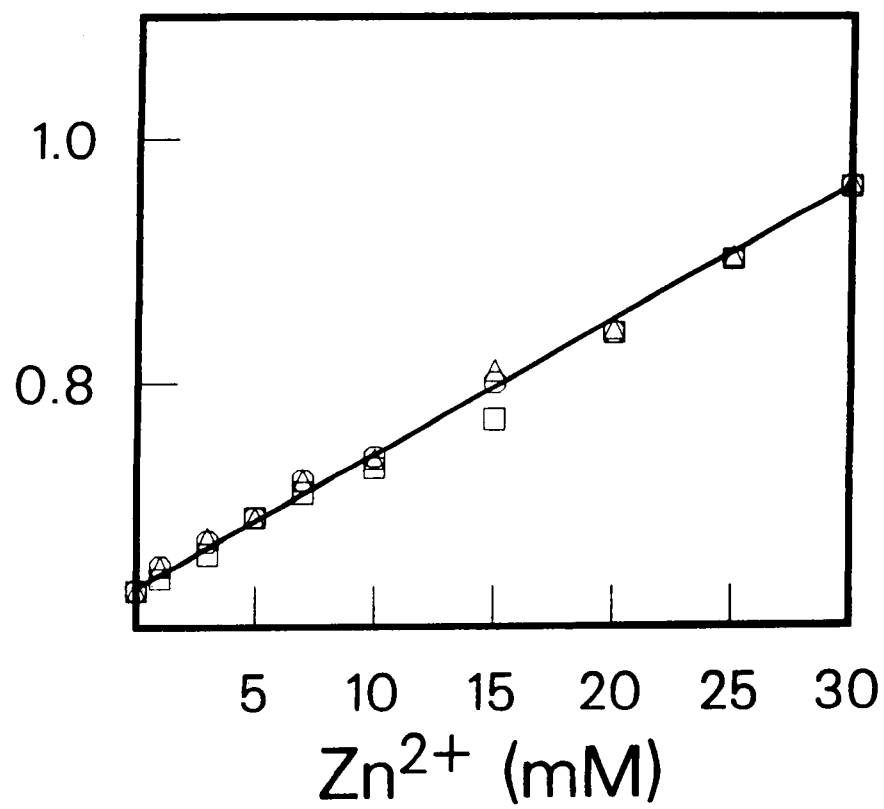
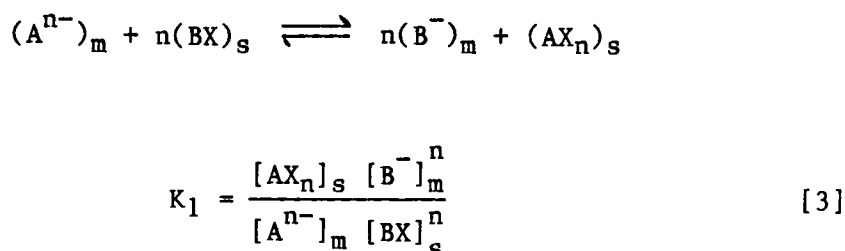
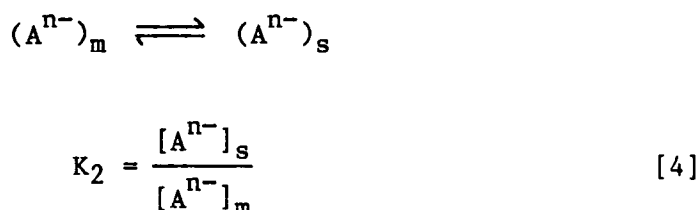


Figure 4

interactions. The ion-exchange reactions are given by equation [3]



The subscripts s and m denote the stationary and mobile phase respectively, A, the phosphate anions of the tRNA, B, the negatively charged counter ions, and X, the quaternary ammonium compound. Hydrophobic sites on the tRNA can also interact with the hydrophobic surface of the column which consists of the C₈₋₁₀ hydrocarbon side-chains of the quaternary ammonium ion and the support bead of RPC-5. These interactions can be represented as:



The overall equilibrium constant K_{eq} , is given by equation 5.

$$K_{eq} = \frac{[tRNAs]_s}{[tRNA]_m} = \frac{[AX_n]_s + [A^{n-}]_s}{[A^{n-}]_m} \quad [5]$$

Combining equations 2-5 gives (eq. 6)

$$k' \propto K_{eq} = K_1 C \left(\frac{1}{[B^-]_m} \right)^n + K_2 \quad [6]$$

where C is the concentration of the ammonium ion in the stationary phase. K_1 is the ion-exchange equilibrium constant and K_2 is the hydrophobic interaction constant which is defined as independent of added salt. The concentration of B, the chloride ion, can be determined from the refractive index of the solution, while k' can be calculated from isocratic retention data using equation 1.

Isocratic chromatography in the presence and absence of Zn^{2+} . Isocratic elution was used to evaluate the chromatographic parameters in equation 6. By determining k' at different fixed concentrations of NaCl the relative contributions of K_1 and K_2 were evaluated. The value of n, the number of chlorides displaced from the chromatographic support upon tRNA^{Phe} binding, can also be calculated from equation 6.

Figures 5a-c show the results of model calculations where the effects of salt concentration on k' were investigated for various assumed values of n, K_1 , and K_2 . Figure 5a shows how varying n (in equation 6), the number of anionic sites on the tRNA interacting with the cationic column, affects the calculated values of k' as a function of NaCl concentration. As a function of increasing n the range of salt concentrations required to reduce k' from a value of 10 to 1 decreases. The effect of increasing K_1 , the ion-exchange parameter, on the calculated k'

Figure 5: Calculated effects of the parameters n , K_1 , and K_2 on the chromatographic capacity ratio, k' , as a function of $[\text{NaCl}]$. The capacity ratio, k' , was calculated from Eq. 6 for a) fixed values of $K_1 = 1.0$, $K_2 = 0.5$ and varying n (1, 3, 5, 10), b) fixed $n = 10$, $K_2 = 0.5$ and varying K_1 (1.0, 2.0, 2.5), and c) fixed $n = 10$, $K_1 = 1.0$ and varying K_2 (0.0, 0.5, 1.0).

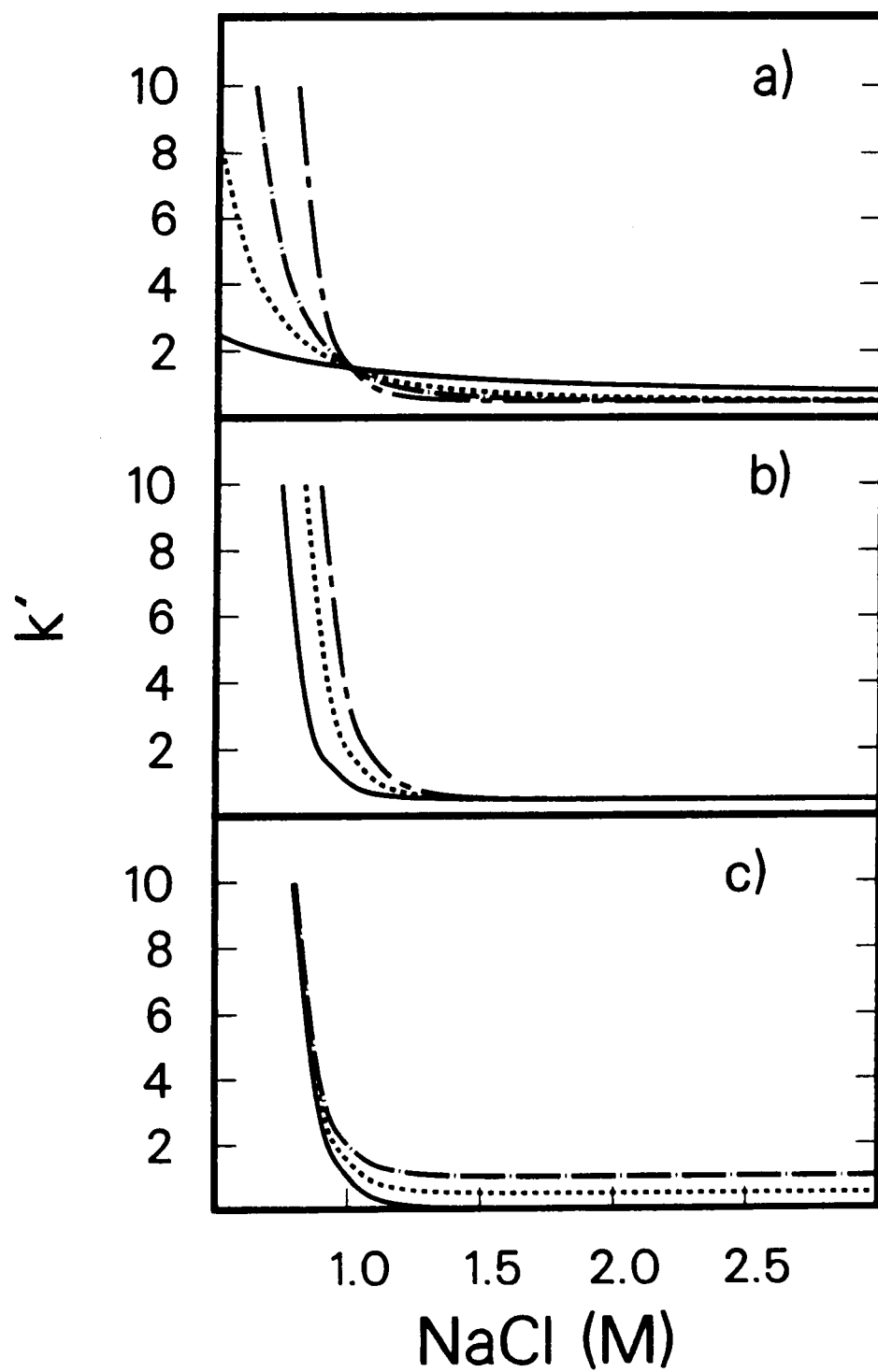


Figure 5

vs [NaCl] curves is to shift them to higher salt concentrations (Figure 5b). The important point here is that n and K_1 are independent parameters, so that altering the number of anionic-cationic interactions does not necessarily affect K_1 , the strength of these interactions. Increasing K_2 , the hydrophobic interaction parameter, increases the value of k' obtained at all salt concentrations (Figure 5c). This is because the effect of K_2 on k' is independent of added salt. According to the model K_2 can be determined easily at salt concentrations that eliminate ionic effects (Figure 5a).

The experimentally determined effect of increasing salt concentrations on k' for isocratic elution of tRNA^{Phe} is shown in Figure 6. At least three effects of Zn^{2+} are apparent. Zn^{2+} displaces the curve to higher $[Cl^-]$. Also Zn^{2+} causes the tRNA^{Phe} to be retained at high salt concentrations. Finally, Zn^{2+} causes k' to vary over a wider range of salt concentration than occurs in the absence of Zn^{2+} . Table 1 summarizes the effects of Zn^{2+} on the chromatographic parameters n , K_1 and K_2 that are calculated by fitting equation 6 to the isocratic elution data. The calculated values of K_1 and K_2 increase in the presence of Zn^{2+} , indicating that affinity of tRNA for the column through the hydrophobic and ion-exchange binding modes are both increased. The presence of Zn^{2+} also decreases n , the number of chloride ions displaced upon tRNA^{Phe} binding, from > 9 to 5. The actual number of chloride ions displaced when no metal is present must be viewed with caution since curves with $n > 9$ are practically

Figure 6: Isocratic elution of tRNA^{Phe} with and without zinc. Yeast tRNA^{Phe} (50-100 μ g) was isocratically eluted from RPC-5 (1.0 $\times$ 10 cm) at 35°C in the presence of 10 mM Zn(OAc)₂ (-▲-) or no divalent ion (-□-), and 10 mM acetate pH 4.6 in various fixed concentration of NaCl. The capacity ratio, k' , was calculated from Eq. 1 where the retention of uridine was taken as V_0 , the excluded volume. The fitted lines were determined with Eq. 6 as the model. The values for the fitted parameters are given in Table 1.

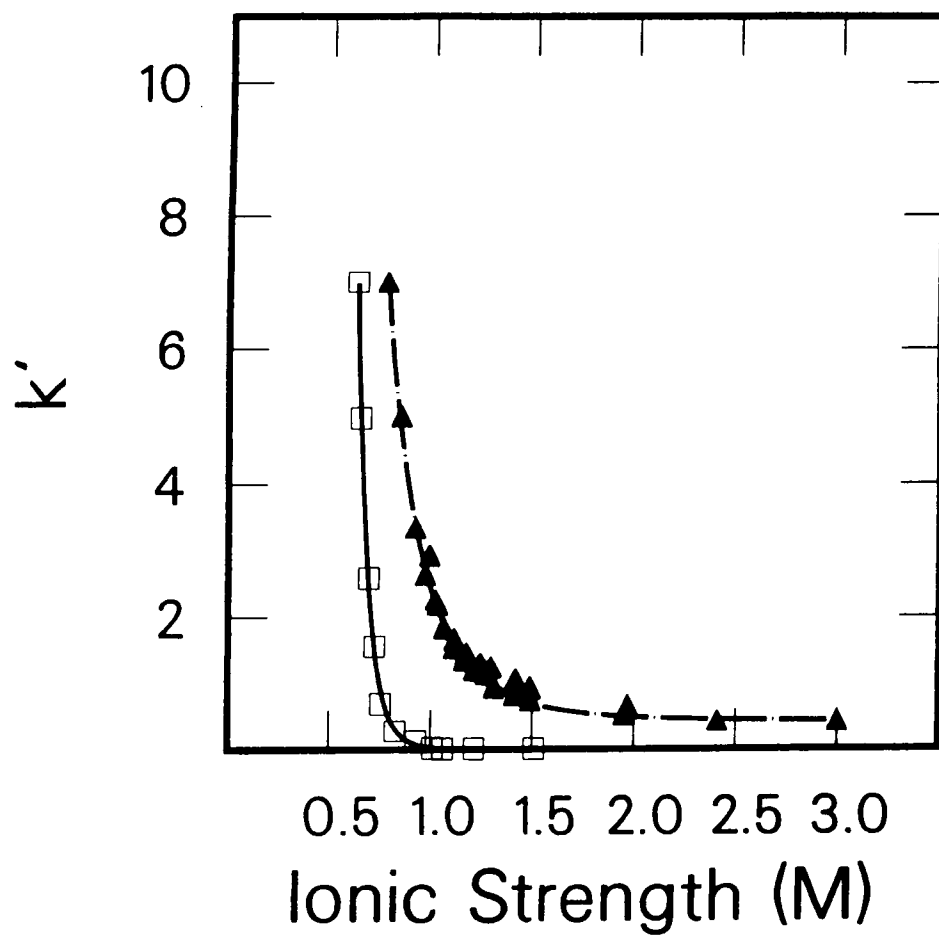


Figure 6

Table 1

Values for the Chromatographic Parameters
Obtained from Equation 6^a

	Ion Exchange	Number of Sites	Hydrophobic
	K_1	n	K_2
-Zn ²⁺ (pH 4.6)	0.035	> 10	-0.002
+Zn ²⁺ (pH 4.6)	1.922	5.0	0.451
+Zn ²⁺ (pH 6.0)	0.32	5.1	0.490

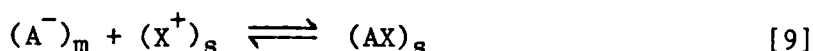
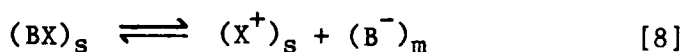
^aEquation 6 $k' = K_1 [\text{NaCl}]^{-n} + K_2$.

indistinguishable though there is clearly a difference in this number with and without Zn^{2+} .

The effect of ΔH on chromatography of yeast tRNA^{Phe}. The effect of temperature on chemical equilibria is described by the Van't Hoff equation. Over the normally accessible temperature range the capacity factor, k' , can be related to temperature by a modified version of the Van't Hoff equation (Colin et al., 1978; Horvath et al., 1976; Riley et al., 1979):

$$\ln k' = -\frac{\Delta H}{RT} + \frac{\Delta S^\circ}{R} + \ln \frac{A}{V_m} \quad [7]$$

where ΔH is the enthalpy of transfer of the solute from the mobile phase to the stationary phase, ΔS° is the associated change in the standard entropy, and A is the total surface area of absorbent in a column with a dead volume V_m . Assuming that ΔH , ΔS° and A/V_m are independent of temperature, eq. 7 suggests the classical linear relationship of $\ln k'$ and $1/T$. For both hydrophobic and ion-exchange processes ΔS° is negative, as is ΔH for the hydrophobic mechanism. The explanation for the sign of ΔH for ion-exchange processes is more complicated, since the ion-exchange mechanism is actually the sum of two reactions:



ΔH is the difference in enthalpy of transfer of X^+ between the two reactions (Dybczyński, 1967), and so can assume either positive, negative, or zero values depending upon the ΔH of transfer for the counter-ion to the solvent. In most, if not all, RPC-5 separations of nucleic acids with Cl^- as the counter-ion, increasing temperature increases retention on RPC-5, implying a positive ΔH .

Figure 7a shows Van't Hoff plots for gradient elution of $tRNA^{Phe}$ in the presence or absence of Zn^{2+} . The slope of the line is $-\Delta H/R$, where ΔH represents the overall chromatographic processes. The apparent ΔH was relatively small and positive, though it is larger (0.9 kcal/mol) in the presence of Zn^{2+} than in its absence (0.3 kcal/mol). In both cases, the Van't Hoff plots were linear, suggesting that these are actually temperature effects on retention and not thermal denaturation of the tRNA.

The effect of temperature on the isocratic elution of $tRNA^{Phe}$ in 10 mM $Zn(OAc)_2$ and 3 M NaCl is shown in Figure 7b. Again, the Van't Hoff plot was linear, but in this case the ΔH was negative and determined to be 1.1 kcal/mol. The fact that ΔH is negative does not prove that this represents hydrophobic interactions, but taken with the earlier observation of the salt independence of retention of $tRNA^{Phe}$ in high salt, it is a strong indication that this indeed is the case.

The effect of pH on RPC-5 chromatography of $tRNA^{Phe}$. Eluant buffer pH plays an important role in retention of nucleic acids

Figure 7: Van't Hoff plot for temperature dependence of RPC-5 chromatography. The effect of increasing temperature on the elution of tRNA^{Phe} in a) the gradient system (legend to Figure 1) with 10 mM Zn²⁺ present (-○-) or no divalent metal (-□-) was determined where k' was approximated as the ionic strength of the peak fraction, or b) the isocratic system in 3 M NaCl; 10 mM acetate pH 4.6 and 10 mM Zn²⁺, where k' was determined as in Figure 6.

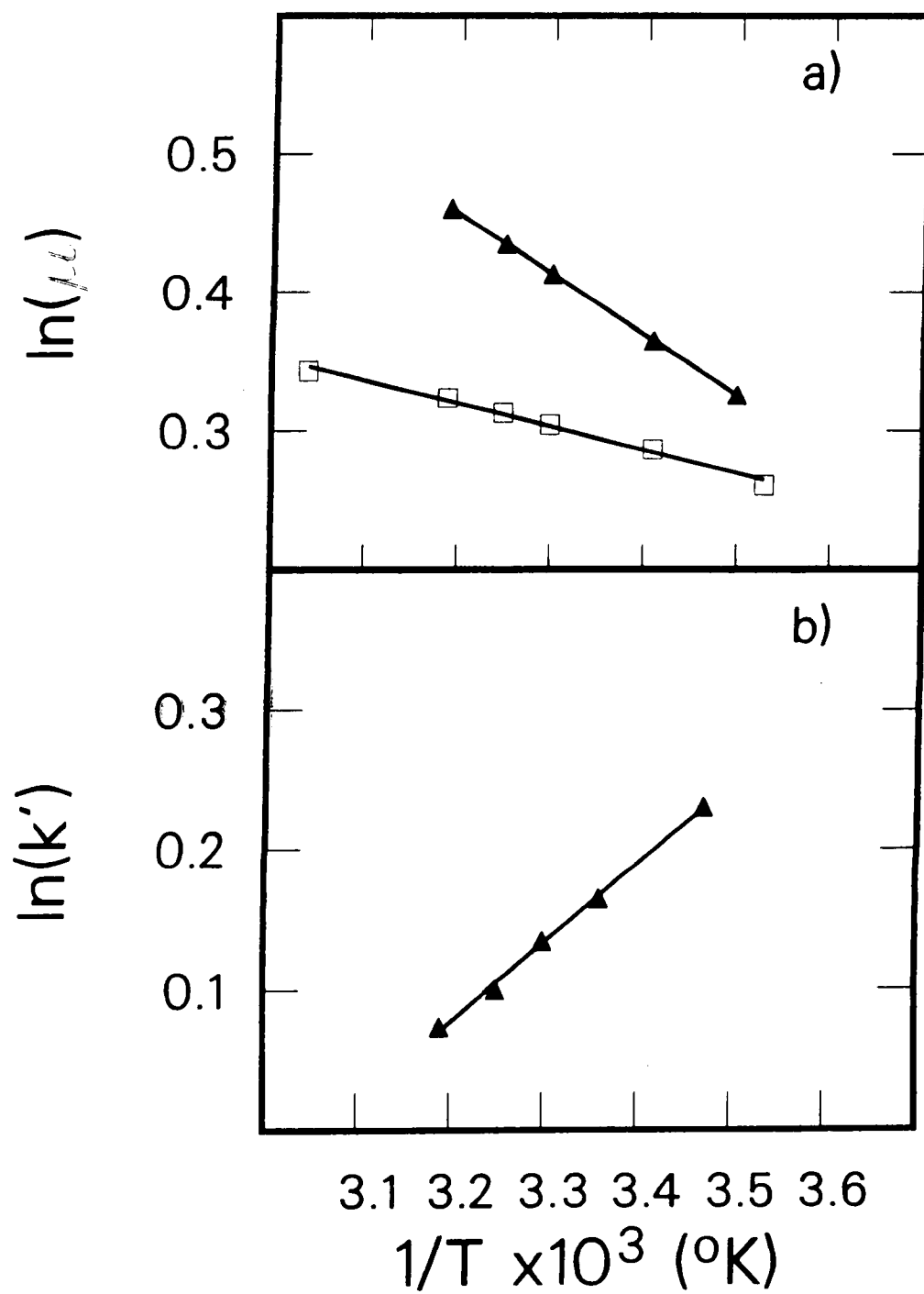


Figure 7

on RPC-5 (Kelmers et al., 1971; Larson et al., 1978). Protonation and deprotonation of nucleic acid bases occur between 3.0 and 10.5, which can have a marked effect upon chromatographic retention. Protons from the N-3 of uracil or thymine, and the N-1 of guanine are removed at alkaline pH. A mildly acidic pH (~4.5) facilitates the protonation of the N-1 of adenine, and the N-3 of cytosine (O'Conner et al., 1981), although the order of protonation for the N-1 of adenine and the N-3 of cytosine is still under debate. At pHs below 3.0, the N-7 of guanine can also be protonated.

