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EFFECT OF ZINC IONS ON tRNA STRUCTURE

I. REVERSED-PHASE CHROMATOGRAPHY

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SUMMARY

The effect of zinc on the chromatographic behavior of four tRNAs was examined on RPC-5 and Aminex A-28 columns. RPC-5 contains dichlorodifluoroethylene beads coated with a quaternary ammonium compound where the substituents are: R_1 = methyl, and R_{2-4} = C_{8-10} hydrocarbons. Aminex A-28 contains quaternary ammonium covalently attached to styrene–divinylbenzene copolymer lattice and R_{1-3} are methyl groups. The retentions of $tRNA^{Val}$, $tRNA^{Ile}$, and $tRNA^{Lys}$ of *E. coli* and yeast $tRNA^{Phe}$ on RPC-5 were all markedly increased by Zn^{2+} ions. In contrast, no increased retention due to Zn^{2+} was observed when $tRNA^{Phe}$ was chromatographed on Aminex A-28. A model for chromatography on RPC-5 is developed which treats the elution behavior of tRNAs from this matrix as the sum of ion-exchange and hydrophobic interactions. The chromatography of tRNA in the presence and absence of Zn^{2+} is interpreted in terms of this model and the effects of sodium chloride concentration, temperature, and pH were explored as the experimental variables. These experiments suggest that in the absence of Zn^{2+} tRNA does not interact appreciably with the hydrophobic surface of the column. The addition of Zn^{2+} has three effects on chromatography: (1) a decrease in the number of anionic sites on the tRNA which interact with the positively charged ammonium ion, (2) an increase in affinity of the tRNA for these ionic sites, and (3) an increase in affinity of tRNA for hydrophobic sites on the column. All three effects were fully reversed by the addition of Cd^{2+} (10 m*M*) or Mg^{2+} (35 m*M*), but only partially reversed at lower concentrations of these competing ions. These results show that chromatography on RCP-5 can be a sensitive physical chemical technique for examination of the structure of tRNA, and probably for other nucleic acids as well.

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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[1,2]. 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. The interactions of metal ions with negatively charged phosphate groups is thought to help stabilize secondary and tertiary structure of 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[3–5]. Rubin *et al.*[4] found five strong binding sites for Zn^{2+} ion in their crystals; two of which had displaced previously bound Mg^{2+}. These two sites represent the most tightly bound of the five Zn^{2+} ions.

The effects of ions on the overall structure of tRNA in solution has also been investigated using fluorescence[6,7], electron spin resonance[8], neutron and X-ray scattering techniques[9,10], light scattering[11,12], and thermal denaturation[13]. 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[14]. 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), and Zn^{2+} (intermediate) and Cd^{2+} (soft) will be compared for their effect on the chromatographic behavior of tRNA. The σp values [softness parameter defined by Pearson[15]] for these divalent ions are 0.167, 0.115, and 0.081 for Mg^{2+}, Zn^{2+}, and Cd^{2+}, respectively.

With respect to RPC-5 chromatography, Hiatt and Jacobson[16] 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 in the presence of guanine or queuine in the "wobble" position of the anticodon[17]. The effects of various cations such as Mg^{2+}, Al^{3+}, and Hg^{2+} on ion-exchange chromatography has been the topic of numerous papers and reviews[18–20].

RPC-5 chromatography has long been used to separate nucleic acids[20–22] and is sensitive to the molecular weight[20,22], base composition[23], and secondary and tertiary structure[22,24] of the nucleic acids. The resolution of nucleic acids on RPC-5 is such that tRNAs that differ only by a methyl group[25,26] or the presence or absence of dihydrouridine[27,28] are separable. Also, $tRNA^{Trp}$ is readily separated from Trp-$tRNA^{Trp}$, presumably because of the hydrophobic amino acid[29,30]. 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 dichlorodifluroethylene in bead form coated with tetraalkyl quaternary ammonium ions[31]. 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.

EXPERIMENTAL

Materials

Plaskon CTFE 2300 (no longer commercially available) was purchased from Allied Chemical Co. (Morristown, NJ, U.S.A.); Voltalef 300LD micropowder from Products Chimiques Ugina-Kuhlman (Paris La-Defense, France; the American distributor is Atochem Polymers, Glen Rock, NJ, U.S.A.); Adogen 464 from Ashland (Columbus, OH, U.S.A.); Aminex A28 from Bio-Rad (Richmond, CA, U.S.A.); yeast $tRNA^{Phe}$ from Boehringer Mannheim, (Indianapolis, IN, U.S.A.) and *E. coli* tRNAs from Subriden RNA (Rolling Bay, WA, U.S.A.). 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[32]. Chromatography was performed at 37°C at pH 4.6 in 10 m*M* sodium acetate buffer except where stated otherwise. The elution was accomplished either by a linear gradient of sodium chloride (0.5–1.1 *M*) or by isocratic elution with the sodium chloride 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.*[32].