Figure 8 shows the pH titration of tRNA chromatography on RPC-5. The chromatography was performed under isocratic conditions with the salt concentration being adjusted to give k' values of 2.0 at pH 4.6 both in the absence (0.62 M NaCl) and presence (1.0 M NaCl) of Zn^{2+} . Eluant pH was not investigated above pH 7.0 since Zn^{2+} solubility is decreased.

In the absence of Zn^{2+} there are at least two distinct pH-dependent transitions and a retention maximum at about pH 4.6. There is an inflection point at 5.2, the apparent pK_{a2} , and another at pH 3.7, the apparent pK_{a1} . Associated with the latter was a decreased sample recovery, so that no sample could be eluted at pH 3.0; the decreased recovery may be due to precipitation or denaturation of the tRNA.

In the presence of 10 mM Zn^{2+} pK_{a2} was unchanged. Retention below pH 4.5 was not affected by pH but the sample recovery

Figure 8: The effect of pH on isocratic elution of tRNA^{Phe}. Chromatography was performed at salt concentrations that would give k' values of 2.0 at pH 4.6: in the absence of Zn^{2+} (○) the NaCl concentration was 0.62 M or in the presence of 10 mM $\text{Zn}(\text{OAc})_2$ (□) it was 1.0 M. The pH of the elution buffer was controlled in the range of pH 2-4 with 10 mM formate buffer, from pH 4-6 with 10 mM acetate buffer, and from pH 6-8 with 10 mM Pipes. All other chromatographic conditions are given in legend to Figure 7.

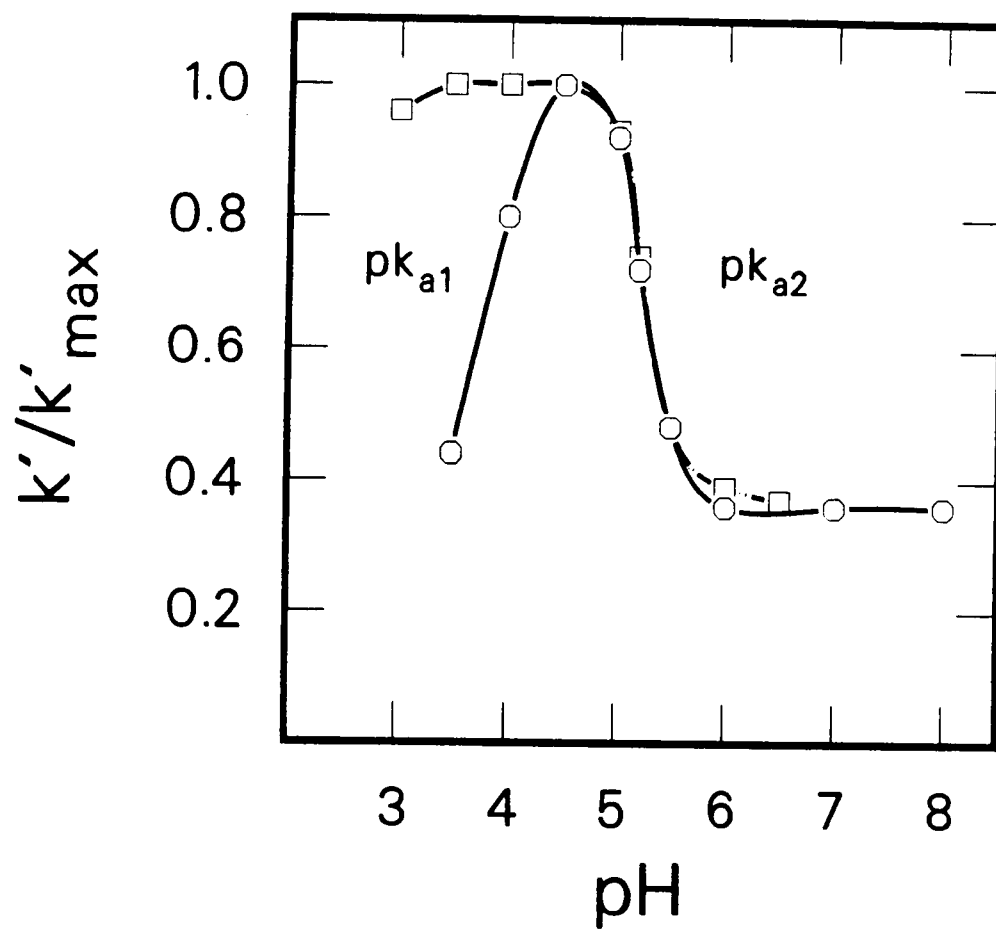


Figure 8

decreased progressively as the pH was lowered below 4.0. No sample could be recovered below pH 3.0.

The effect of pH was further characterized by isocratic chromatography at different fixed levels of NaCl at pH 6.0 in the presence of 10 mM Zn^{2+} . The data for k' vs $[\text{NaCl}]$ obtained were fitted to eq. 6 to determine the chromatographic constants n , K_1 and K_2 , the values of which are given in Table 1. Increasing the pH affected K_1 , the ion-exchange constant, while n and K_2 were unaffected. This result is somewhat of a surprise since at pH 6.0 the overall negative charge on the tRNA would be greater than at pH 4.6, due to protonation of both adenine and cytosine residues at pH 4.6. An explanation for this may be that cytosine protonation destabilizes tRNA structure slightly allowing a greater number of interactions between the tRNA and column.

Restoration of normal chromatographic retention by Cd^{2+} or Mg^{2+} . The ability of Mg^{2+} and Cd^{2+} to restore the normal chromatographic mobility of tRNA^{Phe} was determined in the presence of 10 mM $\text{Zn}(\text{OAc})_2$. Figure 9 shows the functional dependence of the reversal of the Zn^{2+} effect on the concentration of divalent ion. These data clearly show that 10 mM Cd^{2+} , the "soft" ion, completely restores normal retention, while 35 mM Mg^{2+} is required to achieve the same result. In both cases, the restoration of normal retention exhibits a biphasic dependence on metal ion concentration. Both processes appear to

Figure 9: Reversal of zinc effect by cadmium and magnesium. Conditions for chromatography were as described in Figure 1 except that 10 mM $\text{Zn}(\text{OAc})_2$ was present throughout the experiment and various concentrations of either CdCl_2 or MgCl_2 were added at the concentrations shown for "divalent metal".

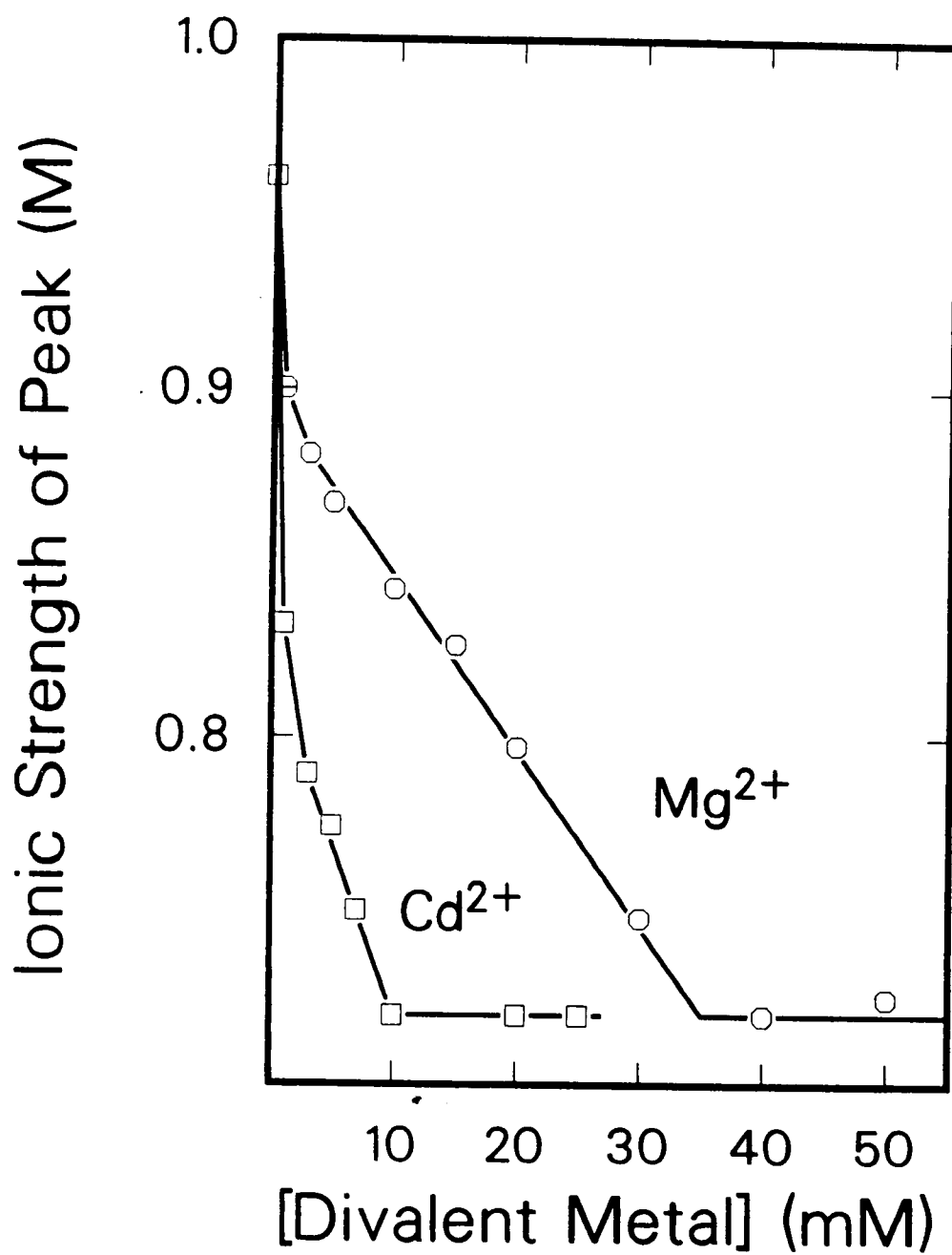


Figure 9

be linear in nature, though the amount of restoration attributed to each portion of the curve differs for Cd^{2+} or Mg^{2+} .

Discussion

Chromatography of tRNA on RPC-5 in the presence or absence of divalent metal ions can be used to explore structural alterations in tRNA. The changes caused by Zn^{2+} and possibly other divalent cations can be explained in terms of three parameters: n , the number of anionic sites on the tRNA that displace chloride ions from the quaternary ammonium ion upon binding; K_1 , the affinity of these anions relative to the counter-ion for the positively charged column; and K_2 , the affinity of the tRNA for the hydrophobic layer of the column.

Analysis of these data using Eq. 6 show that Zn^{2+} affects all three fitted parameters: n , K_1 and K_2 (Table 1). In the absence of Zn^{2+} , the hydrophobic interaction constant, K_2 , was negligible, suggesting that pure hydrophobic interactions do not play a major role in tRNA^{Phe} retention. However, hydrophobic interactions may still affect retention in conjunction with ion exchange interactions. The constant, K_1 , represents the effect of salt on tRNA elution; this is primarily due to ion exchange interactions between the tRNA and the ammonium ion, but it would also include any hydrophobic effects that are coupled to the ion-exchange interactions. This may explain why RPC-5 is

sensitive to the content of the modified nucleoside dihydro-uridine (Waters et al., 1973; Lo et al., 1981), the methyl content of tRNA isoacceptors (Shugart et al., 1968; Bell et al., 1978) and the strong retention of tRNA^{Phe} which contains the hypermodified wyeine base.

The significant increase in the hydrophobic interaction constant, K_2 , in the presence of Zn^{2+} , may explain the difference between RPC-5 columns prepared with Plaskon or Voltalef as the solid support material (Figure 2). In an earlier study (Flanagan et al., 1986), there was little difference between these two supports in gradient separations. Voltalef beads are five times larger than Plaskon, and so columns prepared with Voltalef are effectively one-fifth as long. The difference in the effective length of the column would have little effect upon separations based upon differences in apparent K_1 and n , since these are dependent upon NaCl concentration. However, K_2 is independent of $[NaCl]$, and would not change throughout the separation. The contribution to retention of K_2 would be dependent upon column length. This interpretation is also consistent with the equality of Plaskon and Voltalef in the absence of Zn^{2+} . It is also consistent with the absence of a zinc effect when tRNA is chromatographed on Aminex A-28. The methyl groups may be ineffective, as compared to the C₈₋₁₀ groups in the RPC-5 matrix, in facilitating hydrophobic binding of the tRNA.

All tRNAs which have been examined to date elute at higher NaCl concentrations when Zn ions are present. The dependence of

retention on Zn^{2+} concentration was not the same for all tRNAs investigated. For the E. coli tRNAs (Val, Ile, and Lys) the increase in retention as a function of $[\text{Zn}^{2+}]$ was linear (Figure 3). The increase in retention for tRNA^{Phe} (yeast) was biphasic (Figure 2), and no effect of Zn^{2+} was observed at <1 mM. The E. coli tRNAs responded to all concentrations of Zn^{2+} tested.

The increase in hydrophobic interaction of tRNA^{Phe} with RPC-5 in the presence of Zn^{2+} is probably not due to the "Y base" wyeine. The fluorescence of the Y-base is not affected by addition of Zn^{2+} suggesting that Zn^{2+} has little effect on the environment of this chromophore (Flanagan et al., 1986b). Chromatography of tRNA^{Phe} lacking wyeine was not investigated since removal of this base is believed to alter tRNA solution structure (Ladner & Schweizer, 1974; Kearns et al., 1973; Dananloo et al., 1979; Krzyzosiak & Ciesiolka, 1983).

Two lines of evidence suggest that there are two classes of Zn^{2+} binding sites on tRNA^{Phe} . The data in Figure 2 show a biphasic increase in tRNA retention due to Zn^{2+} . The data in Figure 9 show that Cd^{2+} and Mg^{2+} reverse the Zn^{2+} -induced increase in retention in an apparently biphasic manner. The biphasic nature of these curves suggest that at least two sites may be involved: one site which binds Cd^{2+} and Mg^{2+} strongly, and the other being less sensitive to these ions.

Complete reversal of the Zn^{2+} associated retention can be achieved by the addition of either 10 mM Cd^{2+} or 35 mM Mg^{2+} , and in each limb of the biphasic curves (Figure 9) Cd^{2+} was more

effective than Mg^{2+} at restoring retention. The relative affinities of these three divalent ions for the two classes of Zn^{2+} sites are $Cd^{2+} > Zn^{2+} \approx Mg^{2+}$ in the first limb, while for the site corresponding to the second limb they are $Cd^{2+} > Zn^{2+} > Mg^{2+}$. At least for the second site the relative affinities of these ions appear to be related to their "softness."

Recently, Rubin et al. (1983) reported x-ray diffraction studies of the Zn^{2+} binding sites in yeast tRNA^{Phe}. Five strong binding sites for Zn^{2+} occur in these crystals; two zinc ions displaced previously bound Mg^{2+} . These two sites represented the most tightly bound of the five Zn^{2+} ions. The Zn^{2+} atom which replaced one of the two Mg^{2+} in the dihydrouridine loop (D loop) had the highest occupancy and thus the greatest affinity of all the Zn^{2+} ions. The Zn^{2+} at this location was directly chelated to the N-7 (G-20), O⁶ (G-20), a phosphate oxygen (G-19), and O⁴ (U-59). The location of the Zn^{2+} was displaced nearly 2 Å from the original Mg^{2+} site, primarily due to the direct coordination of N-7 atom of G-20. The geometry of the ligand- Zn^{2+} complex was tetrahedral as opposed to the octahedral arrangement of ligands about the Mg^{2+} ion. The next most tightly bound Zn^{2+} , that replaced a Mg^{2+} , was located in the single stranded region which links the acceptor stem and dihydrouridine stem (P-10 loop). This zinc was in a complex tetrahedral ion from $[Zn(H_2O)_4]^{2+}$ and was hydrogen-bonded to four phosphate oxygens P8, P9, P11, and P12, and perturbed the local conformation

of tRNA^{Phe} around this site. The actual structural alterations in the P-10 loop were not characterized by these workers.

The two Zn²⁺/Mg²⁺ binding sites discussed above may also be the sites at which Zn²⁺ binds to cause the observed increase in retention on RPC-5. First, these are the only sites where Zn²⁺ competes appreciably with Mg²⁺ when both are present in equimolar amounts in the crystal. Second, since neither of these sites contains adenine or cytosine the binding of Zn²⁺ would not be expected to be pH dependent in the range 3.0 to 7.0. Third, both sites are located in regions of the tRNA where extensive intramolecular associations occur; substitution of Zn²⁺ for Mg²⁺ is likely to cause significant structural alterations. Finally, equilibrium binding studies show that strong Mg²⁺ sites have binding constants on the order of 10⁻⁴ M⁻¹ (Schreier & Schimmel, 1975); since these two Mg²⁺ sites are believed to be the most tightly bound (Rubin et al., 1983), and Zn²⁺ displaces both Mg²⁺ in the crystal, the binding constant of Zn²⁺ at these sites must be greater than 10⁻⁴ M⁻¹. In the range of Zn²⁺ concentrations (0 to 20 mM), and tRNA^{Phe} concentrations (0.2 to 2 μM) the Zn²⁺ binding sites would not be saturated. These predictions agree quite well with our data.

Another unpredicted result was the shape of the curve in Figure 2, p. 27, which suggests that the binding of Zn²⁺ is cooperative. Cooperativity is not generally observed at the concentrations of NaCl employed. The three possible explanations are that: a) Zn²⁺ binding to tRNA^{Phe} behaves differently than

any of the divalent metals explored to date, b) the effect of Zn^{2+} on chromatography is non-linear at low $[\text{Zn}^{2+}]$, or c) that the " Zn^{2+} effect" is due to binding of Zn^{2+} at weak sites which are only filled after the strong sites.

Of all five sites in tRNA^{Phe} , the $\text{Zn}^{2+}/\text{Mg}^{2+}$ metal binding site in the D-loop has the greatest affinity for Zn^{2+} (Rubin et al., 1983). This site is similar to sites for Pb^{2+} (Brown et al., 1983; Brown et al., 1984; Sundaralingam et al., 1984) and Mn^{2+} binding (Jack et al., 1977). Indeed, this is probably a binding site for most polyvalent metal ions. The simultaneous binding of Zn^{2+} at the N-7 (G-20), and O^4 (U-59) may account for the high affinity of Zn^{2+} for this site and may represent one of the two sites revealed by RPC-5 for tRNA^{Phe} . Imino-proton NMR spectra of tRNA^{Phe} and tRNA^{Val} are in agreement with this assignment (Chapter IV).

In the cases of E. coli tRNA^{Lys} and tRNA^{Ile} a linear, rather than a biphasic, response to Zn^{2+} occurs and both these tRNAs contain a dihydrouridine instead of a guanine residue at position 20 which cannot serve as a ligand for Zn^{2+} . The valine tRNA, which also shows a linear response to Zn^{2+} has a guanine at position 20 but its D-loop differs from tRNA^{Phe} at position 16 where cytosine is substituted for dihydrouridine. Early crystallographic work (Jack et al., 1976) suggests that the substitution of cytidine for dihydrouridine would have little effect on the D/T Ψ C junction where the Zn^{2+} binding site is located. Hingerty et al. (1980) refined the structure and showed

that D-16 points into the body of the molecule rather than away from it, which allows hydrogen bonding to occur between N-3 (D-16) and O² (C-60). The presence of cytosine at position 16 would alter this region since cytosine will not pair with C-60 and it would also introduce a relatively bulky ammonium ion into this small pocket, and possibly explain why tRNA^{Val} chromatography also exhibits a monophasic dependence on Zn²⁺ concentration like the two other E. coli tRNAs.

CHAPTER III

THE EFFECT OF ZINC ON tRNA STRUCTURE: ABSORBANCE AND FLUORESCENCE SPECTROSCOPIC STUDIES

Introduction

Binding of divalent cations neutralizes the high charge density of the phosphate backbone of tRNA and causes the formation of its native (functional) conformation. Two early studies suggest, that in addition to Mg^{2+} , the cations Zn^{2+} , Cu^{2+} and Mn^{2+} may also be associated with nucleic acids in vivo (Wacker & Vallee, 1959; Belokobylskii et al., 1968). Whether these metals were actually bound in vivo, or during the extraction process, is not known. The affinity of Mg^{2+} for tRNA is less than that of certain other divalent cations, yet Mg^{2+} is the most important biologically. The effect of other divalent ions in Mg^{2+} -dependent reactions involving nucleic acids in vitro such as DNA-directed RNA synthesis, DNA replication and aminoacylation of tRNA, has been investigated. In many instances, these ions had deleterious effects on these reactions (Svensson, 1967; Niyogi et al., 1981a, 1981b; Mildvan & Loeb, 1979).

In previous studies, we have examined the effects of Mg^{2+} , Cd^{2+} and Zn^{2+} on the chromatographic behavior of tRNA and on its ^1H -NMR spectrum (Flanagan & Jacobson, 1986; Flanagan et al., 1986). For chromatography, Zn^{2+} uniquely increases the retention of tRNA on the RPC-5 column, (a quaternary amine $[CH_3NR_3]^+$, where

R=C₈₋₁₀ hydrocarbons), by affecting both the hydrophobic and ionic interactions of the tRNA with the column (Hiatt & Jacobson, 1980; Flanagan & Jacobson, 1986). The structure deduced from a high resolution NMR spectrum suggested that the effects of Zn²⁺ were primarily in the "elbow" region of the L structure (Flanagan et al., 1986).

In this study, various sites on the tRNA (e.g. ring nitrogens of the aromatic bases, or the phosphate oxygens of the backbone) are examined for their ability to bind Cd²⁺, Mg²⁺ and Zn²⁺, using the conditions employed in previous studies (Flanagan & Jacobson, 1986; Flanagan et al., 1986). The ability of these three metal ions to bind at the Mg²⁺ site in the anticodon loop could not be determined in our previous ¹H-NMR study (Flanagan et al., 1986), and is examined here using the fluorescence of the Wye-base (Y). The effects of Cd²⁺, Mg²⁺ and Zn²⁺ on the stability of helical regions in the tRNA are also explored.

Materials and Methods

Purified tRNA^{Phe} (Boehringer-Mannheim) (10 mg/mL) was warmed to 70°C in 10 mM EDTA, pH 7.0. After the solution was allowed to return to room temperature, it was vacuum dialyzed against distilled water. This procedure has been shown to remove the residual metal ions (~0.1 equiv. Mg²⁺/tRNA) [Hyde & Reid, 1985]. The stock tRNA, in distilled water, was stored at -20°C until used. The extinction coefficients for tRNA are 1.59 and

1.89 nmole/A₂₆₀ in distilled water and in high salt at pH 7.0, respectively (Li et al., 1983).

The difference spectra were obtained in a Beckman DU-8 single beam spectrophotometer, using 3-mL quartz cells with a 1 cm path length. The tRNA solutions contained 500 mM NaCl and either 10 mM Tris-HCl, pH 7.4, or 10 mM NaOAc, pH 4.6, as noted. In all experiments the molar concentration of phosphate was 0.2 μ M which gives an absorbance of 1.5 at 260 nm. The added divalent ions are expressed as the ratio, *r*, of molar ion concentration to molar concentration of phosphate in the tRNA.

The Y-base fluorescence was determined in a Perkin-Elmer Fluorometer using anthranilic acid (15 μ M) as the standard. The measurements were carried out at 35°C at an excitation wavelength of 320 nm and an emission wavelength of 440 nm. The tRNA concentration in terms of phosphate residues was ~0.2 μ M in a solution containing 500 mM NaCl and 10 mM NaOAc, pH 4.6 the absorbances of these samples were ~1.6 at 260 nm.

Thermal denaturation profiles were obtained by following the increase in absorption at 260 nm in a Gilford 2000 automatic spectrophotometer equipped with a thermosensor. The data collection and subsequent analysis have been described elsewhere (Cadilla et al., 1986). Temperature increments were obtained by means of a Neslab circulating bath equipped with a Neslab temperature programmer from 20 to 100°C at a rate of 0.5°C/min. The absorbance of the tRNA samples was approximately 1.0 at 260 nm. The samples were dissolved in 500 mM NaCl and 10 mM NaOAc,

pH 4.6. Divalent cations were added in the acetate form, as noted in the figure legends. At each metal ion concentration, the denaturation profiles for three samples were collected independently, and the final melting curve is the average of these. The data are plotted as the change in A_{260} per unit temperature vs. temperature. These differential melting curves were then fit to the sum of three gaussians by a non-linear least squares routine, as described previously (Cadilla et al., 1986).

Results

Ultraviolet difference spectra. Some changes in tRNA structure that are induced by metal ion binding can be detected by UV difference spectroscopy. The largest change in the difference spectra was in the region from 230 to 290 nm and resulted from the presence of each of the four divalent metal ions used in our study (Mg^{2+} , Cd^{2+} , Zn^{2+} or Ni^{2+}) (Figure 10). These ions all reduced the absorbance of purified tRNA^{Phe} throughout this spectral region, with the largest decrease occurring at 254 nm.

In the spectral region between 290 and 305 nm, the transition metals, Zn^{2+} , Cd^{2+} , and Ni^{2+} , had an additional effect on tRNA difference spectra, while Mg^{2+} had no effect. Addition of the transition metals produced an absorption band at 294 nm. The height of the band was ion-dependent in the order $Ni^{2+} > Zn^{2+} >$

Figure 10: Difference spectra of purified tRNA^{Phe} samples after addition of divalent ions. All measurements were performed at 25°C in 500 mM NaCl, 10 mM NaOAc, pH 4.6 with NiCl₂ (—), ZnCl₂ (— • —), CdCl₂ (---) and MgCl₂ (— - —) where [Mg²⁺]/[PO₄] = 1.

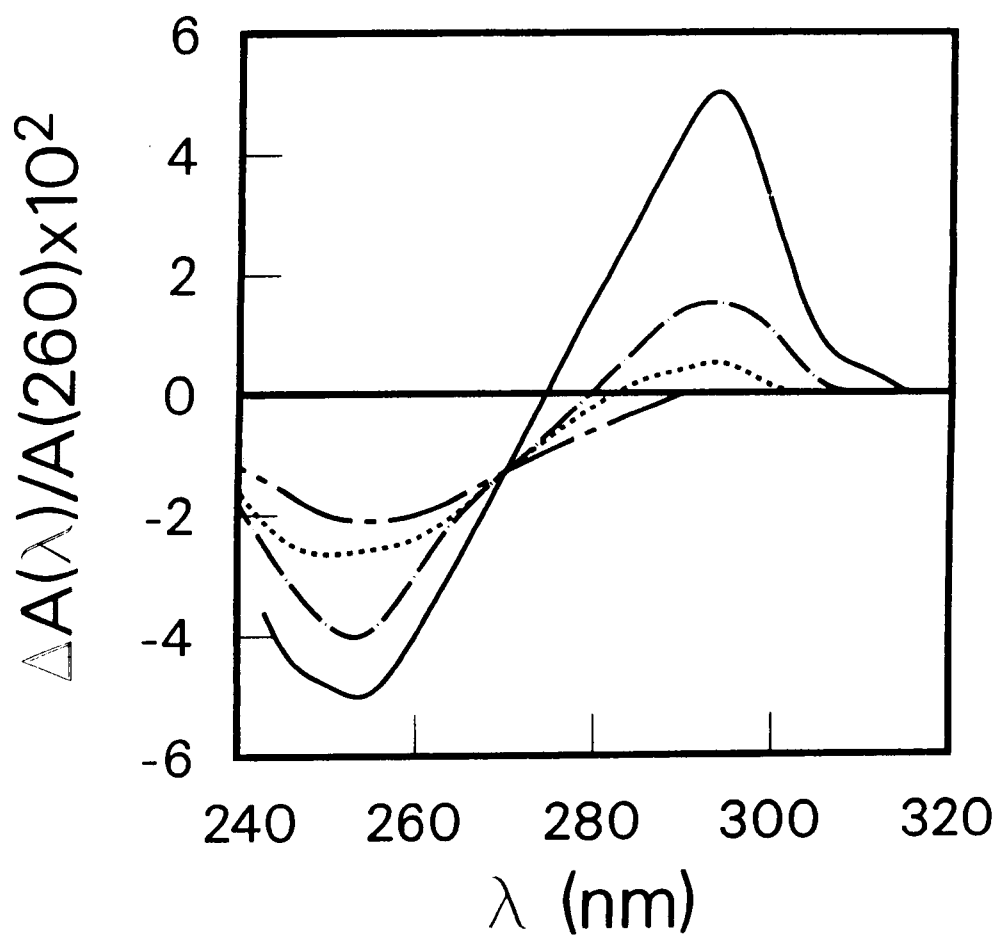


Figure 10

Cd^{2+} , which corresponds to the Irving-Williams series for stability of divalent metal ion complexes (Irving & Williams, 1948).

The binding of Zn^{2+} was compared at pH 7.4 and 4.6 (Figure 11) by measuring the height of the absorption band at 294 nm as a function of Zn^{2+} concentration. The titration curves are identical at the two pH values within experimental error. This is similar to our previous observation in the RPC-5 column chromatography system (Flanagan & Jacobson, 1986), where increasing the pH from 4.6 to 6.5 did not eliminate the increased retention of tRNA due to Zn^{2+} .

Fluorescence of the Wye base in the presence of divalent metal ions. The Wye (Y) base is a reporter for structural changes occurring in the anticodon loop: its fluorescent properties are dependent on the conformation of the anticodon which can be influenced by surrounding solvent conditions. Under the condition of low ionic strength at pH 7.0, the presence of Mg^{2+} , which appears to bind very near the Y-base (Quigley et al., 1978), causes an increase in its fluorescence (Robison & Zimmerman, 1971). This is true also at pH 4.6 in 500 mM NaCl (Figure 12). Either Cd^{2+} or Zn^{2+} , under the same solvent conditions, had a smaller effect on the fluorescence intensity of this base. The effect of Cd^{2+} was somewhat larger than Zn^{2+} .

The small effect of Zn^{2+} on Y-base fluorescence may be due to strong binding of Zn^{2+} at the Mg^{2+} site in the anticodon loop, with structural consequences different from those caused by Mg^{2+} .

Figure 11: The effect of increasing r , $[\text{Zn}^{2+}]/\text{PO}_4$ ratio, on tRNA absorbance at 294 nm in the presence of 500 mM NaCl and 10 mM Tris-HCl, pH 7.4 (■) or 10 mM NaOAc, pH 4.6 (▲).

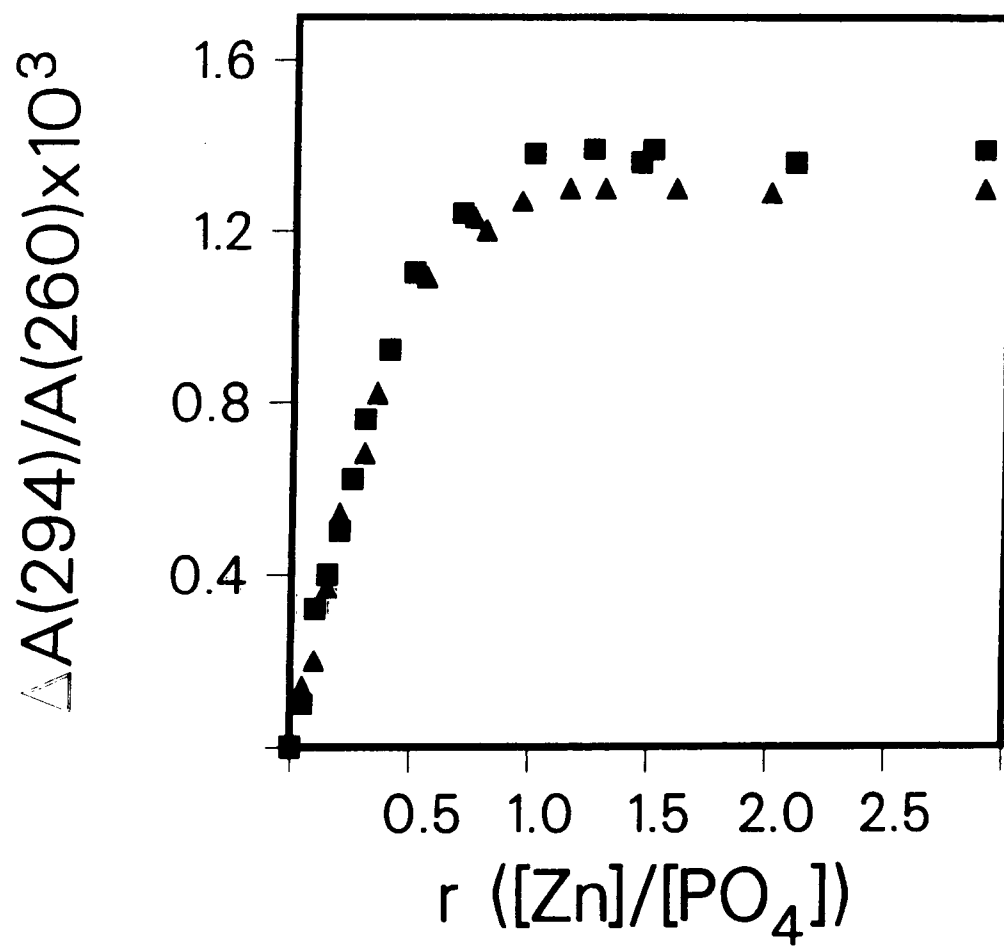


Figure 11

Figure 12: The effect of divalent ions on the fluorescence of the Y-base. Y-base fluorescence was measured in the presence of 500 mM NaCl, 10 mM NaOAc, pH 4.6, and increasing concentrations of ZnCl_2 , CdCl_2 , or MgCl_2 .

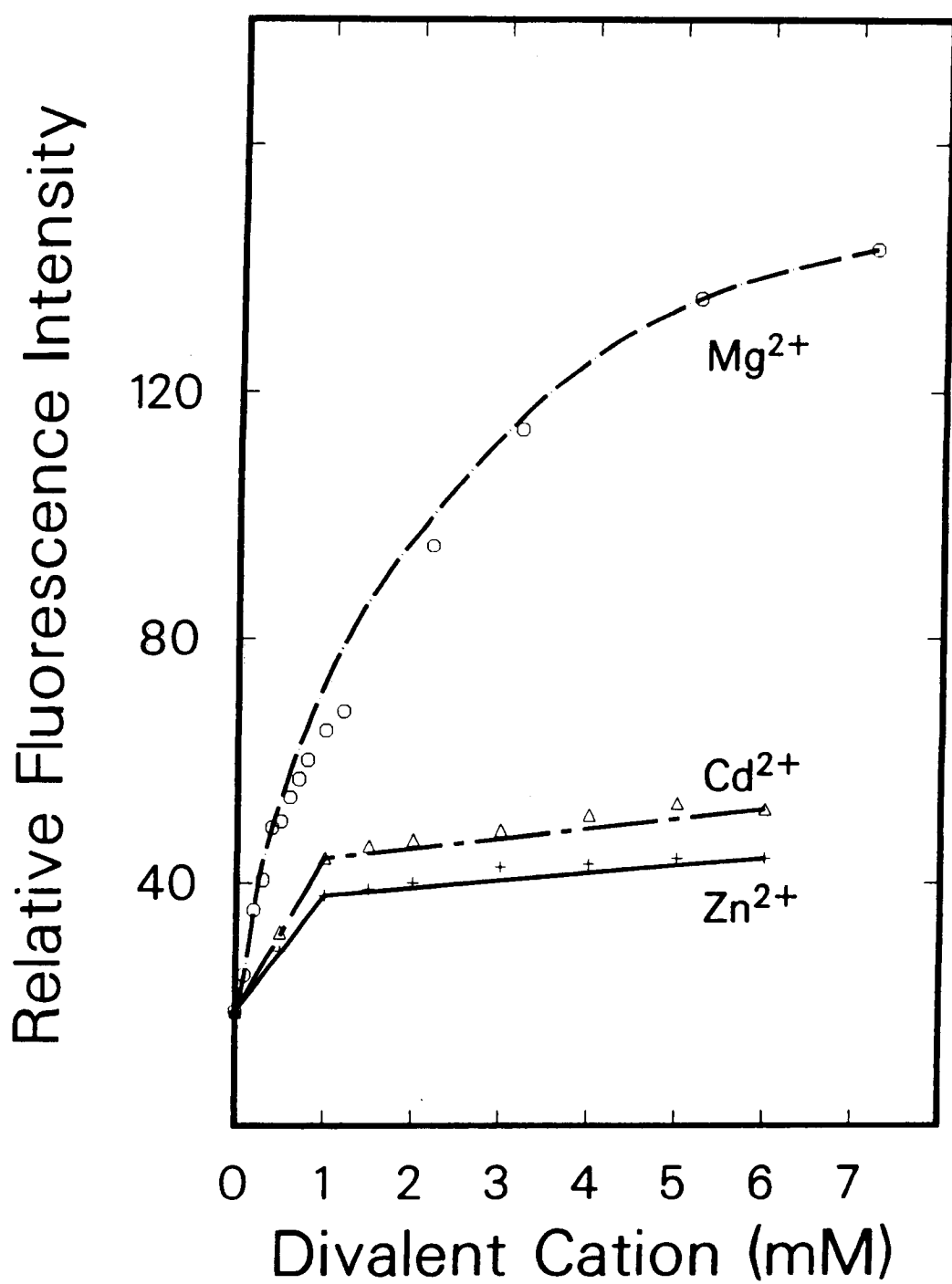


Figure 12

Alternatively, Zn^{2+} may bind elsewhere on the tRNA causing a small perturbation of the structure near the Y-base. To determine which of these two hypotheses is correct, we have titrated tRNA^{Phe} in 10 mM MgCl_2 , 10 mM NaOAc, pH 4.6, and 500 mM NaCl, with increasing concentrations of Zn^{2+} . If Zn^{2+} binds to this site and displaces Mg^{2+} , we would expect the fluorescence intensity to decrease with increasing Zn^{2+} . This, however, was not the case. When the maximal fluorescence of the Y-base was caused by 10 mM Mg^{2+} , the addition of Zn^{2+} , up to 50 mM, caused no decrease in fluorescence intensity (Figure 13). The effect of Zn^{2+} on Y-base fluorescence, in the absence of Mg^{2+} , appears to saturate at much lower divalent ion concentrations than does the effect of Mg^{2+} on fluorescence. These two observations suggest that the binding sites for Mg^{2+} and Zn^{2+} , which give rise to the increase in Y-base fluorescence, are spatially distinct.

The effects of Mg^{2+} , Zn^{2+} , and Cd^{2+} on the thermal denaturation of tRNA^{Phe}. Thermal denaturation of tRNA is accompanied by an increased (hyperchromic) absorption at 260 nm. The melting temperature, or t_m , is a sensitive measure of solute effects on tRNA structure.

Figure 14 represents a comparison of the differential melting profiles of tRNA^{Phe} in 500 mM NaCl, 10 mM NaOAc, pH 4.6, and either no added divalent ions or 10 mM Mg^{2+} , Zn^{2+} , or Cd^{2+} . The melting temperature of this tRNA varied between 57 and 71°C, depending on the nature of the divalent cation present. The t_m

Figure 13: The effect of ZnCl_2 concentration on the Y base fluorescence in 500 mM NaCl, 10 mM NaOAc, pH 4.6, and 10 mM MgCl_2 (○) or the same plus Zn as shown (▲).

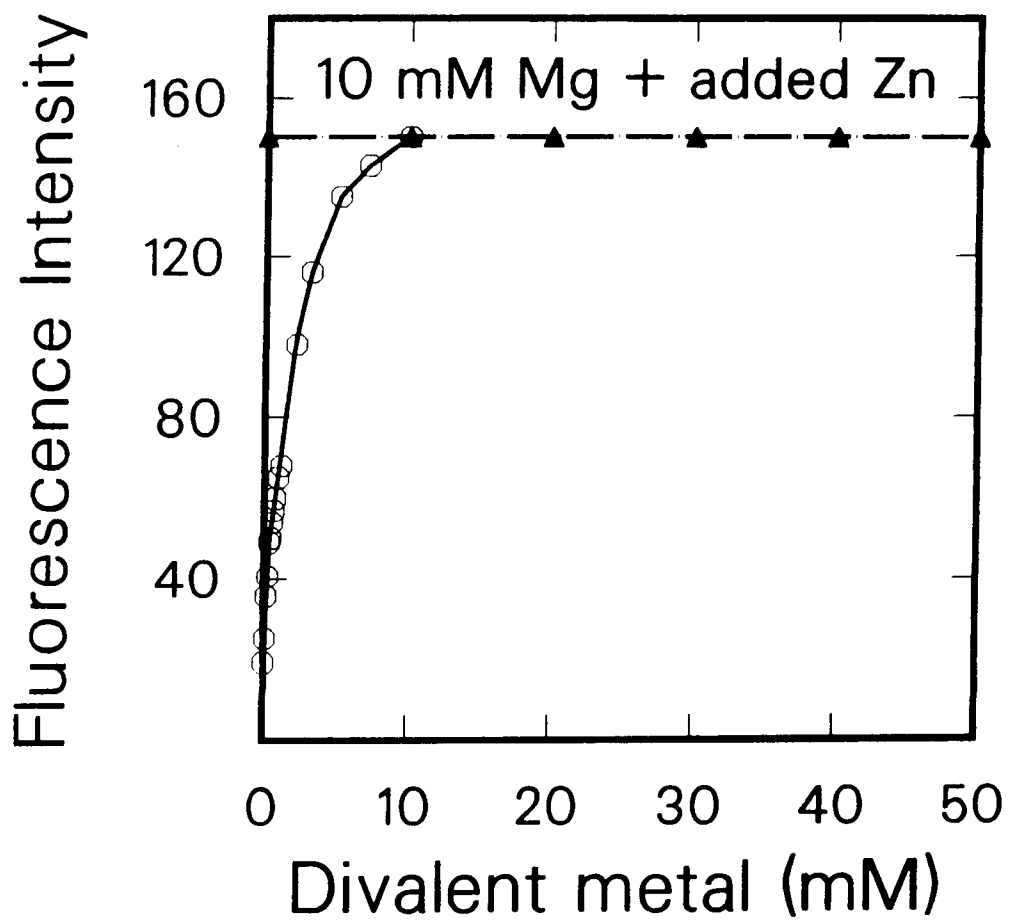


Figure 13

Figure 14: The effect of divalent ions on the differential melting profiles for tRNA^{Phe}. The tRNA^{Phe} was dissolved in 500 mM NaCl, 10 mM NaOAc, pH 4.6, and 10 mM MgCl₂, 10 mM ZnCl₂ or 10 mM CdCl₂. The data are plotted as the first derivative of the change in hyperchromicity with temperature vs. temperature. The change in hyperchromicity at 260 nm was normalized to A₂₆₀ = 1.0 at 20°C.

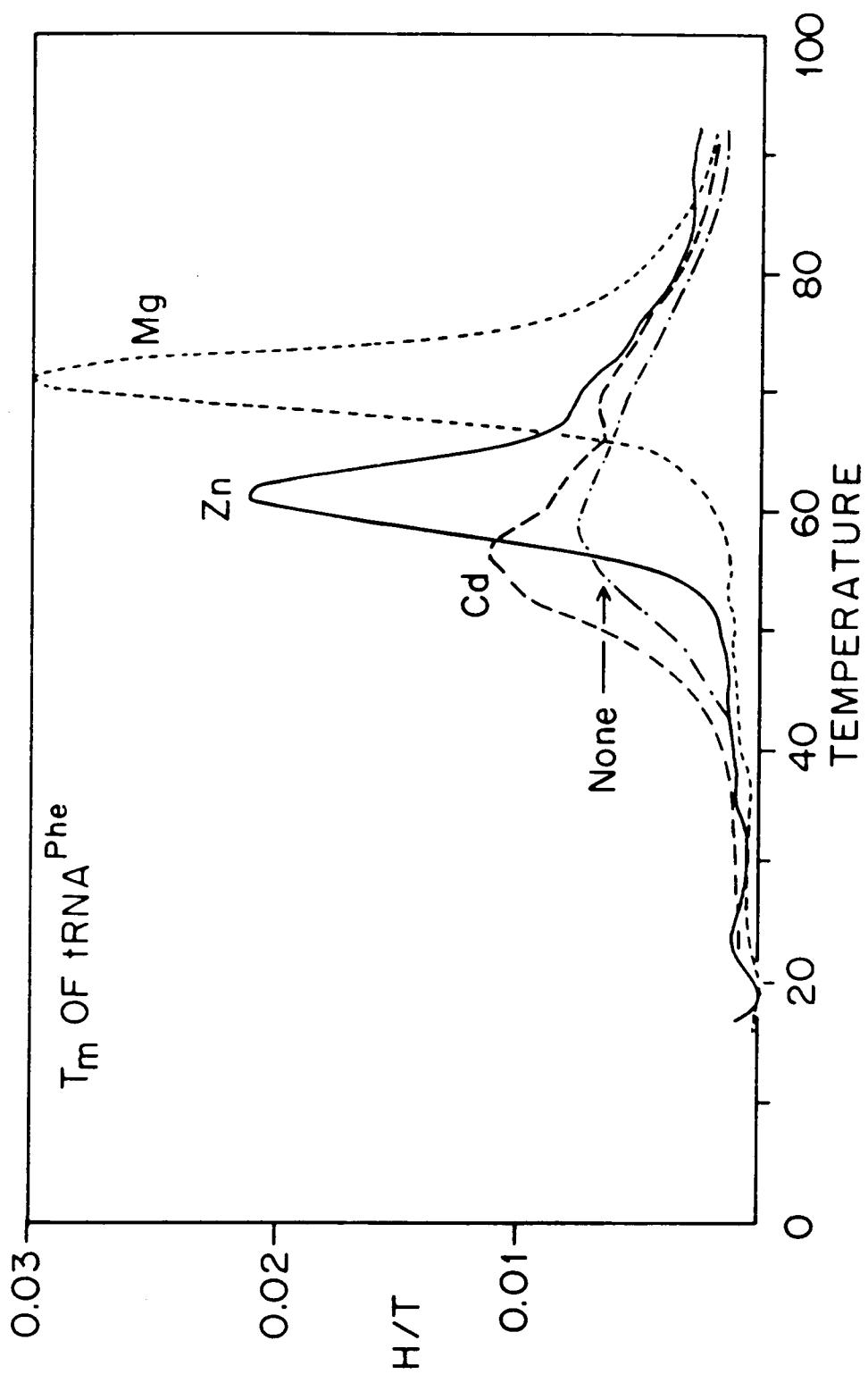


Figure 14

increased in the order Cd^{2+} (57°C) < no added divalent ion (59°C) < Zn^{2+} (61°C) < Mg^{2+} (71°C). These divalent ions also had marked effects on the shapes of the differential melting curves. In the absence of divalent ions the melting transition of tRNA^{Phe} was broad; in contrast, the transition in the presence of 10 mM Mg^{2+} was sharp, almost monophasic. The effects of Cd^{2+} and Zn^{2+} on the melting transition curves were intermediate between those of the Mg^{2+} and of the no divalent ion curves. However, the effect of Zn^{2+} on this transition was more pronounced than that of Cd^{2+} .