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[33]. 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_0, the elution volume of an unretained solute, *e.g.* uridine, by the equation:

$$k' = (V_R - V_0)/V_0 \tag{1}$$

The capacity ratio can also be expressed in terms of the equilibrium distribution constant, K_{eq}:

$$k' = (K_{eq}V_s)/V_m \tag{2}$$

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 $1 < k' < 5$ allowing for the optimal resolution of all solute components[33].

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 tends to reduce peak tailing since the trailing edge of the peak is in a region of lower relative k' than the leading edge. Thus, solute molecules in the band tail migrate faster than those in the center of the band, 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.

EXPERIMENTAL

Gradient elution of $tRNA^{Phe}$ in the presence or absence of Zn^{2+}, Mg^{2+}, Ni^{2+} or Cd^{2+}

Chromatography of yeast $tRNA^{Phe}$ in the presence or absence of divalent metal ions is depicted in Fig. 1. In the presence of 10 m*M* Mg^{2+}, Cd^{2+} or Ni^{2+}, $tRNA^{Phe}$

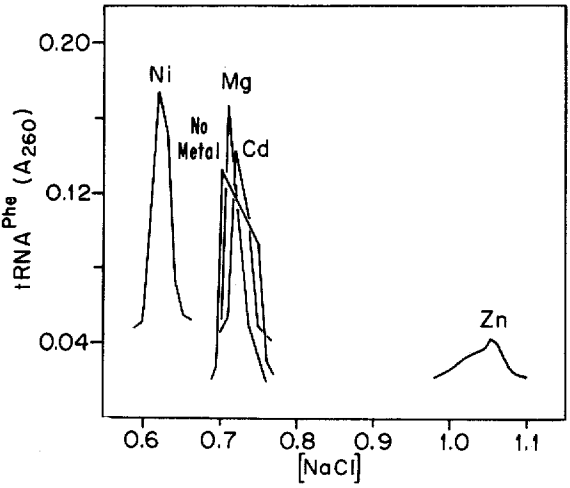

Fig. 1. RPC-5 chromatography of $tRNA^{Phe}$ in the absence of divalent metals and in the presence of Mg^{2+}, Cd^{2+}, Ni^{2+} and Zn^{2+}. Yeast $tRNA^{Phe}$ (100 μg) was chromatographed on RPC-5 (10 × 1 cm) column at 35°C using a 100-ml linear gradient from 0.5 to 1.1 *M* sodium chloride that included 10 m*M* acetate pH 4.6. The flow-rate was 1 ml/min, and 1-ml fractions were collected. The sodium chloride concentration was determined from the refractive index. The divalent cation concentration was 10 m*M* when present.

eluted at the same or lower ionic strength as it did in the absence of divalent ions. However, 10 m*M* Zn^{2+} produced marked changes in chromatographic behavior. Zn^{2+} caused the tRNA to elute as a broad, tailing band which emerged very late in the gradient. The broadened nature of the elution band in the presence of Zn^{2+} 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[34] 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 Zn^{2+}. 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 Zn^{2+} concentration is shown in Fig. 2. At concentrations up to 0.5 m*M*, Zn^{2+} had no effect on yeast $tRNA^{Phe}$ retention. Above 0.5 m*M* Zn^{2+}, the retention was increased in a biphasic manner. The effect of Zn^{2+} was also investigated with RPC-5 in which Voltalef 300LD micropowder replaced the Plaskon as the solid support material[32]. 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 m*M*.

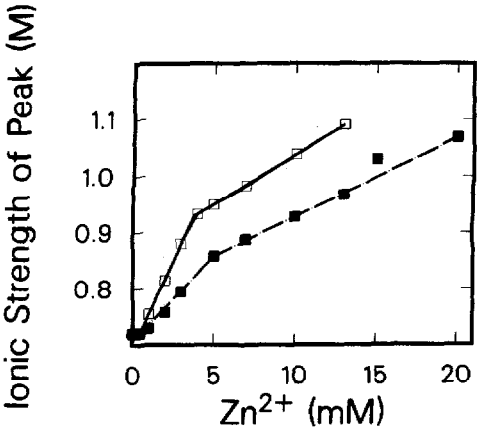

Fig. 2. The dependence of $tRNA^{Phe}$ retention on Zn^{2+} concentration. Two RPC-5 columns were prepared, one with Plaskon (□) and the other with Voltalef (■) powder as the solid support material. Yeast $tRNA^{Phe}$ (50–100 μg) was chromatographed at various concentrations of Zn^{2+} under the conditions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