Figure 15 shows the differential melting curves of tRNA^{Phe} as a function of Zn^{2+} concentration. The data were fit to a model which used three gaussians to describe each differential melting curve. There was the smallest number which gave an acceptable chi square value, and minimized the correlation between residuals. The solid line through the data points is the calculated sum of the individual gaussians. The primary effect of Zn^{2+} was to sharpen the individual transitions that comprise the curve, though it had little effect on their actual t_m 's. This is in contrast to Mg^{2+} , which affected both t_m and the width of the individual transitions. Table 2 summarizes the fitted parameters t_m , h , and w , which correspond to the melting temperature, peak height, and inverse peak width, respectively, for the four thermal denaturations shown in Figure 14.

Figure 15: The effect of increasing Zn^{2+} concentration on the differential melting profiles for tRNA^{Phe} . The data was obtained at: a) 0.1 mM, b) 1 mM, c) 10 mM Zn^{2+} and are represented by the open squares. The solid line was obtained by fitting the data to the sum of three gaussian functions.

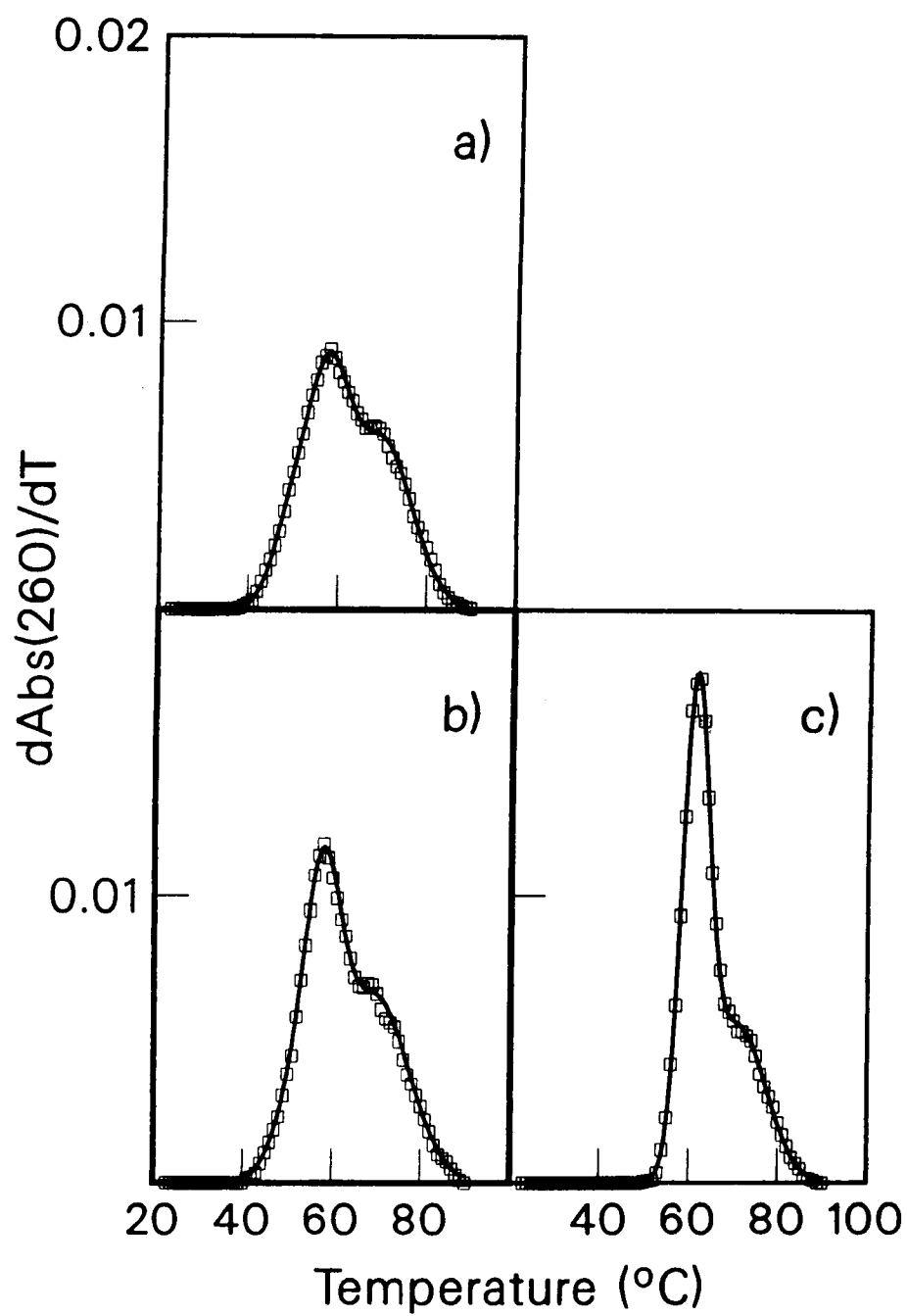


Figure 15

Table 2

Summary of Fitted Parameters^a for Thermal Melting Curves

		t_{m1}	t_{m2}	t_{m3}
0.1 mM Zn ²⁺	a	0.0031	0.0072	0.0058
	b	0.027	0.025	0.011
	t_m	50.2	57.7	69.6
1 mM Zn ²⁺	a	0.0028	0.008	0.006
	b	0.020	0.029	0.011
	t_m	52.2	58.0	69.0
10 mM Zn ²⁺	a	- ^b	0.016	0.0055
	b	-	0.054	0.012
	t_m	-	61.2	71.1

^aThe data were fit to the function $\frac{d(A_{260})}{dT} = \sum_{i=1}^3 a_i e^{-b_i(t_{m_i}-T)}$

^bThis Gaussian was eliminated by the non-linear least squares routine during the fitting process.

Discussion

The effects of Zn^{2+} , Cd^{2+} , and Mg^{2+} on tRNA structure are clearly different as judged by uv difference spectroscopy, fluorescence intensity of the Y base, and thermal melting transitions. The effect of Ni^{2+} , Zn^{2+} , Cd^{2+} and Mg^{2+} at pH 4.6 and 500 mM NaCl, on the absorption of tRNA near 254 nm was similar to that observed by Mazzei and Onori (1984) at pH 7.4 and 150 mM NaCl, using unfractionated E. coli tRNAs. This effect is probably due to increases in base-stacking interactions caused by binding of these cations at sites on the phosphates (Seno et al., 1969; Arco et al., 1980).

The increased absorption of the tRNA at 294 nm is probably due to metal binding to the bases of guanosine or cytosine, since these two nucleosides have significant absorption above 290 nm. The observed increase in absorption here is similar to that seen when the N-7 position of 5'-GMP is protonated. Protonation of the N-3 position of 5'-CMP leads to increased absorbance at ~300 nm (Zimmer & Venner, 1966; Zimmer et al., 1968). Others have attributed the increase in absorbance at 294 nm to binding of metals at the N-7 position (Duane, 1969, 1970). This explanation is also consistent with the observation that Zn^{2+} binding is similar at pHs 7.4 and 4.6, since the pKa for protonation of the N-7 position of guanine in DNA is below 3.0 (O'Conner et al., 1981).

The divalent ion concentrations required to produce the maximum change in tRNA absorbance at 294 nm were in the micromolar concentration range, while the effect on chromatography (Flanagan & Jacobson, 1986), and on the melting transition (Figure 15) were in the mM range. This suggests that the effects on thermal denaturation and chromatography must be due to different metal ion binding sites than those that affect changes in absorbance.

The Y-base fluorescence reflects its hydrophobic environment when the Y-base is stacked between the two adjacent adenine residues, A-39 and A-36, in the anticodon loop. The small, but statistically significant, increase in Y-base fluorescence intensity may be due to the ability of Cd^{2+} and Zn^{2+} to bind in the helical stem of the anticodon arm. Binding of Zn^{2+} has been observed at the N-7 and O⁶ positions of the two adjacent guanine residues, G-42 and G-43, which are located in the major groove of this stem (Rubin et al., 1983).

The lack of a pronounced effect of Zn^{2+} or Cd^{2+} on Y-base fluorescence and the inability of Zn^{2+} to alter the effect of Mg^{2+} on Y-base fluorescence even when Zn^{2+} is present in a four fold excess over Mg^{2+} , suggests that Zn^{2+} and Cd^{2+} do not interact at the Mg^{2+} binding site in the anticodon loop. Also, x-ray crystallographic results suggest that Zn^{2+} does not displace Mg^{2+} from this site (Rubin et al., 1983). Zn^{2+} tends to form tetrahedral complexes while Mg^{2+} generally forms complexes having octahedral geometry. The ionic radius of Zn^{2+} is also

larger than that of Mg^{2+} . Either or both properties may help explain the lack of Zn^{2+} binding at this site.

The total hyperchromicity of all the tRNA samples were similar (~30%), regardless of the nature or concentration of divalent metal ions used, supporting our earlier conclusion that neither Zn^{2+} nor Cd^{2+} causes major alterations in the tertiary structure of tRNA^{Phe} (Flanagan et al., 1986). The effect on t_m was specific for the divalent cation. The overall trend of Cd^{2+} was to lower the t_m , while Mg^{2+} and Zn^{2+} caused an increase. Cd^{2+} is less efficient at neutralizing the charged phosphates of the tRNA backbone than either Mg^{2+} or Zn^{2+} , and it binds to sites on the bases that favor denaturation or random coil formation. Mg^{2+} has very little affinity for sites on the bases and is very effective in neutralizing phosphates; this favors the helical state, thereby increasing the t_m . Zn^{2+} has the largest charge-to-radius ratio of all of the transition elements, and so it is reasonably effective in neutralizing phosphate charges. However, like Cd^{2+} , Zn^{2+} can interact with sites on the bases. These two factors have opposite effects on the melting temperature and tend to cancel each other. Thus, Zn^{2+} elevates the t_m only slightly (~1°C at 10 mM).

All three metal ions, Cd^{2+} , Zn^{2+} and Mg^{2+} , sharpen the individual melting transitions, although to different extents. The effect of these ions on the shape of the differential melting curves is probably a better estimate of their respective contributions to phosphate neutralization than is the actual melting

temperature. The melting temperature reflects the relative affinities of these metals for binding to the helix or coil states. On the other hand, peak sharpening, or the increase in "apparent" cooperativity of these transitions, is directly related to their ability to stabilize individual helical elements. This leads to the order $Mg^{2+} > Zn^{2+} > Cd^{2+}$ for their ability to neutralize phosphates and corresponds to their ranking in the "hard/soft" classification scheme of Pearson (1968).

These observations suggest that the increased retention of tRNA in RPC-5 chromatography is not due to binding of Zn^{2+} at adjacent purines, since these sites were shown to be saturated prior to the range of Zn^{2+} concentrations which have a marked effect on chromatography (Flanagan & Jacobson, 1986). The chromatographic effect also cannot be accounted for by binding of Zn^{2+} at the metal ion site in the anticodon loop. Other sites on the tRNA are involved in the " Zn^{2+} effect" on this chromatography system, e.g., the "elbow" region, as indicated from NMR (Flanagan et al., 1986).

CHAPTER IV

THE EFFECT OF ZINC IONS ON tRNA STRUCTURE:

IMINO-PROTON NMR SPECTROSCOPY

Introduction

The low field ^1H -NMR spectra of tRNAs contain the resonances of the hydrogen-bonded imino-protons of the helical stems and of the tertiary base-pairs (for review, see Reid, 1981). Each imino-proton gives a separate signal and its associated chemical shift is mainly influenced by the ring-current effects of the neighboring base-pairs. The imino-proton spectrum is particularly sensitive to the orientation and distances of the neighboring rings (Arter & Schmidt, 1976). The imino-proton spectrum of yeast tRNA^{Phe} has been assigned independently by two groups using the sequential nuclear Overhauser effects (NOE) technique (Roy & Redfield, 1982; Heerschap et al., 1982, 1983a,b). This region of the E. coli tRNA^{Val}₁ spectrum has been assigned by sequential NOE (Hare & Reid, 1982) and more recently by a two-dimensional NOE technique (Hare et al., 1985).

Studies on the affect of Zn^{2+} on tRNA structure are hampered by the fact that Zn^{2+} , like Pb^{2+} , catalyzes alkaline hydrolysis of RNA (Farkas, 1968; Butzow & Eichhorn, 1975; Werner et al., 1976; Sundaralingam et al., 1984) at neutral pH. The hydrolysis of RNA by metals is not limited to Pb^{2+} and Zn^{2+} ; even Mg^{2+} will

degrade RNA, although the rate of this reaction is generally much less than those of Pb^{2+} and Zn^{2+} (Wintermeyer & Zachau, 1973; Werner et al., 1976). The degradation of RNA by these metals can be suppressed by lowering the pH to below 6.0 without apparently affecting the sites of interaction (Brown et al., 1985).

In this study we have used ^1H -NMR spectroscopy to look for the specific effects of ions on the structure of yeast tRNA^{Phe} and E. coli tRNA₁^{Val}. These experiments were prompted by the observations of Hiatt & Jacobson (1980), and Flanagan & Jacobson (1986) that Zn^{2+} has a unique affect on tRNA structure as judged by the increased retention of tRNAs in the presence of Zn^{2+} in RPC-5 chromatography.

Materials and Methods

Yeast tRNA^{Phe} was purchased from Boehringer Mannheim Biochemical Co. (7941 Castleway, Indianapolis, Indiana 46250); it had an amino acid acceptance of 1460 pmol/A₂₆₀ as determined by the manufacturer. E. coli tRNA₁^{Val} was purchased from Subriden RNA (P. O. Box 121, Rolling Bay, Washington 98061). In all but the initial experiments with tRNA^{Phe}, the residual 1 to 2 Mg^{2+} ions were removed by dissolving the tRNA (7-10 mg) in 10 mL of 10 mM EDTA at pH 7.0 and heating it at 60° for 5 minutes. This solution was allowed to return to room temperature and then vacuum-dialyzed against distilled water to a final volume of

0.25 mL and lyophilized. For the NMR studies, ~8 mg of tRNA was dissolved in 0.3 mL of buffer containing 500 mM NaCl and 10 mM NaOAc, pH 4.6. A 50 μ L aliquot of D₂O was added as a signal lock and 0.2 mM 4,4-dimethyl-4-silapentane-1-sulfonate (DSS) as an internal reference. The final concentration of tRNA was 1 mM. Divalent metal ions were added (Cd²⁺, Mg²⁺, or Zn²⁺) in the chloride form by transferring the tRNA sample to a clean piece of Parafilm and then adding stock metal solutions.

Proton NMR spectra were collected on a Bruker WM 500 FT NMR spectrometer with a 16-bit digitizer resolution, using a modified 21412 Redfield pulse sequence (Redfield, 1978). The spectra were collected in 8 K data channels with a sweep width of 1400 Hz, a pulse length of 205 μ s, and a carrier offset of 4730 Hz downfield of water. Typically 1000 scans were collected for each sample.

Nuclear Overhauser effects (NOEs) were measured by collecting sets of 16 scans with the decoupler alternately on-and-off-resonance. Typically, 4000 scans were collected for each experiment. A saturation pulse between 0.4 to 1.5 s was used, and the decoupler power was adjusted to just saturate the target resonance with minimal spillover to neighboring peaks. The long saturation pulse (1.5 s) was used to facilitate the observation of spatially distant protons via second order NOEs, which can be observed under these conditions.

Results

Removal of residual metal ions. In the presence of the residual 1 to 2 Mg^{2+} ions the imino-proton NMR spectra of yeast tRNA^{Phe} was obtained to provide a baseline against which the effects of added metal ions will be compared. The clover leaf representation of yeast tRNA^{Phe} (RajBhandry & Chang, 1968) is presented in Figure 16.

The removal of residual Mg^{2+} from tRNA^{Phe} affected virtually all of the imino-proton resonances between 9 and 15 ppm. This region is shown for yeast tRNA^{Phe} , in 500 mM NaCl and 10 mM NaOAc (pH 4.6), without removing the residual Mg^{2+} (1 to 2 equiv. Mg^{2+}) in Figure 17a. The spectrum for tRNA^{Phe} treated with warm (50°C) 10 mM EDTA to remove Mg^{2+} (<0.1 equiv. Mg^{2+} , Hyde & Reid, 1985) in the same buffer is shown in Figure 17b. The resonance peaks are lettered from A to Z in accordance with Heerschap et al. (1982). The changes in chemical shifts ranged from 0.01 to 0.4 ppm. We concern ourselves only with those changes that are relatively large (>0.05 ppm), and can be reasonably attributed to a particular imino-proton.

The assignments of Heerschap et al. (1983, 1983a,b) were made at pH 7.0 in two different buffers: buffer 1 contained 80 mM NaCl, 20 mM sodium cacodylate (pH 7.0), 5 mM MgCl_2 , and 0.1 mM EDTA; buffer 2 contained 400 mM NaCl, 20 mM sodium cacodylate (pH 7.0), and 0.2 mM EDTA. The Mg^{2+} in buffer 2 was <0.01 equiv. Mg^{2+} . Our NMR experiments were carried out in

Figure 16: Yeast tRNA^{Phe} arranged in the two-dimensional clover-leaf structure.

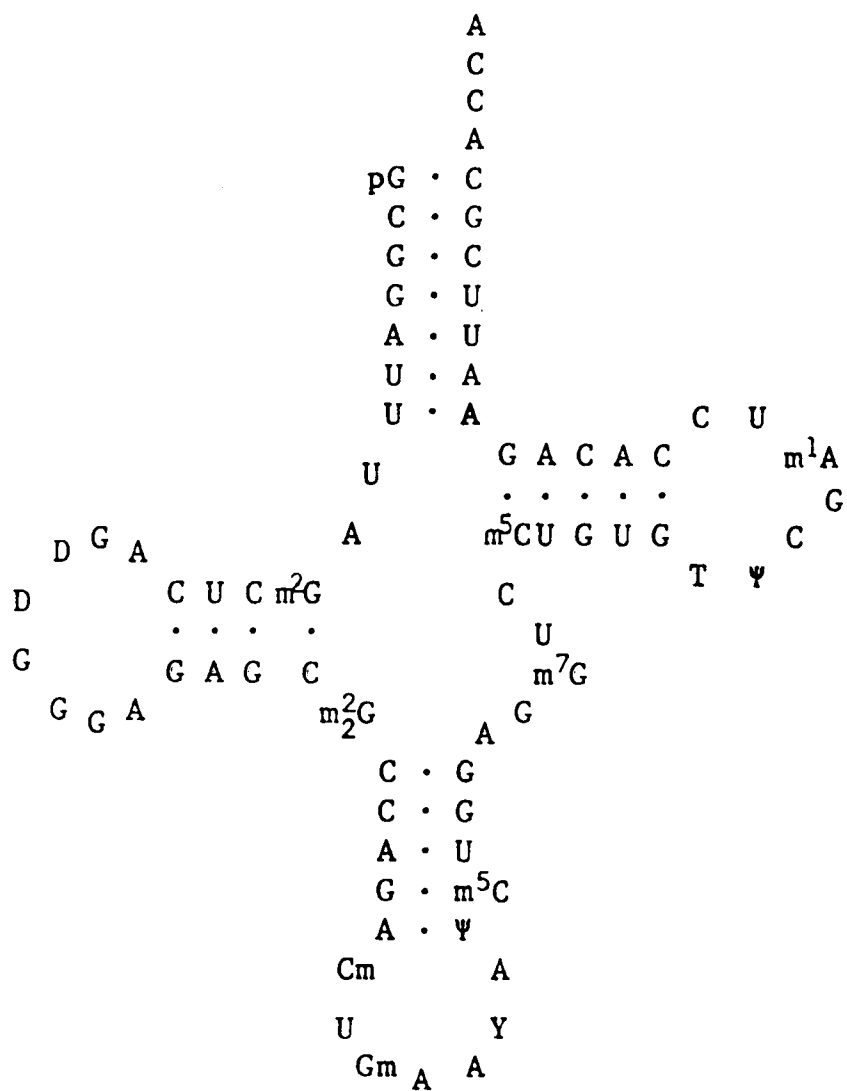


Figure 16

Figure 17: 500-MHz low-field NMR spectrum of yeast tRNA^{Phe}. The tRNA was at 35°C in 500 mM NaCl, 10 mM NaOAc, pH 4.6. (a) in the presence of residual (1 to 2 equiv. Mg²⁺) Mg²⁺, (b) after complete removal of Mg²⁺ ions (<0.1 equiv. Mg²⁺).