Zn^{2+} ions also affected the chromatography of three closely related *E. coli* tRNAs (for valine, lysine and isoleucine) (Fig. 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 $tRNA^{Phe}$ (Fig. 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 interact with the tRNA and affect retention. Aminex A-28

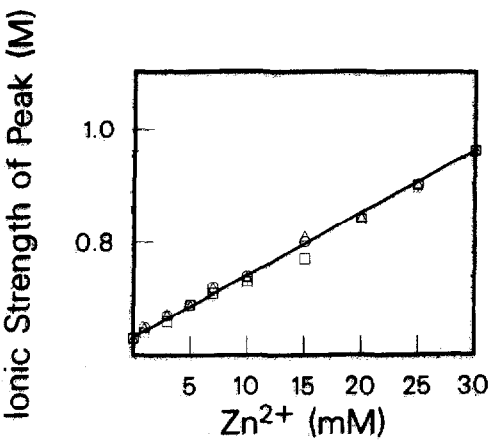

Fig. 3. Effect of Zn^{2+} concentration on ionic strength for elution of three tRNAs. *E. coli* tRNAs valine (□), leucine (△), or isoleucine (○) (50–100 μg) were chromatographed in a 100-ml linear gradient from 0.4 to 1.0 *M* sodium chloride, and increasing fixed concentrations of zinc acetate (all other conditions as in Fig. 1).

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 (Fig. 4). No increased retention due to Zn^{2+} was observed at concentrations up to 40 m*M*.

Isocratic chromatography on RPC-5

The mechanism of chromatography on RPC-5 includes ion-exchange and hydrophobic interactions. The ion-exchange reactions are given by eqn. 3

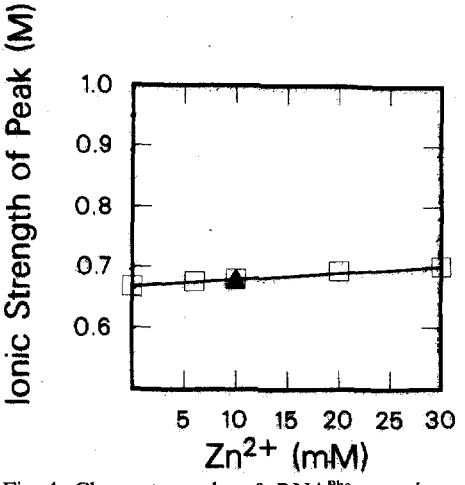

Fig. 4. Chromatography of $tRNA^{Phe}$ at various divalent cation concentrations on Aminex A-28. Yeast $tRNA^{Phe}$ was chromatographed on Aminex A-28 (20 × 1 cm), using the chromatographic conditions in Fig. 1, with various fixed concentrations of zinc acetate (□) or 10 m*M* magnesium acetate (▲).

$$(A^{n-})_m + n(BX)_s \rightleftharpoons n(B^-)_m + (AX_n)_s$$

$$K_1 = \frac{[AX_n]_s\,[B^-]_m^n}{[A^{n-}]_m\,[BX]_s^n} \tag{3}$$

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 C_{8-10} hydrocarbon side-chains of the quaternary ammonium ion and the support bead of RPC-5. These interactions can be represented as:

$$(A^{n-})_m \rightleftharpoons (A^{n-})_s$$

$$K_2 = \frac{[A^{n-}]_s}{[A^{n-}]_m} \tag{4}$$

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

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

Combining eqns. 2–5 gives

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

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 eqn. 1.

Isocratic chromatography in the presence and absence of Zn^{2+}

Isocratic elution was used to evaluate the chromatographic parameters in eqn. 6. By determining k' at different fixed concentrations of sodium chloride 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 eqn. 6.

Fig. 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. Fig. 5a shows how varying n (in eqn. 6), the number of anionic sites on the tRNA interacting with the cationic column, affects the calculated values of k' as a function of sodium chloride 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' *vs.* [NaCl] curves is

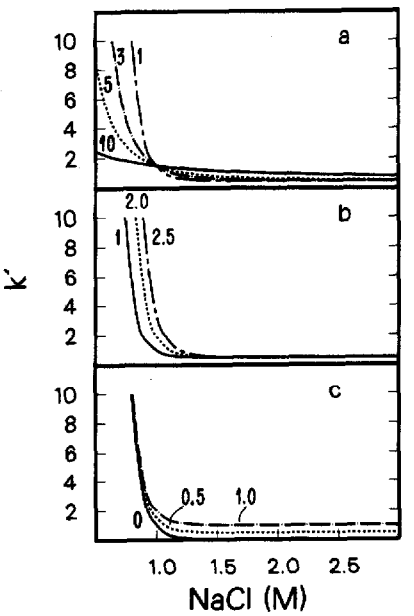

Fig. 5. Calculated effects of the parameters n, K_1, and K_2 on the chromatographic capacity ratio, k', as a function of sodium chloride concentration. k' was calculated from eqn. 6 for (a) fixed values of K_1 = 1.0, K_2 = 0.5 and varying n (1, 3, 5, 10), (b) fixed n = 10, K_2 = 0.5 and varying K_1 (1.0, 2.0, 2.5), and (c) fixed n = 10, K_1 = 1.0 and varying K_2 (0.0, 0.5, 1.0).

to shift them to higher salt concentrations (Fig. 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 (Fig. 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 (Fig. 5a).

The experimentally determined effect of increasing salt concentrations on k' for isocratic elution of $tRNA^{Phe}$ is shown in Fig. 6. At least three effects of Zn^{2+} are apparent. Zn^{2+} displaces the curve to higher chloride concentrations. 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 I summarizes the effects of Zn^{2+} on the chromatographic parameters n, K_1 and K_2 which were calculated by fitting eqn. 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 indistinguishable though there is clearly a difference in this number with and without Zn^{2+}.

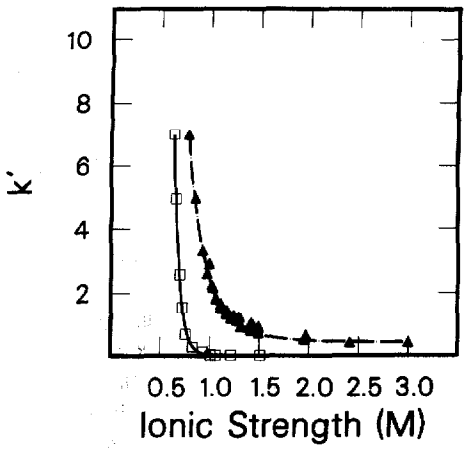

Fig. 6. Isocratic elution of $tRNA^{Phe}$ with and without zinc. Yeast $tRNA^{Phe}$ (50–100 μg) was isocratically eluted from RPC-5 (10 × 1 cm) at 35°C in the presence of 10 mM zinc acetate (▲) or no divalent ion (□), and 10 mM acetate pH 4.6 in various fixed concentrations of sodium chloride. k' was calculated from eqn. 1 where the retention of uridine was taken as V_0. The fitted lines were determined with eqn. 6 as the model. The values for the fitted parameters are given in Table I.

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, k' can be related to temperature by a modified version of the Van 't Hoff equation[35–37]:

$$\ln k' = -\frac{\Delta H}{RT} + \frac{\Delta S^0}{R} + \ln \frac{A}{V_m} \tag{7}$$

where ΔH is the enthalpy of transfer of the solute from the mobile phase to the stationary phase, ΔS^0 is the associated change in the standard entropy, A is the total surface area of adsorbent in a column with a dead volume V_m, R is the gas constant and T is the temperature. Assuming that ΔH, ΔS^0 and A/V_m are independent of temperature, eqn. 7 suggests the classical linear relationship of ln k' and $1/T$. For both hydrophobic and ion-exchange processes, ΔS^0 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:

TABLE I

VALUES FOR THE CHROMATOGRAPHIC PARAMETERS OBTAINED FROM EQN. 6

	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

$$(BX)_s \rightleftharpoons (X^+)_s + (B^-)_m \quad (8)$$

$$(A^-)_m + (X^+)_s \rightleftharpoons (AX)_s \quad (9)$$

ΔH is the difference in enthalpy of transfer of X^+ between the two reactions[38], 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.

Fig. 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 enthalpy change for 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 zinc acetate and 3 M sodium chloride is shown in Fig. 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 hydropohobic 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 may be the case.