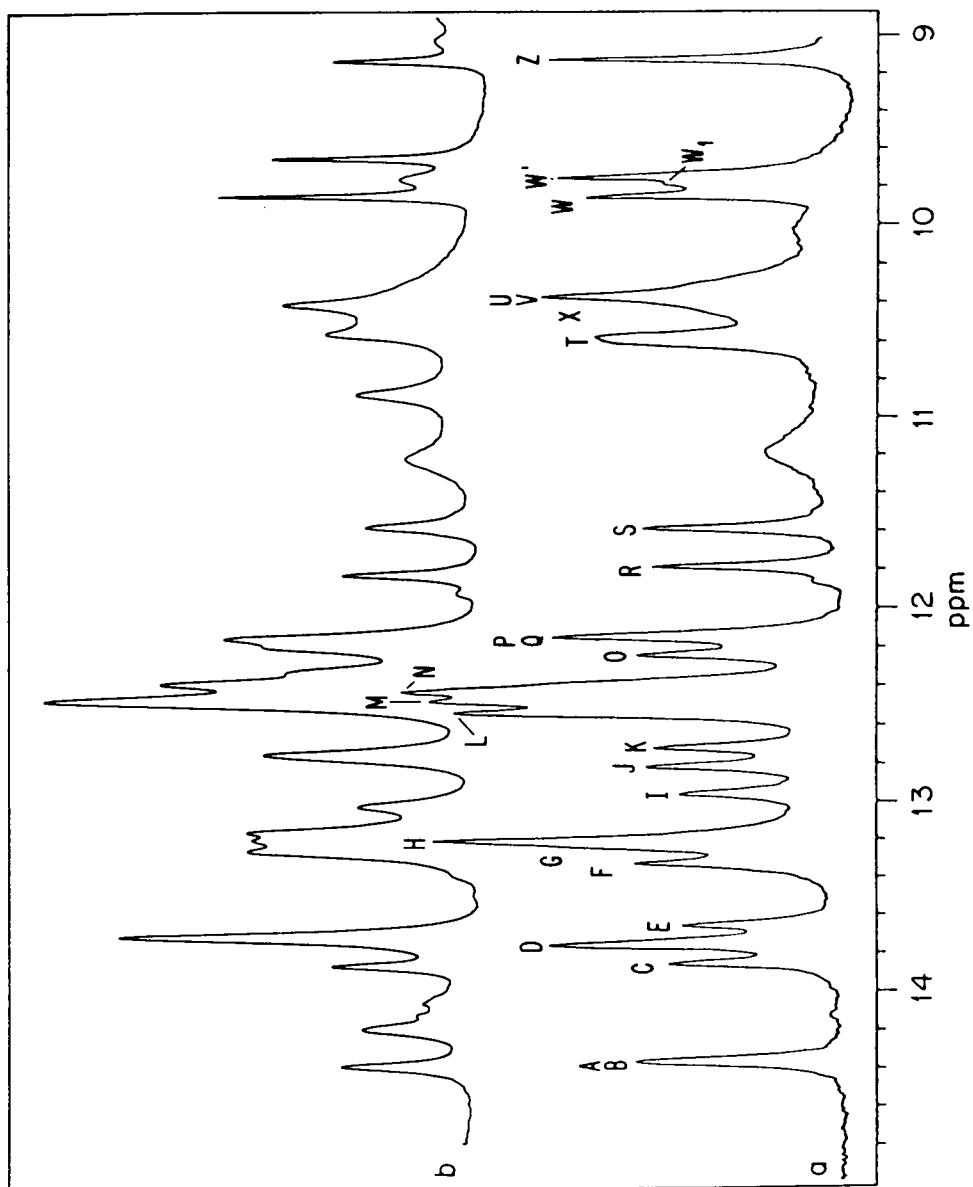


Figure 17

buffers containing 500 mM NaCl and 10 mM NaOAc (pH 4.6). Under these conditions, the spectrum obtained when 1 to 2 Mg^{2+} ions were present (Figure 17a) resembled the spectrum of Heerschap et al. (1982) collected in buffer 1, and our zero Mg^{2+} sample appeared to be similar to their zero Mg^{2+} sample. Wong et al. (1973) also observed that mildly acidic conditions did not produce gross spectral changes in the imino-proton region of yeast tRNA^{Phe}.

Resonances AB, DE, JK and X exhibit relatively large changes in chemical shifts upon removal of Mg^{2+} , as does the region of highly overlapping peaks between 12.6 and 12.0 ppm (LMN). Peaks A and B in the absence of Mg^{2+} correspond to resonance lines containing a single proton each and are found at 14.40 and 14.18 ppm respectively. Resonance A is attributed to U•A-6. Resonance B is probably (U-8)•(A-14), a tertiary base-pair that links the base of the acceptor stem to the dihydrouridine loop (D-loop). These assignments are in agreement with both Heerschap et al. (1982) and Roy & Redfield (1982) in the absence of Mg^{2+} . In the presence of 1 to 2 Mg^{2+} per tRNA, peaks A and B have similar chemical shifts and are seen as a single resonance at 14.40 ppm. Analogously, Roy & Redfield (1982) observed that at pH 7.0, 100 mM NaCl and 15 mM MgCl_2 AB was a single peak at 14.35, while Heerschap et al. (1982), observed a single peak at 14.40 ppm in 80 mM NaCl, 5 mM MgCl at pH 7.0, which they attributed to these protons.

Resonance lines DE are also affected by the removal of the tightly bound Mg^{2+} . In the absence of Mg^{2+} , DE is a single

resonance line at 13.69 ppm, containing at least three imino-protons, C•G-11, U•A-12 and U•A-52, that resonate under this peak (Heerschap et al., 1982, 1983a). Roy & Redfield (1982) originally assigned (T-54)•(m¹A-58) to this peak, which is similar to its location in the spectrum of E. coli tRNA^{Val} (Hare et al., 1985) and E. coli tRNA^{Phe} (Hyde & Reid, 1985). Roy et al. (1984), using (1,3-¹⁵N) uracil-enriched yeast tRNA^{Phe}, discovered that at almost the same ¹⁵N frequency at which A•U base-pairs are decoupled in the ¹⁵N-NMR spectrum, an effect is seen at 12.4 ppm in the imino-proton spectrum; the assignment of (T-54)•(m¹A-58) to this resonance is now consistent with its location as determined by Heerschap et al. (1983b).

Both Heerschap et al. (1982) and Roy & Redfield (1982) noted that in the presence of Mg²⁺ peak DE was resolved into two peaks. Analogously, we find that in the presence of 1 to 2 Mg²⁺, D is located at 13.72 ppm and E at 13.65 ppm. Both groups assigned C•G-11 to peak E while D contained U•A-12, U•A-52 and possibly (T-54)•(m¹A-58).

The addition of Mg²⁺ resolves peak JK (12.76 ppm) into two resonance lines corresponding to 1 proton each at 12.80 ppm (J) and 12.70 ppm (K). Heerschap et al. (1982) assigned the imino-protons of G•C-1 and (m²G-10)•(C-25) to this region while Roy & Redfield (1982) assigned G•C-51 instead of G•C-1 to peak JK. Neither group's assignment for the second imino-proton of peak JK was direct; the alternate assignment of G•C-1 to the highly overlapped region near LMN by Roy & Redfield (1982) is less convincing

since it depends only on the heat-labile nature of one of these resonances. In both *E. coli* tRNA^{Phe} (Hyde & Reid, 1985) and yeast tRNA^{Phe} (Heerschap et al., 1982; Roy & Redfield, 1982), the (m²G-10)•(C-25) base-pair is shifted upfield by increasing Mg²⁺ concentration at pH 7.0, which would explain the upfield shift in one of the components of JK, in the presence of 1 to 2 Mg²⁺.

Peak X exhibits the largest change in chemical shift upon removal of Mg²⁺. In the presence of Mg²⁺, X appears to be a component in the TXUV group (10.3 - 10.6 ppm). In the absence of Mg²⁺, X was shifted downfield by about 0.4 ppm to 10.89 ppm. Both Heerschap et al. (1983b) and Roy & Redfield (1982) have assigned this resonance to the N1-H proton of Ψ-55. The N3-H proton of this base is involved in a hydrogen bond with the phosphate oxygen of G-18; this proton resonates at ~11.6 ppm. The large upfield shift in the presence of 1 to 2 Mg²⁺ is probably due to the displacement of the water molecule that is hydrogen-bonded to the N1-H proton by Mg²⁺ binding (Tropp & Redfield, 1981).

These results are summarized in Table 3, where the identification and chemical shifts of Heerschap et al. (1983b) and Roy & Redfield (1982) are included for comparison. It is interesting to note that the chemical shifts obtained from our data (pH 4.6) are very similar to those observed in the other studies, even though the conditions used in each study were different. The list of resonances in Table 3 are not meant as an exhaustive list of those affected by Mg²⁺. For example, Table 3 does not include the region for peak, LMN, which contains a

Table 3

Summary of Chemical Shifts in tRNA^{Phe} that are Affected by the Presence of Mg²⁺

Base-pair	Peak	Resonances (ppm)			
		1-2 Mg ²⁺ per tRNA ^a	~0 Mg ²⁺ per tRNA ^b	Heerschap et al. Buffer 1 ^c Buffer 2 ^d	Roy & Redfield ^e
(A-6)•(U-67)	A	14.40	14.40	14.40 14.46	14.35
(A-8)•(U-14)	B	14.40	14.18	14.40 14.20	14.20
(U-52)•(A-62) (U-12)•(A-23) (T-54)•(m ¹ A-58)	D	13.72	13.69	13.80 13.73	13.70
(C-11)•(G-24)	E	13.65	13.69	13.65 13.73	13.70
(G-1)•(C-72)	J	12.80	12.76	12.84 12.78	---

(Table 3 continued)

Table 3 (continued)

Base-pair	Peak	Resonances (ppm)			
		1-2 Mg ²⁺ per tRNA ^a	~0 Mg ²⁺ per tRNA ^b	Heerschap et al. Buffer 1 ^c Buffer 2 ^d	Roy & Redfield ^e
(m ² G-10)•(C-25)	K	12.70	12.76	12.74 12.77	12.75
NH-1 (Ψ-55)	X	10.89	10.05	11.00 10.48	11.00

^aBuffer: 500 mM NaCl, 10 mM NaOAc, pH 4.6, at 35°C.

^bBuffer: 500 mM NaCl, 10 mM NaOAc, pH 4.6, tRNA sample treated as in Hyde & Reid (1985) to remove residual Mg²⁺, at 35°C.

^cBuffer 1: 80 mM NaCl, 30 mM cacodylate, pH 7.0, 5 mM MgCl₂, 0.1 mM EDTA, at 23°C.

^dBuffer 2: 400 mM NaCl, 30 mM cacodylate, pH 7.0, 0.2 mM EDTA (residual Mg²⁺ <0.01 equiv.), at 23°C.

^e100 mM NaCl, 10 mM phosphate, pH 7.0, 1 mM EDTA at 20°C.

number of highly overlapped resonances, some of which are dramatically affected. Our assignments must be taken as tentative due to the lack of NOE data to corroborate them.

Comparison of the imino-proton spectrum of yeast tRNA^{Phe} in the presence of MgCl₂, CdCl₂ and ZnCl₂. Figure 18 shows the spectra obtained from yeast tRNA^{Phe} with no divalent metal ion (Figure 18a), 10 mM MgCl₂ (Figure 18b), 10 mM CdCl₂ (Figure 18c), or 8 mM ZnCl₂ (Figure 18d). In addition to the added metal ion, the samples also contained 1 to 2 tightly bound Mg²⁺ per tRNA. This situation is similar to the recent X-ray crystallographic study of tRNA^{Phe} in the presence of ZnCl₂ (Rubin et al., 1983), where both Zn²⁺ and Mg²⁺ were present in the crystal.

There appear to be very few changes in the imino-proton spectra that may be ascribed to an individual divalent ion. In general, if one metal ion affected a resonance so did the other two, although not necessarily in the same manner. A specific effect is caused by Zn²⁺ on the peaks designated WW', at 9.83 and 9.75 ppm respectively (Figure 18d). The addition of 8 mM Zn²⁺ causes this peak to collapse into a single, albeit asymmetric, peak at 9.80 ppm. The peaks W and W' are the only obvious NMR spectral feature altered on lowering the pH. At pH 7.0 WW' is seen as a hump at 9.75 ppm, whereas at lower pH two very sharp side peaks appeared with a central hump between them (Choi & Redfield, 1985).

Figure 18: Effect of various metal ions on the low-field NMR spectra of 1 mM yeast tRNA^{Phe}. Conditions were the same as for Figure 2a. (a) residual Mg²⁺, (b) 10 mM added MgCl₂, (c) 10 mM added CdCl₂, and (d) 8 mM added ZnCl₂.

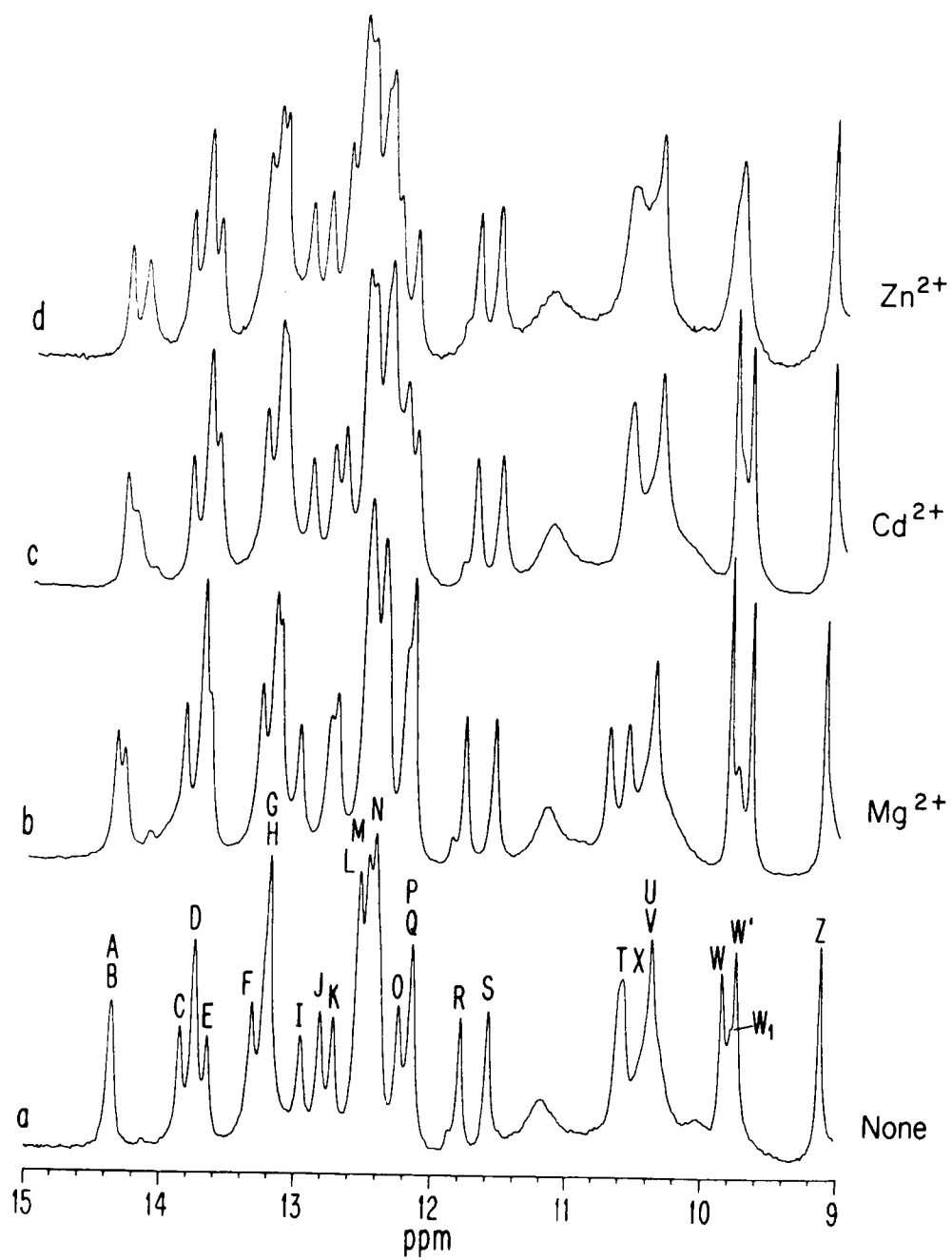


Figure 18

One of the protons assigned to the peak WW' is the N1-H of G-18 (Heerschap et al., 1983b; Choi & Redfield, 1985). This proton probably accounts for the central hump. The upfield component of WW' (9.62 ppm in Figure 18a) was recently assigned to the tertiary base-triple (U-12)•(A-23)•(A-9). The amino-proton of A-23, which is hydrogen bonded to the N-7 of A-9, is thought to be shifted downfield from the usual amino-proton region by the ring current of A-9 (Choi & Redfield, 1985). The increased stability of this peak, WW', at lower pH is probably due to the protonation of N-1 (A-9) and the decreased general base catalysis of NH (G-18). The protonated form of A-9 can interact electrostatically with nearby phosphates, stabilizing residues A-23 and A-9 in a salt bridge (Steinmetz-Kayne et al., 1977).

The effect of 8 mM Zn^{2+} on (U-12)•(A-23)•(A-9) is to shift this resonance at peak WW' downfield by 0.05 ppm from the position in the spectrum obtained without added divalent metal ion, or 0.13 ppm downfield from its position in 10 mM Mg^{2+} . Zn^{2+} also appears to broaden this resonance, which may indicate an increased rate of exchange under these conditions.

Zn^{2+} also has a marked effect on the OPQ region of the imino-proton spectra (12.3 to 12.0 ppm in Figure 18d). There are three protons in this region: G•C-3, (G-15)•(C-48) and C•G-28 (Heerschap et al., 1982, 1983b; Roy & Redfield, 1982). In the absence of divalent ions, peak O, G•C-3 is downfield of PQ, Mg^{2+} causes G•C-3 to shift upfield toward peak PQ. Eight mM Zn^{2+} , however, may cause peak O and one of the protons in peak PQ to be

shifted downfield. Two lines of indirect evidence suggest that the proton in PQ that moves downfield is due to (G-15)•(C-48). First, the imino-proton of this base-pair is near one of the Mg^{2+} binding sites in the crystal (Holbrook et al., 1977) and it has also been observed to be affected by low concentrations of Mn^{2+} and Co^{2+} (Hurd et al., 1979). Second, base-pair (U-8)•(A-14), the spatially adjacent neighbor of (G-15)•(C-48), is also affected by Zn^{2+} (shifted upfield), although peak GH (A•U-29), the neighbor of C•G-28, may also be affected (shifted downfield). Under these conditions we could not observe an NOE from (U-8)•(A-14) to (G-15)•(C-48) to confirm our assignment. The magnitude of the change in chemical shift for the proton from (G-15)•(C-48) is relatively large, at least 0.2 ppm, and it could be as large as 0.3 ppm. An accurate determination of the chemical shift for this resonance was complicated by the large number of protons resonating in this region. Cd^{2+} also appears to perturb the (G-15)•(C-48) environment (Figure 18c), though the change in chemical shift was only about ~0.1 ppm. Mg^{2+} had little or no effect on peak PQ.

With no added metal ions, peaks A and B represent single proton resonances at 14.40 and 14.18 ppm respectively (Figure 17b). The presence of small amounts of Mg^{2+} collapses these resonances into a single peak at 14.36 ppm. Addition of 10 mM $MgCl_2$, 10 mM $CdCl_2$ or 8 mM $ZnCl_2$ shifts one of these protons upfield with Zn^{2+} being the most effective and Cd^{2+} the least. The mobile proton in terms of chemical shift has generally been attributed to

(U-8)•(A-14), a tertiary base-pair. We have confirmed this assignment by measuring the NOE from the mobile peak B, which gives rise to NOE peaks at 12.93 ppm, 13.65 ppm, as well as a very strong NOE to the aromatic region (data not shown). The strong NOE to the aromatic region confirms that this is an A•U base-pair. The NOE to 12.93 ppm (C•G-13) and the weak NOE to 13.65 (U•A-12) suggest that peak B is indeed due to the imino-proton of (U-8)•(A-14). Our inability to detect an NOE to (G-15)•(C-48) under these conditions (35°C, 500 mM NaCl and 10 mM NaOAc, pH 4.6) is in accord with the findings of Heerschap et al. (1983b), who could only observe an NOE to the (G-15)•(C-48) proton at 10°C.

An interesting phenomenon not related to the effect of Zn^{2+} can be seen in the two Mg^{2+} spectra (Figure 17a, 18b). A comparison of Figure 17a (1-2 equiv. Mg^{2+}), Figure 17b (<0.1 equiv. Mg^{2+}), and Figure 18b (11 equiv. Mg^{2+}) shows that a number of resonances undergo U-shaped titration curves. In particular, peak B shifts downfield to 14.40 ppm upon addition of 1 to 2 Mg^{2+} (Figure 17a), and then upfield to 14.31 ppm at 10 mM MgCl_2 . Peak X is another example of this behavior; it shifts upfield from 10.89 ppm ($-\text{Mg}^{2+}$) to 10.5 ppm when 1 to 2 Mg^{2+} are present, and then back downfield to 10.72 ppm at 10 mM Mg^{2+} . These are two of the most prominent examples. The U-shaped titration curve cannot be explained by any previous Mg^{2+} titration curves at pH 7.0 (Hyde & Reid, 1985; Reid et al., 1979) and is apparently the result of a pH effect. At present we have no

explanation for this phenomenon; further study will be required to determine the nature of this behavior.

Titration of yeast tRNA^{Phe} with Zn²⁺. The effect of increasing the concentration of Zn²⁺ from 0 to 8 mM on the imino-proton spectra at 37°C is shown in Figure 19 with a summary in Figure 20. Most of the peaks shift slightly with Zn²⁺. The largest shifts are experienced by peaks B, M, P and W'. Peak B moves upfield while M, P and W' are shifted downfield. The chemical change occurs progressively with Zn²⁺ for peaks B and P, which shift in opposite directions; both apparently reach completion at about 4 mM Zn²⁺. Here, as in earlier work with Mg²⁺ (Hyde & Reid, 1985b), effects of metal ions are seen in all the stem structures of the tRNA, but each effect is a very local one. Even protons in the same base are affected differently. For example, peaks X and R, which are due to the N1-H and N3-H proton of Ψ-55, respectively, exhibit quite different behavior. Peak X shifts continuously downfield with increasing Zn²⁺, while R appears to shift upfield slightly at concentrations up to ~4 mM Zn²⁺.

In only one case, peaks B and P, did Zn²⁺ cause a change in the chemical shifts of neighboring base-pairs. Peak B shifts upfield by 0.13 ppm (Figure 19) and is assigned to (U-8)•(A-14). Peak P shifts downfield by 0.25 ppm and, as discussed above, this resonance has been tentatively assigned to (G-15)•(C-48). As suggested above, both shifts appear to have been completed at about 4 mM Zn²⁺. Base-pair (G-15)•(C-48) is the terminal

Figure 19: Effect of increasing concentrations of Zn^{2+} on low-field NMR spectra of 1 mM yeast tRNA^{Phe}. Conditions were as for Figure 2a. (a) 0 mM, (b) 2 mM, (c) 4 mM, (d) 6 mM and (e) 8 mM added ZnCl_2 .