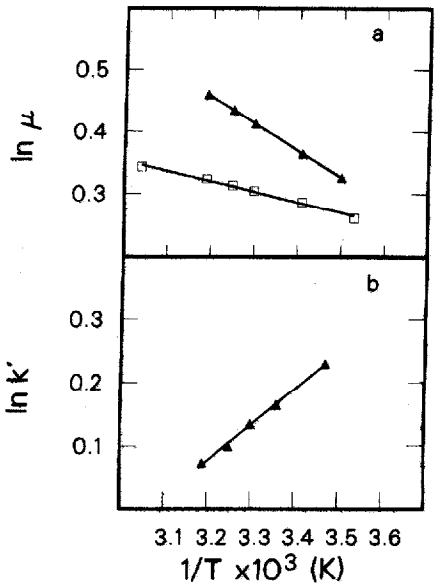

Fig. 7. Van 't Hoff plots for temperature dependence of RPC-5 chromatography. The effect of increasing temperature on the elution of $tRNA^{Phe}$ in (a) the gradient system (see Fig. 1) with 10 mM Zn^{2+} present (▲) or no divalent metal (□) was determined where k' was approximated as the ionic strength (μ) of the peak fraction, or (b) the isocratic system in 3 M sodium chloride; 10 mM acetate pH 4.6 and 10 mM Zn^{2+}, where k' was determined as in Fig. 6.

The effect of pH on RPC-5 chromatography of $tRNA^{Phe}$

Eluent buffer pH plays an important role in retention of nucleic acids on RPC-5[20,21]. Protonation and deprotonation of nucleic acid bases 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. Mildly acidic pH (*ca.* 4.5) facilitates the protonation of the N-1 of adenine, and the N-3 of cytosine[39]. At pH values below 3.0, the N-7 of guanine can also be protonated.

Fig. 8 shows the pH titration of tRNA chromatography on RPC-5. 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* sodium chloride) and presence (1.0 *M* sodium chloride) of Zn^{2+}. Conditions above pH 7 were not investigated since the solubility of Zn^{2+} decreases sharply in that region.

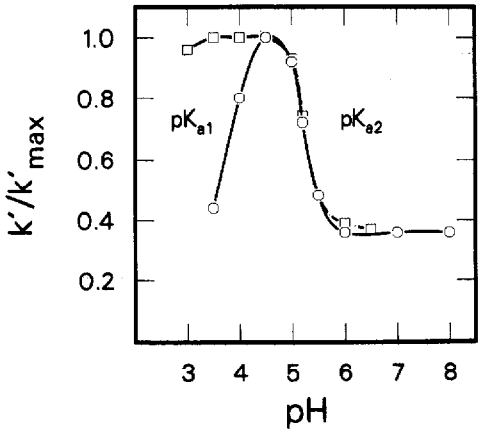

Fig. 8. The effect of pH on isocratic elution of $tRNA^{Phe}$. Chromatography was performed at salt concentrations that would give k' values of 2.0 at pH 4.6: in the absence of Zn^{2+} (○), the sodium chloride concentration was 0.62 *M*, and in the presence of 10 m*M* zinc acetate (□), it was 1.0 *M*. The pH of the elution buffer was controlled in the range of pH 2–4 with 10 m*M* formate buffer, from pH 4–6 with 10 m*M* acetate buffer, and from pH 6–8 with 10 m*M* piperazine-N,N-bis(2-ethano sulfonic acid) (PIPES). All other chromatographic conditions are as in Fig. 6.

In the absence of Zn^{2+}, there are at least two distinct pH-dependent transitions that yield 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 m*M* Zn^{2+}, pK_{a2} was unchanged. Retention below pH 4.5 was not affected by pH but the sample recovery 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 sodium chloride at pH 6.0 in the presence of 10 m*M* Zn^{2+}. The data for k' *vs.* [NaCl] were fitted to eqn. 6 to determine the chromatographic constants n, K_1 and K_2, the values of which are given in Table I. Increasing the pH affected K_1, the ion-exchange constant, while n and K_2 were unaffected. This result

is somewhat surprising since, at pH 6.0 tRNA has a greater net negative charge than at pH 4.6, due to protonation of both adenine and cytosine residues below pH 5.0. An explanation for this may be that base protonation destabilizes tRNA structure slightly, allowing a greater number of interactions between the tRNA and the 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 m*M* zinc acetate. Fig. 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 m*M* Cd^{2+}, the "soft" ion, completely restores normal retention, while 35 m*M* 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 be linear in nature, though the amount of restoration attributed to each portion of the curve differs between Cd^{2+} and Mg^{2+}.

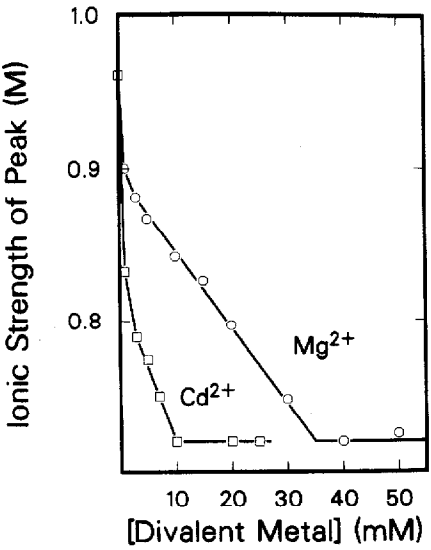

Fig. 9. Reversal of zinc effect by cadmium and magnesium. Conditions for chromatography were as in Fig. 1, except that 10 m*M* zinc acetate was present throughout the experiment and various concentrations of either cadmium chloride or magnesium chloride were added at the concentrations shown for "divalent metal".