Figure 20: Summary of chemical shifts of peaks in Figure 19 at various concentrations of zinc.

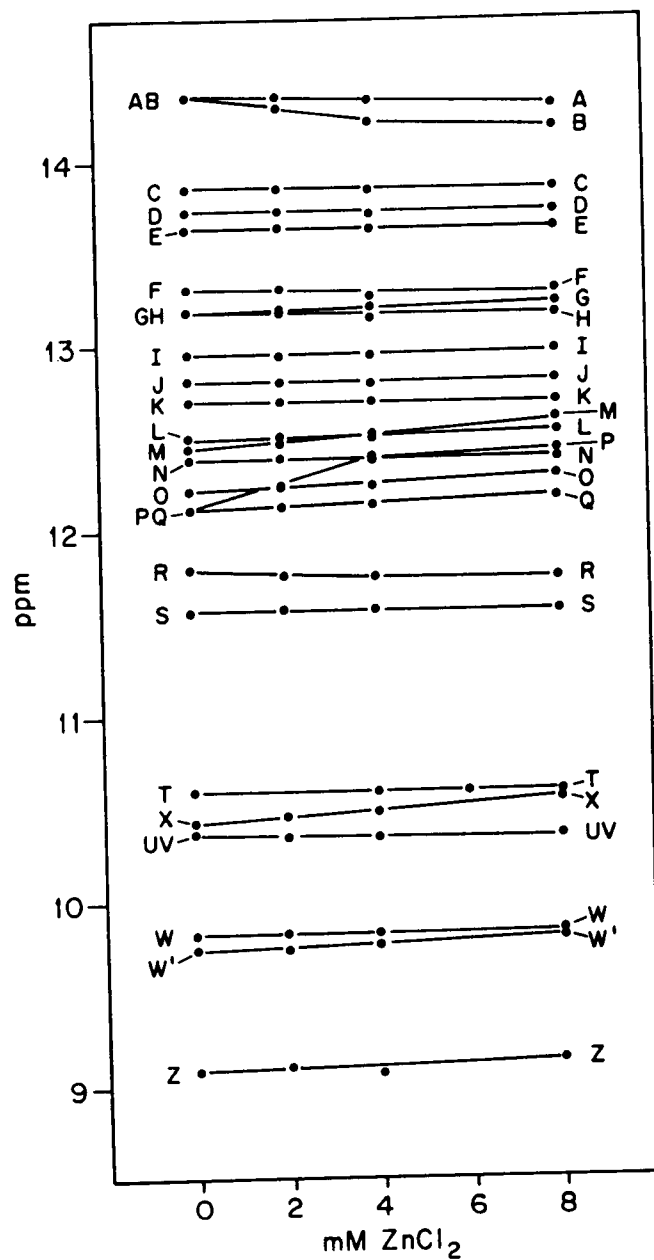


Figure 20

base-pair in the augmented D-helix and would not be expected to affect any other resonance. Base-pair (U-8)•(A-14), however, is also stacked on C•G-13, Peak I, which does not appear to be affected by Zn^{2+} .

The effect of Zn^{2+} on tRNA^{Phe} imino-proton spectrum in the absence of Mg^{2+} . Figure 21a-c shows the imino-proton spectra of yeast tRNA^{Phe} at concentrations of Zn^{2+} from 0 to 10 mM. Unlike the previous titration, (Figure 19a-e), the residual Mg^{2+} had been removed (<0.1 equiv. Mg^{2+}). The effect of added Zn^{2+} on each resonance is summarized in Figure 22, which shows that the major effects of Zn^{2+} are on resonances D, L_1 , and P, all shifted downfield by Zn^{2+} . The effects on all three resonances are seen at the lowest Zn^{2+} concentrations. By far the largest change in chemical shift was that of P. Resonance peaks P and Q correspond to a single two-proton peak at 12.15 ppm in the absence of all divalent metal ions. The addition of 4 mM Zn^{2+} appears to shift one proton downfield to about 12.5 ppm. The exact location of this resonance cannot be determined, since it has moved into the highly overlapped region between 12.6 and 12.4 ppm. It appears, however, that this resonance is shifted by at least 0.3 ppm. This resonance was also affected by Zn^{2+} when 1 to 2 Mg^{2+} ions were present (Figure 19a). As shown above, we attribute peak P to (G-15)•(C-48).

Another relatively large downfield shift (~ 0.2 ppm) occurred with resonance L_1 (Figure 21). It appears that one proton in the

Figure 21: Effect of increasing concentrations of Zn^{2+} on the low-field NMR spectra of 1 mM yeast tRNA^{Phe}. Conditions were as in Figure 2b. (a) 0 mM, (b) 2 mM, (c) 4 mM, (d) 6 mM and (e) 10 mM added ZnCl_2 .

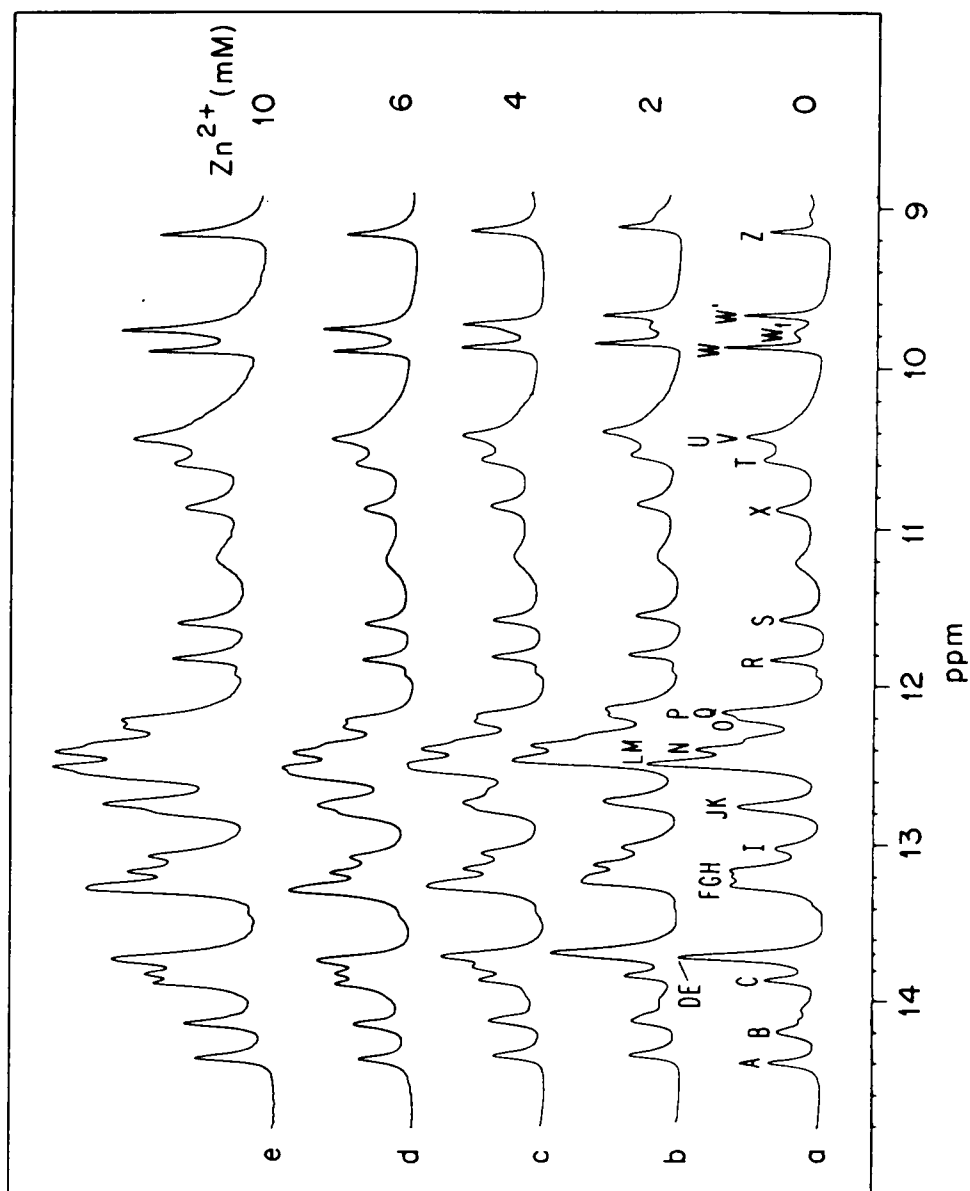


Figure 21

Figure 22: Summary of chemical shifts of peaks in Figure 21 at various concentrations of zinc.

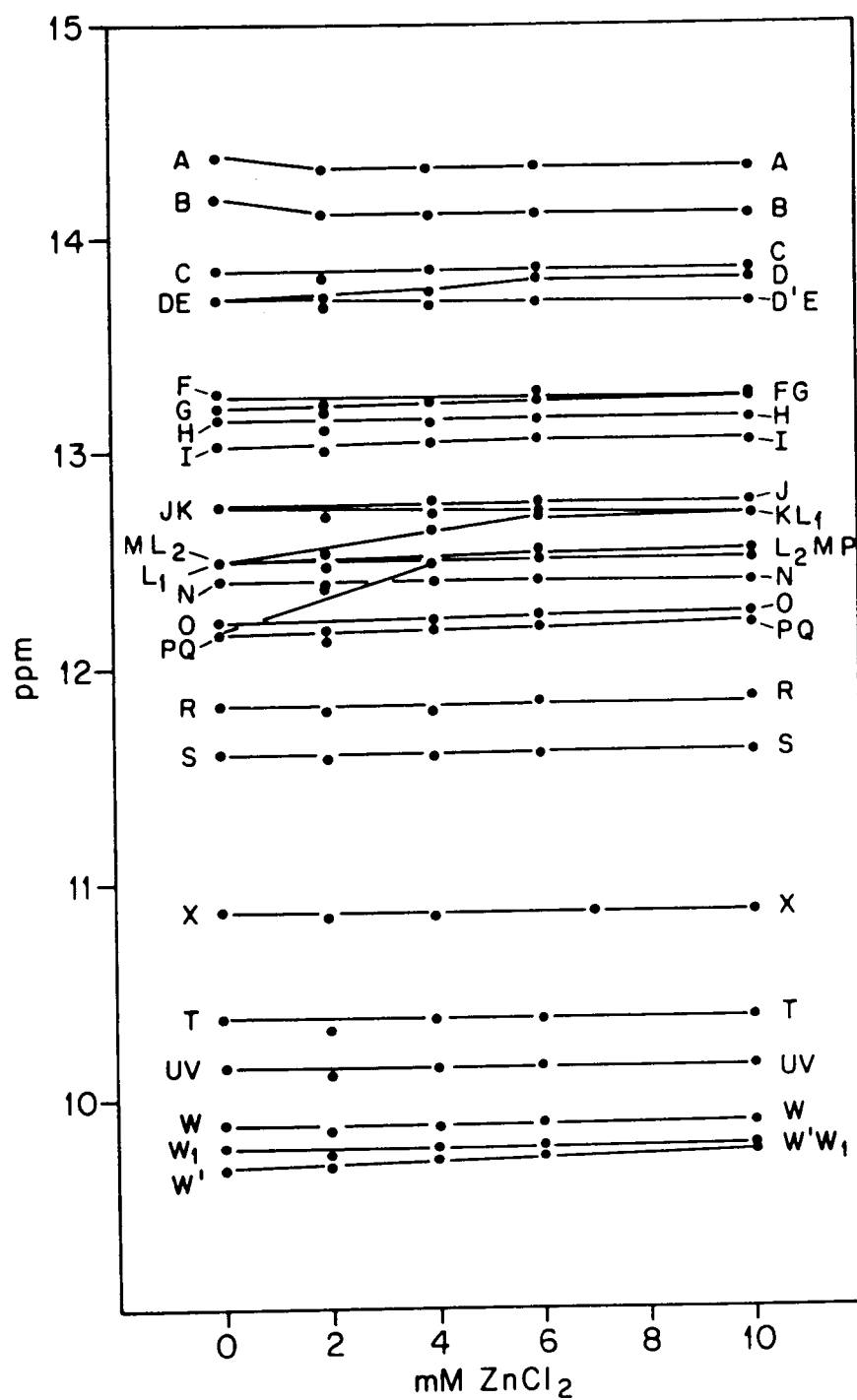


Figure 22

LMN grouping is shifted downfield into peak K (12.70 ppm). There are numerous protons resonating between 12.6 and 12.4 ppm including: C•G-43, G•C-30, (G-19)•(C-56), C•G-2, m⁵C•G-49, G•C-53, G•C-51 and possibly (T-54)•(m¹A-58). Without supporting NOE data it is impossible to draw any conclusion about the imino-proton that gives rise to the resonance at L₁. It is interesting to note that in the presence of 1 to 2 Mg²⁺ there is also a resonance from this region shifted upfield to ~12.7 ppm (Figure 18d, resonance M).

Addition of 10 equivalents of Zn²⁺ per tRNA shifts one proton from peak DE downfield to 13.80 ppm (Figure 21). Peak DE contains base-pairs U•A-12, U•A-52, and C•G-11. Base-pair U•A-12 is involved in a tertiary interaction with the amino group of A-9, the upfield component of WW₁W' at 9.67 ppm. The resonance of the amino-proton from A-9 is also affected by Zn²⁺ suggesting that the component of D affected by Zn²⁺ may be U•A-12. However, the amino proton of A-9 is also shifted 0.08 ppm downfield by Zn²⁺ when 1 to 2 Mg²⁺ are present and no component of D is similarly affected (Figure 20 peaks W' and D respectively). At present, we cannot assign conclusively any of the three protons of peak DE to base-pairs affected by Zn²⁺.

The residual tightly-bound Mg²⁺ not only has a marked effect on the imino-proton spectrum but also has an effect on the change caused by zinc. The spectrum of yeast tRNA^{Phe} with no residual Mg²⁺ and 10 mM Zn²⁺ (Figure 21e) is significantly different from that obtained when 1 to 2 mM Mg²⁺ are present with 8 mM Zn²⁺

(Figure 20). In the latter, peak X is located in the group of resonances TXUV near 10.50 ppm while in the absence of all divalent ions (Figure 21a) or with 10 mM Zn^{2+} and no Mg^{2+} (Figure 21e). Peak X is located at 10.87 ppm. With no added metal ion and 1 to 2 Mg^{2+} per tRNA (Figure 19a), X is also near 10.50 ppm. These observations suggest that peak X (N1-H of Ψ -55) is insensitive to Zn^{2+} , although it is affected by Mg^{2+} . The changes in chemical shift of this proton, N1-H of Ψ -55, occur without significantly affecting the chemical shift of peak S, the NH-3 proton of Ψ -55, which is hydrogen-bonded to the phosphate of m^1A -58.

The presence of Mg^{2+} influences how Zn^{2+} affects the resolution of peak WW'. In the absence of divalent ions peaks WW' are separated by 0.2 ppm; 10 mM Zn^{2+} reduces this by 0.12 ppm, primarily by shifting peak W' downfield (Figure 21e). In the presence of 1 to 2 Mg^{2+} ions per tRNA peaks WW' are separated by 0.12 ppm (Figure 19a); the addition of 8 mM Zn^{2+} causes the resolution of these peaks to deteriorate further with WW' resonances being separated by only 0.02 ppm (Figure 19d). Peak W' has been assigned to the amino proton of A-9 which participates in a tertiary interaction with U $\cdot$ A-12 (Choi & Redfield, 1985).

Because of the highly overlapped nature of the resonances in yeast tRNA^{Phe} imino-proton spectrum, especially in the absence of any Mg^{2+} , potential data are lost. In particular, the region between 12.4 to 12.0 ppm, Zn^{2+} appears to have some interesting effects in the absence of residual Mg^{2+} , although we cannot interpret these effects.

Effect of Zn^{2+} concentration on the imino-proton spectra of *E. coli* tRNA^{Val}. Under optimal solvent conditions for the ^1H -NMR spectrum of *E. coli* tRNA₁^{Val}, 22 peaks corresponding to 27 protons can be resolved (Hare et al., 1985), allowing a more detailed examination of effects caused by metal ions. Figure 23 shows the cloverleaf representation of *E. coli* tRNA₁^{Val}. The acceptor stem of this tRNA is symmetric about the central A•U base-pair, A•U-29, hence, the assignments of base-pairs 27, 31 and 28,30 cannot be obtained independently (Hare et al., 1985).

Figure 23, included as a reference, was obtained from *E. coli* tRNA^{Val} in 10 mM sodium phosphate, pH 7.0 buffer and contained ~ 7.5 equiv Mg^{2+} per tRNA. Each peak of this spectrum was assigned to a base-pair by Hare et al. (1985). For the purpose of comparison, we labeled the imino-proton resonances in this region from A to Z (Figure 24a); these designations are similar to the convention of Hare & Reid (1982).

The spectra (Figure 24a-e) were all obtained in 500 mM NaCl, and 10 mM NaOAc pH 4.6 buffer at various concentrations of added Zn^{2+} . The tRNA^{Val} sample (Figure 24a) contained < 0.1 equiv. Mg^{2+} per tRNA and resembles the zero-added Mg^{2+} sample of Reid et al. (1979), which was obtained at pH 7.0. Again, no gross spectral changes due to lowering the pH to 4.6 are observed.

Zn^{2+} primarily affects four resonances: C, L', R and T. All four peaks, C, L', R and T, are shifted downfield, at increasing Zn^{2+} concentration. The effects on these four resonances are seen, even at the lowest Zn^{2+} concentration

Figure 23: Imino-proton spectrum of E. coli tRNA^{Val}. The tRNA was dissolved in buffer to give maximum resolution and data were obtained at 35°C after partial removal of residual bound magnesium ion to obtain maximum peak resolution. Each peak is labeled with its base-pair identity as assigned by Hare et al., 1985. Inset: Sequence of E. coli tRNA^{Val} arranged in the two-dimensional cloverleaf structure. [Data used by permission of Hare et al. (1985)].

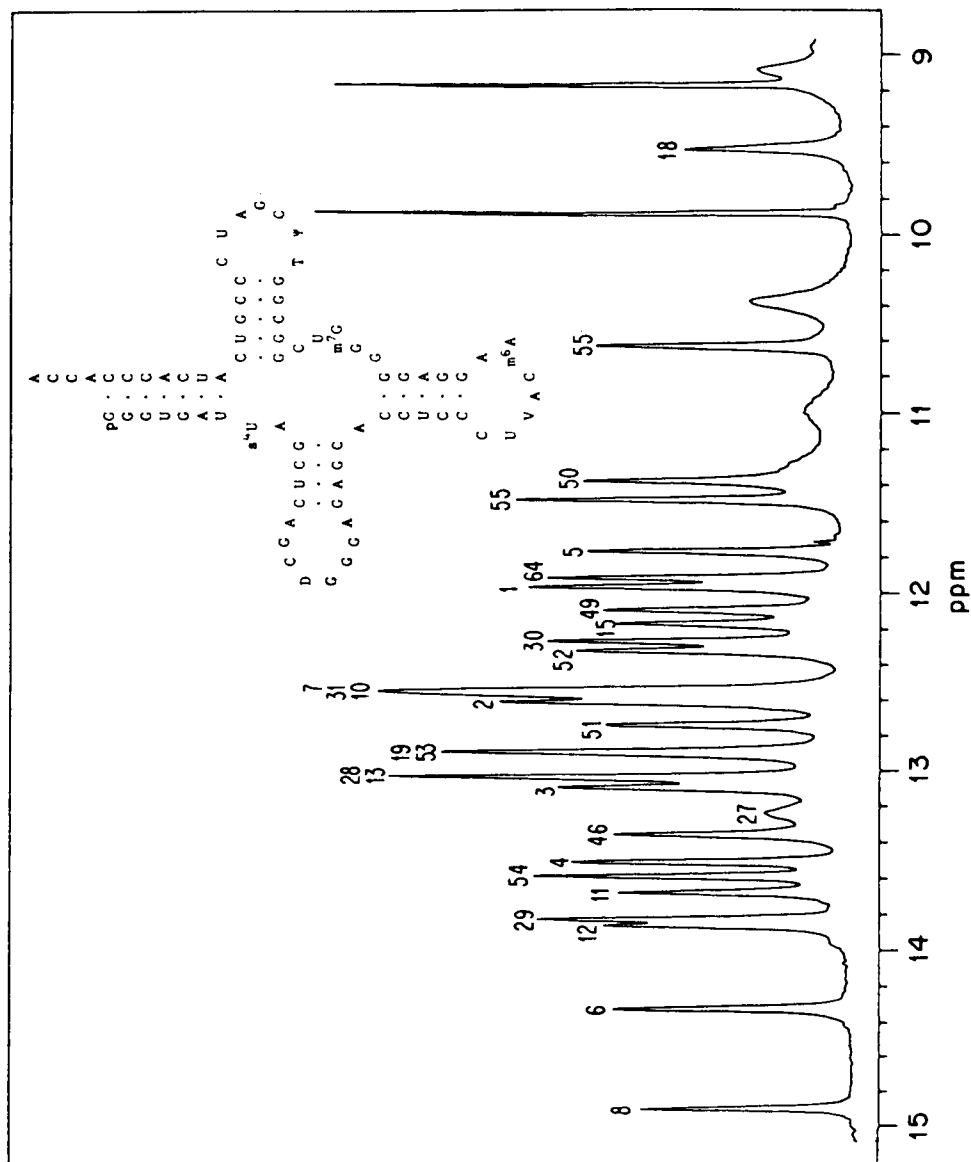


Figure 23

Figure 24: Effect of increasing concentrations of Zn^{2+} on the low-field NMR spectra of 1 mM E. coli tRNA^{Val}. Conditions were as in Figure 2b. (a) 0 mM, (b) 2 mM, (c) 4 mM, (d) 6 mM and (e) 8 mM added ZnCl_2 .

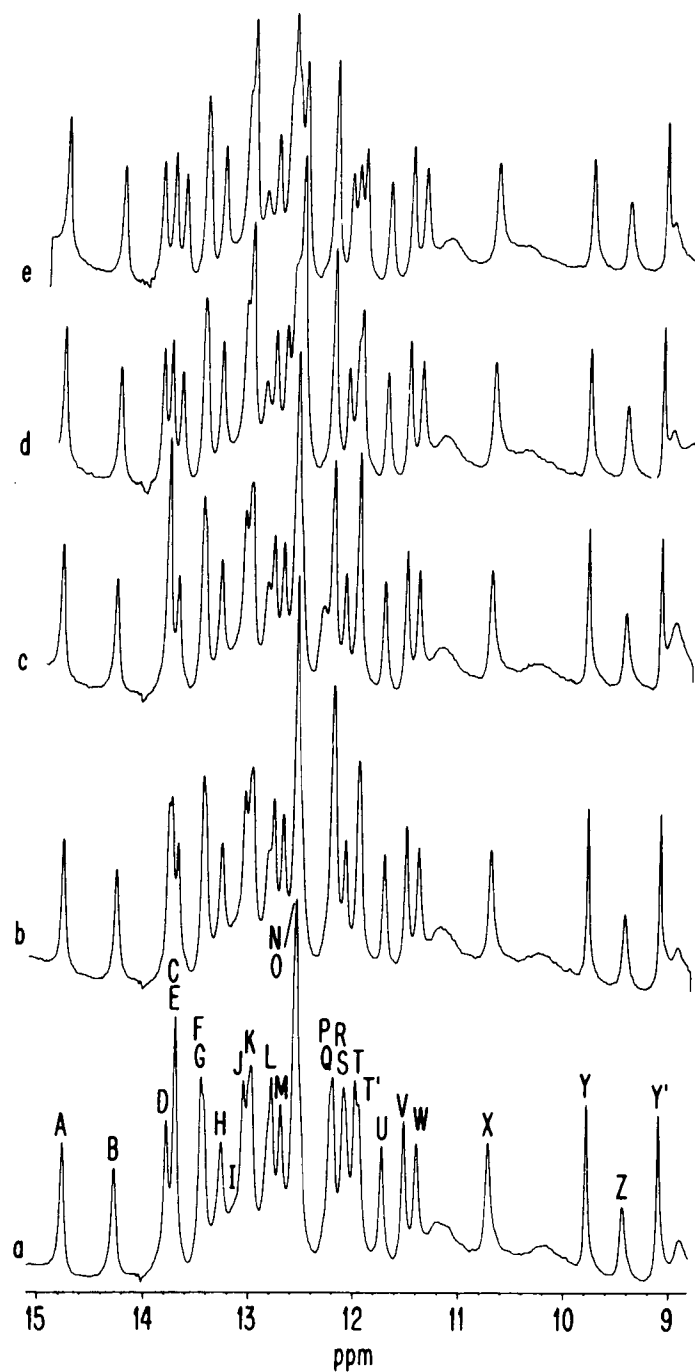


Figure 24

2 mM Figure 22b). Two other protons originating from peak 0 are also affected by Zn^{2+} , although it appears that at least one is affected only at concentrations above 4 mM. Figure 25 summarizes the effects of Zn^{2+} on all the resonances in the region.

The largest change observed upon addition of Zn^{2+} was on peak R, which is located at 12.08 ppm in the absence of Zn^{2+} and is shifted upfield to near 12.7 ppm in 8 mM Zn^{2+} . This is by far the largest change in chemical shift observed in our study (~ 0.6 ppm). The effect of Zn^{2+} on this resonance was not linear. The two protons believed to resonate under peak RS in the absence of divalent ions are G•C-49 and the tertiary base-pair (G-15)•(C-48) (Figure 24a). To determine which of these protons was affected by Zn^{2+} , we measured the NOE connectivity of peak A, ($\text{s}^4\text{U}-8$)•(A-14), which is located near (G-15)•(C-48). Although these base-pairs are spatially adjacent, the actual distance between imino-protons approaches the limit of detection for a first-order NOE ($\sim 5\text{\AA}$). To optimize our chances of observing an NOE to this proton we decreased the temperature to 17°C and used a 1.5 s saturation pulse. Under these conditions second order NOEs may be observed.

Figure 26 shows the results of this experiment. Pre-saturation of A, ($\text{s}^4\text{U}-8$)•(A-14), gave rise to NOEs at ~ 13.1 , ~ 8.6 , ~ 12.7 and possibly 13.3 ppm. The strong NOE to the aromatic proton region (8.6 ppm) shows that peak A is due to an A•U base-pair. The relatively large NOE to 13.1 ppm is in the region where C•G-13 is expected to resonate. Since the imino-proton in

Figure 25: Summary of chemical shifts of peaks in Figure 24 at various concentrations of zinc.

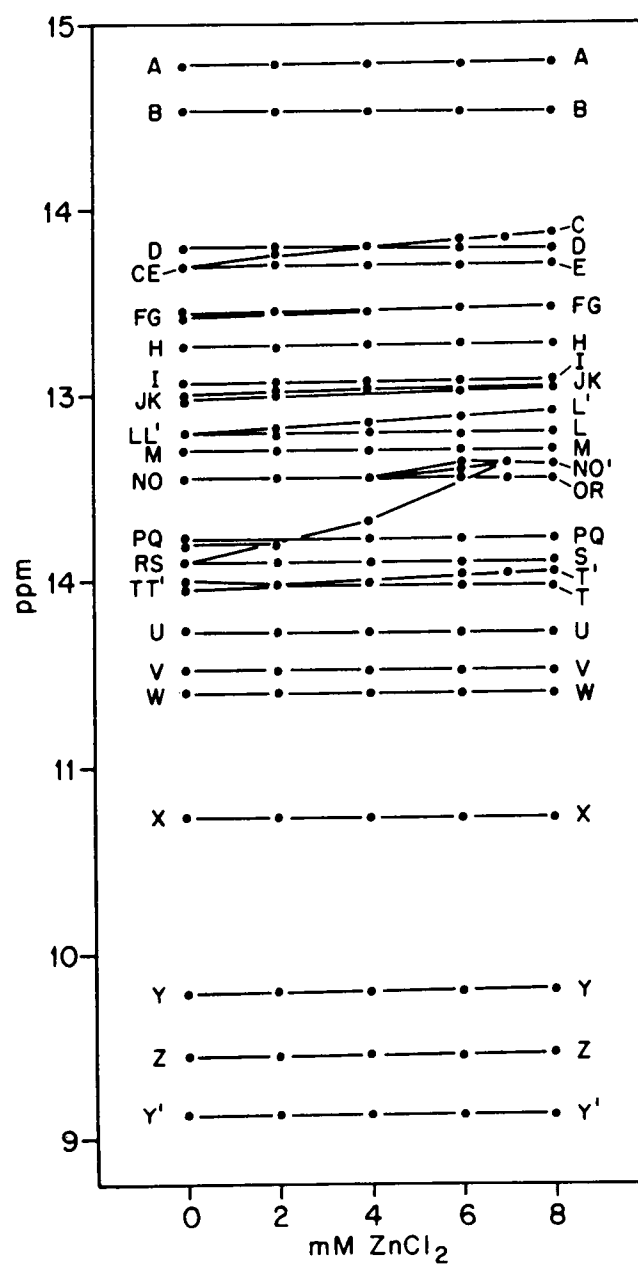


Figure 25

Figure 26: One-dimensional NOEs produced by selective saturation of peak A of Figure 24. Peak A was continuously saturated for 1.5 s. The sample contains 1 mM E. coli tRNA^{Val}₁, 500 mM NaCl, 10 mM NaOAc, pH 4.6, and 7 mM ZnCl₂ at 17°C.

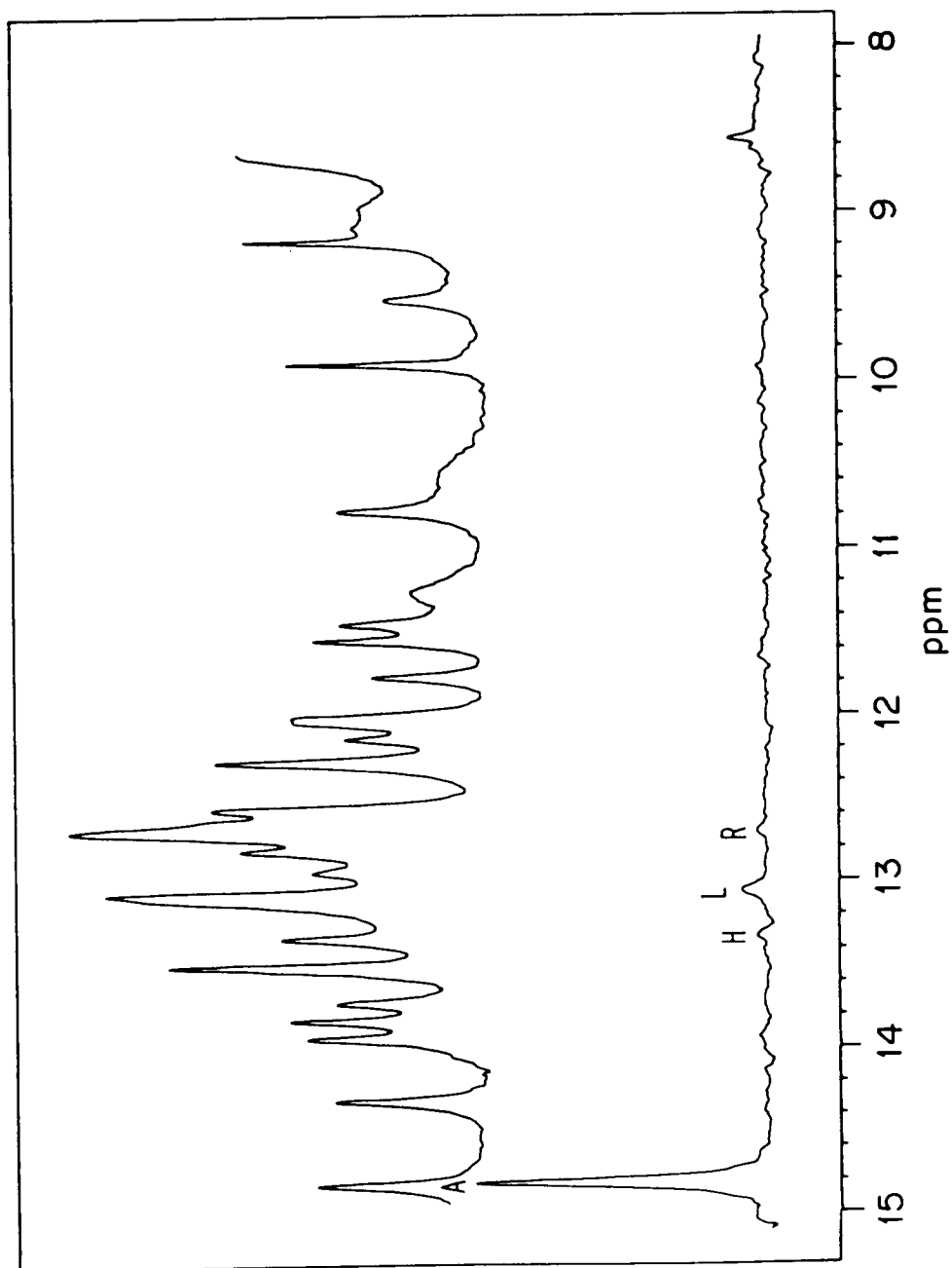


Figure 26

this base-pair is believed to be closer to (s^4U-8)(A-14) than (G-15)(C-48), it is reasonable to conclude that this NOE is due to C•G-13. The weak NOE at 12.7 ppm is probably due to a second-order effect. The next closest imino-proton to (s^4U-8)(A-14) is that of G-15 and thus we believe that the 12.7 ppm (peak R) NOE is due to the imino-proton of (G-15)(C-48). Hare et al. (1985) performed a similar experiment and showed that (G-15)(C-48) was found at ~12.1 ppm (corresponds to our peak RS), although their study was conducted at pH 7.0 with no divalent ions present. Using this information, and our observation that one proton from RS is downfield shifted to near 12.7 ppm, we conclude that the presence of Zn^{2+} shifts the imino-proton of (G-15)(C-48) to lower field strengths.

Peak C was also affected by Zn^{2+} . In 8 mM Zn^{2+} , this peak was shifted downfield by 0.17 ppm from its location with no metal present (Figure 24). Unlike the effect of Zn^{2+} on peak R, the chemical shift of C was linear with increasing Zn^{2+} concentrations (Figure 25). The three imino-protons assigned to peak CDE are C•G-11, U•A-12, and U•A-29 (Hare et al., 1985). Above 6 mM Zn^{2+} three peaks, each corresponding to a single proton, are observed.

To determine which of the protons is affected by Zn^{2+} , we examined the NOE connectivity of the most upfield of these three protons in 7 mM Zn^{2+} (Figure 27). No NOE was observed to the aromatic region (7-9 ppm) of the spectrum which means that this resonance is due to a G•C and not an A•U base-pair. Therefore,

Figure 27: One-dimensional NOE produced by selective saturation of peak E of Figure 24. The sample was the same as used in Figure 26 except the spectrum was recorded at 37°C.

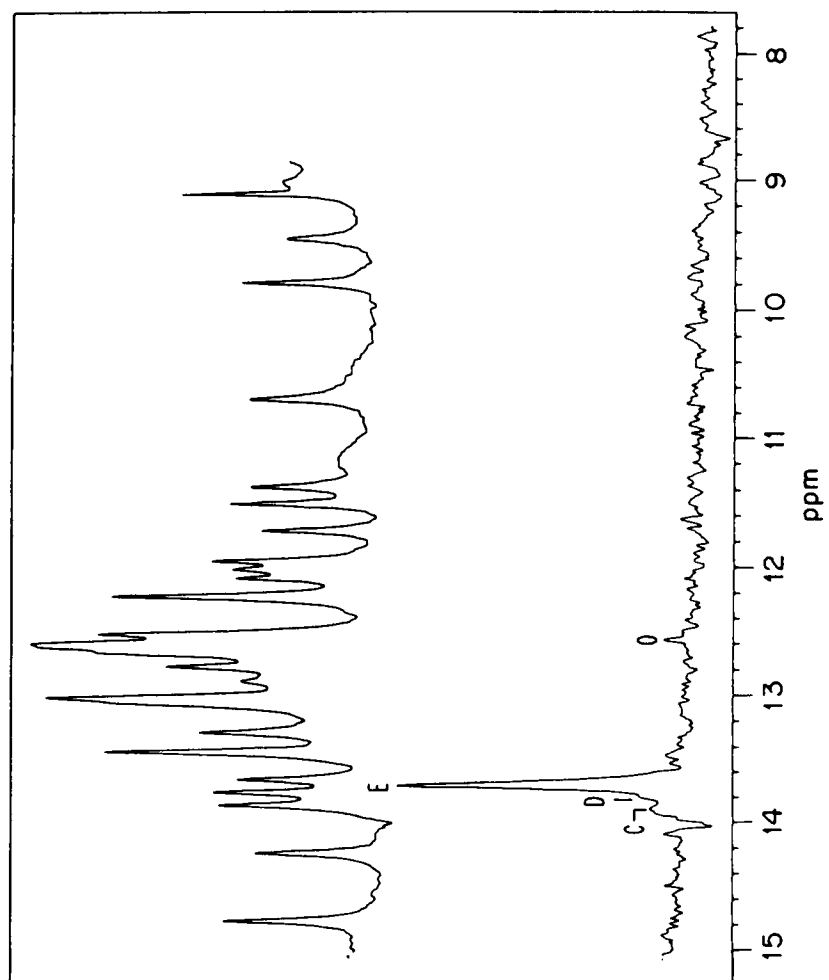


Figure 27

the upfield component, peak E, is probably due to C•G-11. This is consistent with its normal location in the ^1H -NMR spectrum.

The assignment of C is complicated by the fact that peaks C and D both were affected by irradiation of E. We suggest that D arises from direct irradiation spillover from E and that C is due to an NOE from C•G-11 and is therefore U•A-12. This interpretation is consistent with the observation of a NOE, albeit weak, to ~ 12.57 ppm. If this peak does in fact represent an NOE, it would be due to G•C-10. Two indirect pieces of evidence support these assignments: a) a similar effect of Zn^{2+} is seen for peak D in yeast tRNA^{Phe} (Figure 21) and this resonance is due to either C•G-11, U•A-12, or U•A-52, but not U•A-29; b) from the crystal structure of yeast tRNA^{Phe} in the presence of Zn^{2+} (Rubin et al., 1983) there is no Zn^{2+} binding site near U•A-29 while U•A-12 is very near one of the strongest sites. No one piece of evidence is conclusive, although the evidence, if taken together, supports the assignment of Peak C to U•A-12, D to U•A-29 and E to G•C-11 in E. coli $\text{tRNA}_1^{\text{Val}}$.

Peak L was also affected by Zn^{2+} . When no divalent metal is present, L is a single resonance line at 12.80 ppm; Zn^{2+} causes one proton to shift downfield from peak L to peak L' at 12.91 ppm. The imino-protons of G•C-53 and the tertiary base-pair (G-19)•(C-56) are assigned to this region. It is not clear which of the two protons are affected by Zn^{2+} . Neither of the spatially nearby base-pairs of G•C-53, namely peaks F [(T-54)•(A-58)] or P [C•G-13], are affected by Zn^{2+} . While for

(G-19)•(C-56) only the 5' neighbor peak X (G-18)•(Ψ-55) can be directly monitored; it too is unaffected by Zn^{2+} . The 3' neighbor of (G-19)•(C-56) is G-20, which is not involved in a base-pair and therefore does not give rise to a resonance in the imino-proton region.

Here, as in the previous two cases, we must resort to ancillary data to tentatively assign the resonance of peak L. In yeast tRNA^{Phe}, Zn^{2+} has been shown to interact directly with the phosphate of G-19, while no Zn^{2+} was found in the vicinity of G•C-53 (Rubin et al., 1983). So we tentatively assign the component of L that is affected by Zn^{2+} to (G-19)•(C-56).

Peak T' was shifted downfield by Zn^{2+} . This is probably due to an effect on the imino-proton of G•C-1. The other imino-proton assigned to this region is from U-64, which is involved in a G•U base-pair with G-50. The imino-proton of G-50, peak W, is unaffected by Zn^{2+} . To test this hypothesis we performed an NOE experiment where the most upfield component of the peak in 8 mM Zn^{2+} (i.e. peak T) was pre-saturated (Figure 28). Pre-saturation of T gives rise to a strong NOE to W, the imino-proton of G-50, suggesting that peak T is indeed due to U-64, while peak T', which has now moved downfield, is due to the imino-proton of G•C-1. Peak T also appears to give rise to two other NOEs, one at 12.75 ppm and the other at 12.1 ppm. The peak at 12.75, though not particularly strong, is probably due to C•G-51, the 3' neighbor of U-64. The NOE to 12.1 ppm may be due to direct spillover, although spillover to the central peak, T', does not

Figure 28: One-dimensional NOE produced by selective saturation of peak T. The sample was the same as that used in Figure 26.

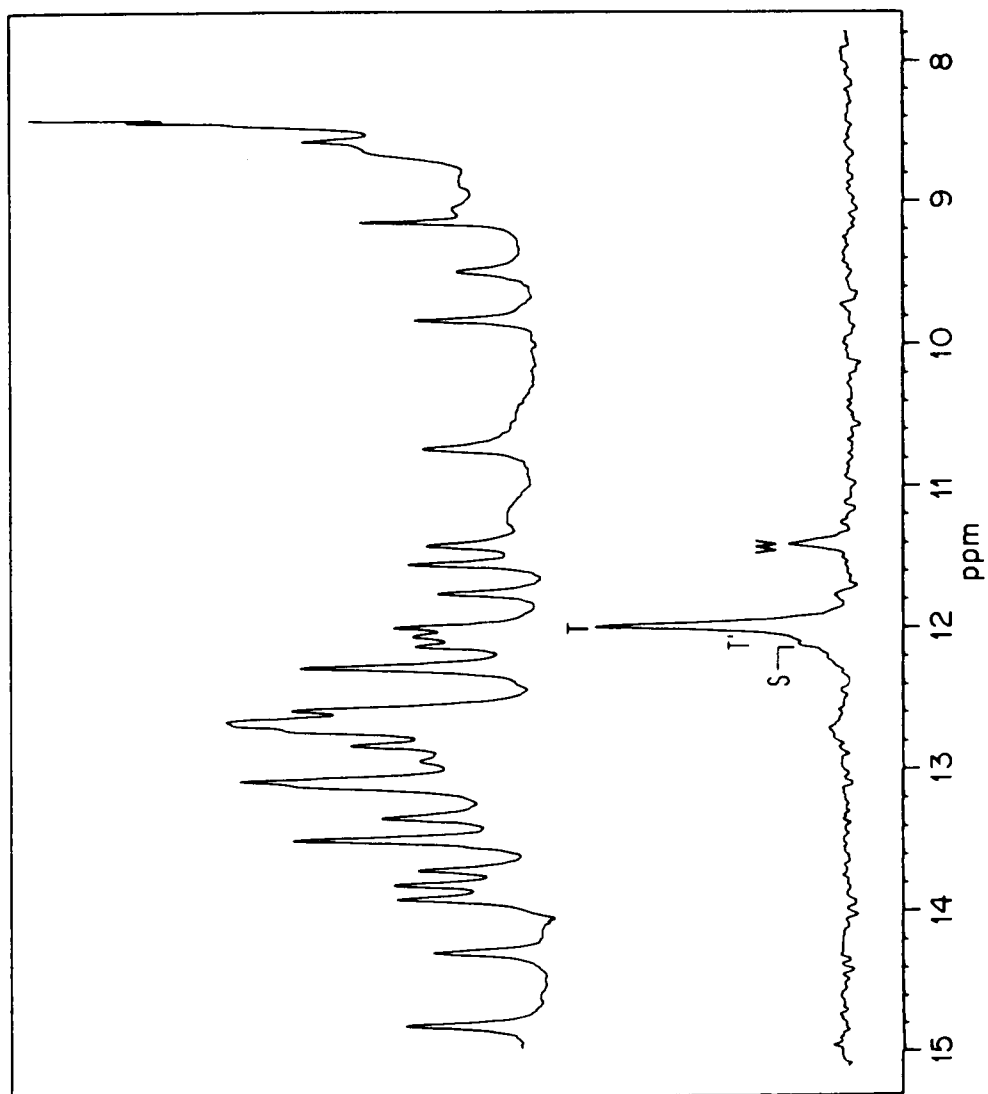


Figure 28

appear to be large. If this peak is an authentic NOE, it would confirm our assignment of this resonance to G•C-49 the 5' neighbor of (G-50)•(U-64).

The assignment of G•C-1 to the resonance in peak T'T, which was affected by Zn^{2+} , may help explain the effect of Zn^{2+} on peak NO' (Figure 25). Two protons in NO' were perturbed by Zn^{2+} , one is probably G•C-10 while the other component could not be assigned. The imino-protons that are believed to comprise peak NO' in the absence of metal ions are U•A-7, G•C-2, and G•C-10. G•C-2 is spatially near G•C-1 and the effect of Zn^{2+} on G•C-1 may also be experienced by G•C-2. However, at least one of the components of peak NO' is affected only at concentrations of Zn^{2+} above 4 mM; the effect of Zn^{2+} on G•C-1 is seen at all Zn^{2+} concentrations.