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 the three parameters n, K_1 and K_2.

Analysis of these data using eqn. 6 shows that Zn^{2+} affects all three fitted

parameters (Table I). 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 under these conditions. The constant K_1, represents the effect of salt concentration 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 the sensitivity of RPC-5 to the dihydrouridine content[27,28], the methyl content of tRNA isoacceptors[25,26] and the strong retention of yeast $tRNA^{Phe}$ which contains the hypermodified wyeine base.

The increase in 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 (Fig. 2). In an earlier study[32], there was little difference between these two supports in gradient separations. Voltalef beads are five times larger than Plaskon, thus columns prepared with Voltalef are effectively one-fifth as long. The column length would have little effect upon separations based upon differences in apparent K_1 and n, since these variables depend primarily upon sodium chloride concentration. However, K_2 is independent of sodium chloride concentration, and would not change throughout the separation. Therefore its contribution to retention would be dependent upon column length. This interpretation is consistent with the equality of Plaskon and Voltalef in the absence of Zn^{2+}, and the absence of a zinc effect for tRNA chromatographed on Aminex A-28. The methyl groups of Aminex A-28 may be ineffective, as compared to the C_{8-10} groups in the RPC-5 matrix, in facilitating hydrophobic binding of the tRNA.

All tRNAs which have been examined to date elute at higher sodium chloride concentrations when Zn^{2+} is 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 Zn^{2+} concentration was linear (Fig. 3). The increase in retention for $tRNA^{Phe}$ (yeast) was biphasic (Fig. 2), and no effect of Zn^{2+} was observed at <1 m*M*. 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[40]. Chromatography of $tRNA^{Phe}$ lacking wyeine was not investigated since removal of this base is believed to alter tRNA solution structure[41–44].

Two lines of evidence suggest that there are two classes of Zn^{2+} binding sites on $tRNA^{Phe}$. The data in Fig. 2 show a biphasic increase in tRNA retention due to Zn^{2+}. The data in Fig. 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 m*M* Cd^{2+} or 35 m*M* Mg^{2+}. In each limb of the biphasic curves (Fig. 9), Cd^{2+} was more effective than Mg^{2+} in 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".

Complexes of the metal (M) ions Zn^{2+} and Cd^{2+} with Cl^- can produce the species MCl^+, MCl_2, MCl_3^-, and MCl_4^{2-}. In 0.5 *M* sodium chloride zinc will exist primarily as Zn^{2+} and $ZnCl^+$ whereas cadmium has a greater tendency to form the chloride complexes[45]. At 1 *M* sodium chloride the appearance of $ZnCl_3^-$ will result in a slight decrease in Zn^{2+}. Neither $ZnCl_3^-$ or $ZnCl_4^{2-}$ can be expected to interact with the highly anionic tRNA molecule; the binding constant of tRNA for Zn^{2+} is probably several orders of magnitude greater than for any of the other species. We have not taken the various chlorinated species of zinc into account in our calculations since (a) the formation constants for these complexes are not known with much certainty[46] and (b) the effect of tRNA on these equilibria is not known. The local concentration of tRNA in the chromatographic band can be appreciable. In the case of cadmium, the same reservations are applicable but it may be that the concentration of Cd^{2+} is affected to a greater extent than is the case for Zn^{2+}. As a result the Cd^{2+} concentration may be somewhat less than the total cadmium in the experiment shown in Fig. 9.

Rubin *et al.*[4] 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+} ion which replaced one of the two Mg^{2+} ions 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), O^6 (G-20), a phosphate oxygen (G-19), and O^4 (U-59) where the parenthetic units refer to the identity of the nucleotide and its position in the linear sequence in the tRNA. 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+}. 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 Zn^{2+}/Mg^{2+} binding sites discussed above may also be those to which Zn^{2+} binds causing the observed increase in retention on RPC-5. First, these are the only sites where Zn^{2+} competes appreciably with Mg^{2+} when both are present in equimolar amounts in the crystal. Second, since neither of these sites contains adenine or cytosine, the binding of Zn^{2+} 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 Zn^{2+} for Mg^{2+} is likely to cause significant structural alterations. Finally, equilibrium binding studies show that strong Mg^{2+} sites have binding constants on the order of 10^{-4} M^{-1} (ref. 47); since these two Mg^{2+} sites are believed to be the most tightly bound[4] and Zn^{2+} displaces both Mg^{2+} in the crystal, the binding constant of Zn^{2+} at these sites must be greater than 10^{-4} M^{-1}. In the range of Zn^{2+} concentrations (0 to 20 m*M*) and $tRNA^{Phe}$

concentrations (0.2 to 2 μM), the Zn^{2+} binding sites would not be saturated. These predictions agree quite well with our data.

Another unpredicted result was the shape of the curve in Fig. 2 which suggests that the binding of Zn^{2+} is cooperative. Cooperativity is not generally observed at the sodium chloride concentrations employed. The three possible explanations are: (a) Zn^{2+} is bound to $tRNA^{Phe}$ differently than any of the divalent metals explored to date, (b) the effect of Zn^{2+} on chromatography is non-linear at low $[Zn^{2+}]$, 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 Zn^{2+}/Mg^{2+} metal binding site in the D loop has the greatest affinity for Zn^{2+} (ref. 4). This site is similar to sites for Pb^{2+} (refs. 5, 48 and 49) and Mn^{2+} (ref. 50) binding. 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[51].

In the cases of *E. coli* $tRNA^{Lys}$ and $tRNA^{Ile}$, there is a linear, rather than a biphasic, response to Zn^{2+}. 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[50] suggested that the substitution of cytidine for dihydrouridine would have little effect on the interaction between the D loop and the TψC loop where the Zn^{2+} binding site is located. Hingerty *et al.*[52] 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 O^2 (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. This possibly explains why $tRNA^{Val}$ chromatography also exhibits a monophasic dependence on Zn^{2+} concentration like the two other *E. coli* tRNAs.

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REFERENCES

1 A. S. Mildvan and L. A. Loeb, *Crit. Rev. Chem.*, 6 (1979) 219–244.
2 T. G. Spiro (Editor), in *Metal Ions in Biology*, Vol. I, Wiley, New York, 1980.
3 M. M. Teeter, G. H. Quigley and A. Rich, in T. G. Spiro (Editor), *Metal Ions in Biology*, Vol. I, Wiley, New York, 1980, pp. 145–177.
4 J. R. Rubin, J. Wang and M. Sundarlingam, *Biochim. Biophys. Acta*, 756 (1983) 111–118.
5 R. S. Brown, B. E. Hingerty, J. C. Dewan and A. Klug, *Nature (London)*, 303 (1983) 543–546.
6 M. Ehrenberg, R. Rigler and W. Wintermeyer, *Biochemistry*, 18 (1979) 4588–4599.