Discussion

One effect Zn^{2+} has on tRNA structure is to alter the chromatographic behavior of tRNA on RPC-5 (Hiatt & Jacobson, 1980). The nature of this alteration is not, as yet, completely understood. One explanation that has been suggested for the aberrant chromatographic behavior is that Zn^{2+} disrupts the hydrogen-bonded structure of tRNA, allowing 8 to 10 previously "hidden" negative charges to interact with the column. Clearly, this is not the case since the number of resonances in the imino-proton spectra of both tRNA^{Phe} and $\text{tRNA}_1^{\text{Val}}$ are the same in

the presence or absence of Zn^{2+} . It is argued elsewhere (Flanagan & Jacobson, 1986) that hydrophobic forces play a significant role in the increased retention of tRNA on RPC-5 due to Zn^{2+} . The basis for these conclusions, as well as the nature of some possible Zn^{2+} binding sites in solution, will be discussed in light of the known crystal structure of tRNA^{Phe} in the presence of Zn^{2+} (Rubin et al., 1983).

In Table 4 we summarize the zinc-induced changes in the imino-proton spectra of yeast tRNA^{Phe} in the presence of 1 to 2 Mg^{2+} ions per tRNA, and of yeast tRNA^{Phe} and E. coli tRNA₁^{Val} in the absence of Mg^{2+} . Zn^{2+} effects both tRNAs similarly, with the majority of affected resonances occurring in the D-arm.

In all three experiments, tRNA^{Phe} with and without 1 to 2 tightly bound Mg^{2+} and tRNA₁^{Val} without Mg^{2+} , Zn^{2+} had its largest effect on the imino-proton of (G-15)•(C-48), a reversed Watson-Crick type base-pair that forms the base of the augmented D-helix. In the absence of Mg^{2+} the base-pair (U-8)•(A-14) in tRNA^{Phe} or (s⁴U-8)•(A-14) in tRNA₁^{Val}, that is spatially adjacent to (G-15)•(C-48), is not affected by Zn^{2+} . These observations suggest that Zn^{2+} interacts near the (G-15)•(C-48) base-pair in solution.

The imino-proton of (G-19)•(C-56) like that of (G-15)•(C-48) is affected by Zn^{2+} in all three experiments. The (G-19)•(C-56) base-pair links the TΨC loop to the D-loop forming the corner of the L-structure. One of the four strong Mg^{2+} binding sites is located near this base-pair; here, Mg^{2+} is coordinated directly

Table 4

Summary of the Effects of Zn^{2+} on the Imino-proton
Resonances in Yeast tRNA^{Phe} and E. coli $\text{tRNA}_1^{\text{Val}}$

tRNA^{Phe}				tRNA^{Val}	
1 to 2 Mg^{2+}		No Mg^{2+} Present		No Mg^{2+} Present	
Peak	Assignment	Peak	Assignment	Peak	Assignment
B	(U-8)•(A-14)	D	(U-12)•(A-23)	C	(U-12)•(A-23)
G	?	G	?		
M	(G-19)•(C-56)	L'	(G-19)•(C-56)	L'	(G-19)•(C-56)
		K	(G-10)•(C-25)	N ^a	(G-2)•(C-71)
				O ^a	(G-10)•(C-25)
P	(G-15)•(C-48)	P	(G-15)•(C-48)	R	(G-15)•(C-48)
				T	(G-1)•(C-72)
X	N1-H of Ψ -55				
W'	amino-proton of A-9	W'	amino-proton of A-9		

^aThis effect appears only above 4 mM Zn^{2+} .

to the phosphate oxygen of G-19 and, through water molecules, to the N-7 and O⁶ of G-20, the O⁴ of U-59 and the N-3 of C-60. Zn²⁺ appears to bind at this site displacing Mg²⁺ and altering the arrangement of the ligands, possibly due to direct chelation of N-7 and O⁶ of G-20. We are unable to observe an effect on G-20 or U-59 directly since these residues are not involved in base-pairing interactions and don't give rise to resonances in the imino-proton region.

Both (G-15)•(C-48) and (G-19)•(C-56) are invariant base-pairs that are important in stabilizing the tertiary folding of tRNA. The imino-protons of these base-pairs have been shown to be sensitive to the paramagnetic divalent cations Co²⁺ and Mn²⁺ (Hurd et al., 1979). The Mg²⁺ binding site near G-19 is also a site of Pb²⁺ interaction; the hydrated Pb²⁺ ion bound here appears to catalyze the alkaline hydrolysis of the polynucleotide backbone at the dihydrouridine residues at positions 17 and 18 (Sundaralingam et al., 1984; Brown et al., 1985). Preliminary experiments in this laboratory suggest that Zn²⁺ ions can also facilitate the cleavage of tRNA at this site (unpublished data). These observations strongly suggest that Zn²⁺ can bind to at least 2 sites on the tRNA, one near (G-15)•(C-48) and the other near (G-19)•(C-56).

In the absence of Mg²⁺, Zn²⁺ affects the imino-proton resonances of base-pairs U•A-12 and G•C-10 in both tRNA^{Phe} and tRNA^{Val}. However, the presence of 1 to 2 strongly bound Mg²⁺ ions "protects" these resonances from Zn²⁺ (Table 4). Both

U•A-12 and G•C-10 are located near the Mg^{2+} site in the Ψ -10 loop (Hingerty et al., 1978). The Mg^{2+} at this site is coordinated to the phosphate oxygens of U-8, A-9, and C-11. The effect of Zn^{2+} on these resonances may be due to the tetrahedral geometry of the Zn^{2+} complex as compared to the octahedral Mg^{2+} complex (Rubin et al., 1983). In solution the affinity of Zn^{2+} for this site appears to be less than that of Mg^{2+} .

In contrast to the imino-protons of U•A-12 and G•C-10, the N1-H proton of Ψ -55 and the imino-proton of (U-8)•(A-14) are only affected by Zn^{2+} when Mg^{2+} is present (Table 4). The changes in chemical shift of the N1-H proton of Ψ -55 (resonance peak X, Figures 17 to 22) are probably due to the presence or absence of a water molecule that can hydrogen-bond there (Tropp & Redfield, 1981). In the hydrogen-bonded state peak X resonates at ~11 ppm and when the interaction is disrupted it shifts upfield to ~10.5 ppm (Tropp & Redfield, 1981; Heerschap et al., 1983b). The (U-8)•(A-14) base-pair, which is tertiary interaction, links the base of the acceptor stem to the D-stem and anchors the elbow of the L-structure. The chemical shifts of both these resonances in 8 mM Zn^{2+} and 1 to 2 Mg^{2+} per tRNA are intermediate between their positions in 1 to 2 Mg^{2+} per tRNA (Figure 18a) and 10 mM Mg^{2+} (Figure 18b). This suggests that the effects of Zn^{2+} on these resonances are indirect; binding of Zn^{2+} to the tRNA displaces one more Mg^{2+} that can now bind to other sites on the tRNA. The site or sites of Mg^{2+} interaction that lead to these effects are unknown. Two possibilities are: a) the site where Mg^{2+} is

coordinated to the phosphates of G-20 and A-21 (Hingerty et al., 1978; Quigley et al., 1978) or b) the site where Mg^{2+} coordinates to the phosphate oxygens of A-14 and G-57.

Zn^{2+} affects both yeast $tRNA^{Phe}$ and E. coli $tRNA^{Val}$ in much the same manner (Columns 2 and 3, Table 4). However, there are at least two differences between these two tRNAs. Peak W' in yeast $tRNA^{Phe}$ is affected by Zn^{2+} while Y' the equivalent resonance in E. coli $tRNA_1^{Val}$ is not. Peak W' has been assigned to the amino-proton of A-9 that is involved in the tertiary base-triple (U-12)•(A-23)•(A-9) (Choi & Redfield, 1985). Since the NOE connectivities of peak Y' in $tRNA_1^{Val}$ (Hare et al., 1985) are similar to those of W' in $tRNA^{Phe}$ we conclude that Y' is also due to the amino-proton of A-9. The reason for the lack of Zn^{2+} effect on this resonance in $tRNA_1^{Val}$ is unknown. However, it does appear that the effect of Zn^{2+} on the two components of the base triple, U•A-12 and (A-23)•(A-9), is independent in $tRNA_1^{Val}$ since peak C, U•A-12, is affected by Zn^{2+} while A-9 is not and in $tRNA^{Phe}$ that contains 1 to 2 Mg^{2+} per tRNA, Zn^{2+} effects only peak W', (A-23)•(A-9).

One effect of Zn^{2+} that is unique to $tRNA_1^{Val}$ is the upfield shift of the resonances associated with G•C-1 and G•C-2. These resonances are the terminal base-pairs in the acceptor stem. Zn^{2+} may affect these resonances independently, since G•C-2, a component of peak NO, appears to be affected only at concentrations above 4 mM while G•C-1 is affected at all Zn^{2+} concentrations (Figure 25). This would appear to rule out the possibility

that Zn^{2+} binds to these adjacent guanines by chelation of their N-7 and O⁶ positions. At present the cause of these effects is unknown.

Recently, the crystal structure of yeast tRNA^{Phe} in the presence of Zn^{2+} was reported (Rubin et al., 1983). These authors observed five Zn^{2+} binding sites, two of which directly replace previously bound Mg^{2+} (Table 5). From these data it appears that Zn^{2+} prefers binding at the N-7 and O⁶ positions of purines, in particular to adjacent purines located in the major-groove of helical regions. The authors noted that, due to the geometry of Zn^{2+} complexes (tetrahedral) and to the preference of Zn^{2+} for sites on the bases, the arrangement of the ligands at these sites differs substantially from those bound to Mg^{2+} . The exact nature of these differences was not elaborated.

The potential binding sites that we have observed in solution [near (G-19)•(C-56), the P-10 loop and (G-15)•(C-48)] correspond quite well to the zinc binding sites of Rubin et al. (1983), sites Zn(1), Zn(2), and Zn(3). In this study we did not observe changes in the imino-proton spectra corresponding to zinc sites, Zn(4) and Zn(5), however, using UV-difference spectroscopy, binding sites of this type were observed (Flanagan et al., 1986b). One difference between the imino-proton NMR spectral data and the crystal data is that Zn(2), which binds in the P-10 loop, can displace Mg^{2+} in the crystal, but not in solution. Further investigation will be required to obtain a detailed understanding of the effects of Zn^{2+} on the molecular structure of tRNA.

Table 5

A Comparison of Mg^a and Zn^b Binding Sites in tRNA^{Phe}

Zn ²⁺		Mg ²⁺	
Ligand	Coordination	Ligand	Coordination
Zn(1)	N-7 (G-20)	Mg(3)	N-7 (G-20)
	O ⁶ (G-20)		O ⁶ (G-20)
	O2P (G-19)		O2P (G-19)
	O ⁴ (U-59)		O ⁴ (U-59)
			N-3 (C-60)
Zn(2)	O2P (U-8)	Mg(1)	O2P (U-8)
	O2P (A-9)		O2P (A-9)
	O1P (C-11)		O1P (C-11)
	O2P (C-11)		
	O1P (U-12)		O1P (U-12)
		Mg(2)	O2P (G-20)
			O1P (A-21)
		Mg(4)	O2P (A-14)
			O2P (G-57)
Zn(3)	N-7 (G-15)		
	O ⁶ (G-15)		
	O ⁴ (U-8)		
	O1P (A-14)		
Zn(4)	N-7 (G-65)		
	O ⁶ (G-65)		
	O ⁶ (A-64)		
	N-7 (A-64)		
Zn(5)	N-7 (G-43)		
	O ⁶ (G-43)		
	N-7 (G-42)		
	O ⁶ (G-42)		

^aHingerty et al., 1978.^bRubin et al., 1983.

CHAPTER V

DISCUSSION

One goal of this study was to elucidate some of the effects of divalent metal ions on tRNA structure and correlate them with physicochemical parameter/parameters describing metal chemistry such as $\log K$ or the Irving-Williams stability order. A secondary goal was to clarify the mechanism of nucleic acid chromatography on RPC-5; so that, the mechanisms by which Zn^{2+} caused increased retention of tRNA by RPC-5 columns could be understood.

The chromatographic retention of tRNA on RPC-5 can be explained by two independent interactions between the tRNA and column. First, anionic phosphates of the tRNA can interact electrostatically with the cationic quaternary ammonium ion (Adogen 464); second, hydrophobic interactions between aromatic bases of the tRNA and the column's organic surface can occur. This surface is composed of the hydrocarbon side chains of the quaternary ammonium ion, which are 8 to 10 carbons in length, and the hydrophobic support material (Votalef or Plaskon).

Zinc effects both the electrostatic and hydrophobic components of tRNA retention on RPC-5. In the absence of Zn^{2+} the hydrophobic association between tRNA and RPC-5 was minimal. Even yeast tRNA^{Phe}, containing the hydrophobic Y-base, was not retained in high salt (3 M NaCl) where retention is almost exclusively due to hydrophobic processes. Zinc increased the hydrophobic interactions of tRNA with RPC-5, as well as the ionic interaction

with the cationic quaternary ammonium ion. Surprisingly, this occurred with a net decrease in the total number charged sites on the tRNA involved in ion-exchange interactions with the column.

Cadmium and Mg^{2+} reversed the effect of Zn^{2+} on chromatography. Cadmium completely reversed the effect of 10 mM Zn^{2+} when present in equimolar amounts, while 35 mM Mg^{2+} was required to restore normal chromatographic retention. These data suggest that the relative affinities of Mg^{2+} , Cd^{2+} and Zn^{2+} for the binding site/sites on the tRNA that are responsible for the "Zn effect" in chromatography are $Cd^{2+} > Zn^{2+} > Mg^{2+}$, a series that represents decreasing softness of the metal ions.

To investigate the locations of Zn^{2+} binding sites on tRNA we examined the effects of Zn^{2+} on the imino-proton ^1H -NMR spectra, of both yeast tRNA^{Phe} and *E. coli* tRNA^{Val}. The largest change in the imino-proton spectra due to Zn^{2+} was on the resonance assigned to (G-15)•(C-48), a tertiary interaction that is the terminal base-pair of the augmented D-helix. For both tRNAs the change in chemical shift was as large as 0.5 ppm.

By analogy to the Zn^{2+} ion binding sites observed in the crystal structure of tRNA^{Phe} (Rubin et al., 1983), the effect of Zn^{2+} on the imino-proton of base-pair (G-15)•(C-48) may be due to binding at the N-7 and O⁶ positions of G-15, O⁴ of U-8 and O1P of A-14. Although Zn^{2+} was able to displace Mg^{2+} from some sites (Rubin et al., 1983), the Zn^{2+} ion bound to the G-15 site was not of this type. The resonance assigned to (U-8)•(A-14), a base-pair that is stacked on (G-15)•(C-48), was not effected. These

observations suggest that Zn^{2+} perturbs the orientation of G-15 relative to position 16, a dihydrouridine residue in tRNA^{Phe} or a cytosine in tRNA^{Val} . We propose that alterations of this type would have little, if any, effect on the chemical shift of the (U-8)•(A-14) imino-proton. We could not test this hypothesis, however, since neither D-16 (tRNA^{Phe}) nor C-16 (tRNA^{Val}) are involved in base-pairs and thus lack imino-proton resonances in this region of the spectrum.

Zinc ions also affect the imino-proton resonances corresponding to (U-12)•(A-23) and (G-10)•(C-25). Rubin et al. (1983) observed that Zn^{2+} could displace Mg^{2+} from the P-10 loop, which may account for these effects. From their data they could not conclude what specific changes Zn^{2+} had on the arrangement of ligands at this site. The Zn^{2+} ion bound here has a tetrahedral coordination geometry, while Mg^{2+} ion has an octahedral geometry. This difference may account for the observed effects of Zn^{2+} on the imino-proton resonances of (U-12)•(A-23). The affinity of Zn^{2+} for this site seems to be greater than that of Mg^{2+} both in crystals (Rubin et al., 1983) and in solution. The affinity of Zn^{2+} for this site may be due to an inherent preference of the ligand arrangement at this site for tetrahedral coordination. This idea is consistent with Zn^{2+} coordination with five phosphate oxygens, while Mg^{2+} binds only four (Hingerty et al., 1978; Rubin et al., 1983).

Finally, there appears to be a Zn^{2+} binding site at the bend in the tRNA ribose-phosphate backbone near G-19 and G-20. This is

indicated by the pronounced effect of Zn^{2+} on the imino-proton resonance of (G-19)•(C-56). Rubin et al. (1983) observed that Zn^{2+} displaced the Mg^{2+} ion bound here. In the crystal Zn^{2+} was bound to the N-7 and O⁶ positions of G-20, the O2P of G-19, and O⁴ of U-59. In our NMR experiment the effect of this Zn^{2+} could be reversed when 1 to 2 Mg^{2+} per tRNA were present, suggesting that the affinity of Zn^{2+} for this site was smaller than for Mg^{2+} . In contrast, Rubin et al. (1983) found that, in the crystal, Zn^{2+} had a greater affinity for this site. An explanation for this may be that in our study the pH was 4.6, while in the crystal structure it was 7.0; however, none of the groups on the tRNA that interact with Zn^{2+} appear to have a pKa in this region.

The majority of the effects of Zn^{2+} appear to involve base-pairs in the D-arm that are involved in stabilizing the "elbow" of the L structure. Transfer RNA structure when Zn^{2+} is present is not very different than when Mg^{2+} is the divalent ion, since no base-pairs are disrupted. However, Zn^{2+} may not stabilize individual structural elements (e.g. anticodon stem, D-stem or acceptor stem) to the same degree as Mg^{2+} . We investigated this possibility by observing the thermal denaturation profiles of tRNA^{Phe} in the presence of Mg^{2+} , Cd^{2+} and Zn^{2+} . Both Mg^{2+} and Zn^{2+} increased the melting temperatures of tRNA, although, for Zn^{2+} it was only about (1°C). Cadmium ions decreased the melting temperature slightly (2°C) as compared to no divalent ion present. The effects of these ions on melting temperature probably

represents the relative affinities of these cations for sites on the bases and not their abilities to stabilize tRNA structure.

The stabilizing effect of these divalent ions is indicated by the widths of the individual transitions; since these directly reflect the apparent cooperativity of the melting transition and hence the stability of the structure. All three metal ions sharpened the melting transitions as compared to those obtained in the absence of divalent metal. Magnesium, however, was more effective at decreasing the half widths of the melting transitions than either Cd^{2+} or Zn^{2+} ; and Cd^{2+} was the least effective.

A conclusion, that can be drawn from this study, is that Zn^{2+} generally binds more tightly to tRNA than does Mg^{2+} . This has been observed by other authors (Duane, 1969; Duane, 1970; Mazzei & Onori, 1984) and is consistent with their ranking in the Irving-Williams order for metal-complex stability coefficients (Irving & Williams, 1948). At some sites, however, there are exceptions such as the inability of Zn^{2+} to displace Mg^{2+} from its binding site in the anticodon loop even when present in a four fold excess. Another exception is the lack of Zn^{2+} binding at one of the Mg^{2+} sites in the D-loop (Rubin et al., 1983). Exceptions such as these point out the complex nature of metal ion tRNA interactions, which are dependent upon both the chemical properties of the metal ions involved and constraints imposed by tRNA conformation.

Transition metals generally bind more tightly to tRNA than do alkaline earth metals; they also show more diversity in the

types of ligands that they bind. Transition metals can interact with both the anionic phosphate oxygens and sites on the bases, such as the O⁶ and N-7 positions of adenine and guanine. Alkaline earth metals appear to interact directly only with ionic sites. Binding at bases can lead to complexes with tRNA which are unique to transition metals. In particular, the "soft" metals bind to adjacent purines located in the major grooves. Apparently, the exposed N-7 and O⁶ positions of these adjacent purines are ideally suited for complex formation with "soft" metals. Base binding also plays a role in many of the complexes formed by transition metals with "traditional" Mg²⁺ sites. At these sites Mg²⁺ is bound to bases through water molecules; replacement of Mg²⁺ by metals such as Pb²⁺, Mn²⁺, Co²⁺, or Zn²⁺, generally results in the direct coordination of these metals to the bases (Rubin et al., 1983; Brown et al., 1985).

Under our experimental conditions (500 mM NaCl, 10 mM NaOAc, pH 4.6), binding to bases appears to be favored. These sites (e.g. N-7 of guanine) were saturated at about 1 μ M divalent metal ion, while sites where phosphate neutralization played a major role saturate in the 10 to 20 mM range. This preference for binding to bases is probably due to the high Na⁺ concentrations present in our experiments. Na⁺ can compete with divalent metal ions for sites on the phosphate backbone of tRNA (Leroy & Gueron, 1977), while it would not interact, to any appreciable extent, with the bases under any circumstances.

The ability of metal ions to stabilize tRNA structure seems to be related to their ranking in the "hard/soft" classification scheme and not on the strength of their interactions with the tRNA. From the standpoint of electronegativity, Zn^{2+} is the most electropositive of the three and therefore should be the best at neutralizing the anionic phosphate sites. This is exemplified by the stability coefficients for their complex formation with hydroxide ion. The natural logarithm of the formation coefficient for $\text{Zn}(\text{OH})^{1+}$ was 5.0 while for $\text{Mg}(\text{OH})^{1+}$ it was 2.6. However, the outer electrons of Zn^{2+} are shielded to a greater degree than those of Mg^{2+} from the nuclear charge. The increased shielding of these electrons allows Zn^{2+} to be more polarizable than Mg^{2+} and to form complexes with more covalent character. In mixed complexes, where Zn^{2+} simultaneously interacts with relatively "soft" and "hard" ligands, the ability of Zn^{2+} to neutralize anionic ligands may be decreased by these non-ionic interactions.

An anomaly, which at present, cannot be rationalized, is the ability of Cd^{2+} to completely restore normal chromatographic retention, of tRNA on RPC-5, when present in equimolar concentrations with Zn^{2+} . This is unexpected since, in general, Cd^{2+} complexes are less stable than Zn^{2+} complexes. An exception to this are complexes of Cd^{2+} with very soft ligands such as thiocyanate, where the formation coefficient for its complex with Cd^{2+} was 1.9, while with Zn^{2+} it was 1.3. In our system, however, this might correspond to binding at the bases of adjacent purines,

which are saturated at metal ion concentrations well below those required for a marked effect of Zn^{2+} on chromatography.

The molecular resolution of these studies was relatively "coarse" in comparison with x-ray crystallographic studies; however, in some instances limitations of tRNA structural analysis by x-ray crystallography can be circumvented. In particular, the tRNA structures did not have to be performed in the presence of Mg^{2+} and spermine, nor was it required that changes in tRNA structure, due to the added metal ion, be small, as in the case of the Zn^{2+} -tRNA structure of Rubin et al. (1983). The solution structure of tRNA is generally similar to the structure deduced from x-ray diffraction.

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