7 R. Ehrlich, J. F. Lefèvre and P. Remy, *Eur. J. Biochem.*, 103 (1980) 145–153.
8 V. Nötnig-Laslo, I. Weygand-Durasević and Z. Kućan, *J. Biomol. Struct. Dynamics*, 2 (1985) 941–951.
9 I. Pilz, O. Kratky, F. v. d. Haar and F. Cramer, *Eur. J. Biochem.*, 18 (1971) 436–441.
10 Z. Q. Li, R. Giége, B. Jacrot, R. Oberthur, J. C. Thierry and G. Zaccai, *Biochemistry*, 22 (1983) 4380–4388.
11 T. Olson, M. J. Fournier, K. H. Langley and N. C. Ford, *J. Mol. Biol.*, 102 (1976) 193–203.
12 D. C. Frintzinger and M. J. Fournier, *Biochemistry*, 21 (1982) 2118–2127.
13 G. L. Eichhorn and V. A. Shin, *J. Am. Chem. Soc.*, 90 (1968) 7323–7328.
14 K. B. Jacobson and J. E. Turner, *Toxicology*, 16 (1980) 1–37.
15 R. G. Pearson, *J. Am. Chem. Soc.*, 85 (1963) 3533–3539.
16 V. S. Hiatt and K. B. Jacobson, *Toxicol. Appl. Pharmacol.*, 56 (1980) 116–126.
17 K. B. Jacobson and E. H. Lee, *Biochem. Biophys. Res. Commun.*, 123 (1984) 1027–1032.
18 R. M. Kothari, *Chromatogr. Rev.*, 12 (1970) 127–155.
19 R. M. Kothari and M. W. Taylor, *J. Chromatogr.*, 73 (1972) 463–478.
20 A. D. Kelmers, H. O. Weeren, J. F. Weiss, R. L. Pearson, M. P. Stulberg, and G. D. Novelli, *Methods Enzymol.*, 20 (1971) 9–34.
21 J. E. Larson, S. C. Hardies, R. K. Patient and R. D. Wells, *J. Biol. Chem.*, 254 (1979) 5535–5541.
22 R. D. Wells, S. C. Hardies, G. T. Horn, B. Klein, J. E. Larson, S. K. Neuendorf, N. Panayotatos, R. K. Patient and E. Selsing, *Methods Enzymol.*, 65 (1980) 327–347.
23 R. K. Patient, S. C. Hardies, J. E. Larson, R. B. Inman, L. F. Maquat and R. D. Wells, *J. Biol. Chem.*, 254 (1979) 5548–5554.
24 A. N. Best, D. P. Allison and G. D. Novelli, *Anal. Biochem.*, 114 (1981) 235–243.
25 L. R. Shugart, G. D. Novelli and M. P. Stulberg, *Biochem. Biophys. Acta*, 157 (1968) 83–90.
26 J. B. Bell, K. B. Jacobson and L. R. Shugart, *Can. J. Biochem.*, 56 (1978) 51–59.
27 L. C. Waters, L. R. Shugart, W.-K. Yang and A. N. Best, *Arch. Biochem. Biophys.*, 156 (1973) 780–793.
28 R. Y. C. Lo, J. B. Bell and K. L. Roy, *Nucl. Acids Res.*, 10 (1982) 889–902.
29 L. C. Waters, B. C. Mullin, T. Ho and W. K. Yang, *Proc. Natl. Acad. Sci. U.S.A.*, 72 (1975) 2155–2159.
30 L. C. Waters, W.-K. Yang, B. C. Mullin and J. L. Nichols, *J. Biol. Chem.* 250 (1975) 6627–6629.
31 R. L. Pearson, J. F. Weiss and A. D. Kelmers, *Biochim. Biophys. Acta*, 228 (1971) 770–774.
32 J. M. Flanagan, R. K. Fujimura and K. B. Jacobson, *Anal. Biochem.*, 153 (1986) 299–304.
33 L. R. Snyder and J. J. Kirkland, *Introduction to Modern Liquid Chromatography*, Wiley, New York, 2nd ed., 1979.
34 A. Sokolowski and K.-G. Wahlund, *J. Chromatogr.*, 189 (1980) 299–316.
35 Cs. Horváth, W. Melander and I. Molnár, *J. Chromatogr.*, 125 (1976) 129–156.
36 H. Colin, J. C. Diez-Masa, G. Guiochon, T. Czajkowska and I. Miedziak, *J. Chromatogr.*, 167 (1978) 41–65.
37 C. M. Riley, E. Tomlinson and T. M. Jefferies, *J. Chromatogr.*, 185 (1979) 197–224.
38 R. Dybczyński, *J. Chromatogr.*, 31 (1967) 155–170.
39 T. O'Connor, S. Mansy, M. Bina, D. R. McMillin, M. A. Bruck and R. S. Tobias, *Biophys. Chem.*, 15 (1981) 53–64.
40 J. M. Flanagan, E. H. Lee and K. B. Jacobson, *Biopolymers*, (1986) submitted for publication.
41 D. R. Kearns, K. L. Wong and Y. P. Wong, *Proc. Natl. Acad. Sci. U.S.A.*, 70 (1973) 3843–3846.
42 J. E. Ladner and M. P. Schweizer, *Nucl. Acids Res.*, 1 (1974) 183–192.
43 P. Davanloo, M. Sprinzl and F. Cramer, *Biochemistry*, 18 (1979) 3189–3199.
44 W. J. Krzyzosiah and J. Ciesiolka, *Nucl. Acids. Res.*, 11 (1983) 6913–6921.
45 L. G. Sillen and A. E. Martell, *Stability Constants of Metal Ion Complexes*, The Chemical Society, London, 1971.
46 C. F. Baes, Jr., personal communication.
47 A. A. Schreier and P. R. Schimmel, *J. Mol. Biol.*, 93 (1975) 323–329.
48 M. Sundarlingam, J. R. Rubin and J. F. Cannon, *Int. J. Quantum Chem.*, 11 (1984) 355–366.
49 R. S. Brown, J. C. Dewan and A. Klug, *Biochemistry*, 24 (1985) 4785–4801.
50 A. Jack, J. E. Ladner, D. Rhodes, R. S. Brown and A. Klug, *J. Mol. Biol.*., 111 (1977) 315–328.
51 J. M. Flanagan, D. R. Hare, B. R. Reid and K. B. Jacobson, in preparation.
52 B. E. Hingerty, R. S. Brown and A. Jack, in R. Srinivasan (Editor), *Biomolecular Structure, Conformation, Function and Evolution*, Vol. 1, Pergamon Press, Oxford, 1980, pp. 293–302.