Binding of Spermidine to Transfer Ribonucleic Acid†

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ABSTRACT: The binding of spermidine to yeast $tRNA^{Phe}$ and *Escherichia coli* $tRNA_2^{Glu}$ at low and high ionic strength was studied by equilibrium dialysis. Once corrected for the expected Donnan effect, the binding at low ionic strength obeys the simple relationship of equivalent binding sites, and cooperative binding of spermidine to tRNA could not be detected. At low ionic strength (0.013 M Na^+ ion), $tRNA^{Phe}$ (yeast) has 13.9 ± 2.3 strong spermidine binding sites per molecule with $K_d = 1.39 \times 10^{-6}$ M and a few weak spermidine binding sites which were inaccessible to experimentation; $tRNA_2^{Glu}$ (*E. coli*) has 14.8 ± 1.6 strong spermidine binding sites and 4.0 ± 0.1 weak spermidine binding sites with $K_d = 1.4 \times 10^{-6}$ M and $K_d = 1.23 \times 10^{-4}$ M, respectively. At high ionic strength (0.12 M monovalent cation) and 0.01 M Mg^{2+}, $tRNA^{Phe}$ (yeast) has approximately 13 strong spermidine binding sites with an apparent $K_d = 3.4 \times 10^{-3}$ M while the dimeric complex $tRNA^{Phe}$·$tRNA_2^{Glu}$ has 10.4 ± 1.2 strong spermidine binding sites per monomer with an apparent $K_d = 2.0 \times 10^{-3}$ M. In the presence of increasing Na^+ ion or K^+ ion concentration, spermidine binding data do not fit a model for competitive binding to tRNA by monovalent cations. Rather, analysis of binding data by the Debye–Hückel approximation results in a good fit of experimental data, indicating that monovalent cations form a counterion atmosphere about tRNA, thus decreasing electrostatic interactions. On the basis of equilibrium binding analyses, it is proposed that the binding of spermidine to tRNA occurs predominantly by electrostatic forces.

The polyanionic nature of transfer ribonucleic acid (tRNA) allows for the binding of multivalent cations which thus neutralize the high charge density and confer a native (functional) conformation on the macromolecule. Recent crystallographic analyses (Holbrook et al., 1977; Jack et al., 1977; Quigley et al., 1978) of purified tRNA species indicate that metal ions and polyamines can bind to specific sites on the crystallized tRNA molecule, but results from numerous studies of tRNA solutions are conflicting with regard to the nature, specificity, and number of cation binding sites (Crothers & Cole, 1978; Schimmel & Redfield, 1980).

A number of reports (Cohn et al., 1969; Danchin & Guéron, 1970; Sander & Ts'o, 1971; Danchin, 1972; Rialdi et al., 1972; Schreier & Schimmel, 1974, 1975; Wolfson & Kearns, 1974; Römer & Hach, 1975; Sakai et al., 1975; Bina-Stein & Stein, 1976; Stein & Crothers, 1976) established that tRNA has at least two classes of independent binding sites for multivalent cations. The biphasic nature of Scatchard plots of binding data indicates a class of sites with strong binding affinity for small cations and a second class of sites with weak binding affinity. At low ionic strength (<20 mM Na^+ ion) nonnative tRNA appears to bind one to five multivalent cations (Mg^{2+}, Mn^{2+}, Co^{2+}, spermidine, and spermine) in a cooperative manner (Cohn et al., 1969; Sander & Ts'o, 1971; Danchin, 1972; Lynch & Schimmel, 1974; Schreier & Schimmel, 1974, 1975). By such a mechanism the binding of the first small cation would facilitate the binding of one to four additional cations to strong binding sites as the macromolecule undergoes a significant conformational change to a more compact, native structure. Crothers and co-workers have pointed out that cooperative binding of cations is an expected event since a correlation exists between initial cation binding and tRNA conformational change (Cole et al., 1972; Stein & Crothers, 1976).

On the other hand, several investigators have reported that binding of multivalent cations to native tRNA is noncooperative at high ionic strength (>30 mM Na^+ ion) and it is not associated with any major change in macromolecular structure (Römer & Hach, 1975; Bina-Stein & Stein, 1976; Stein & Crothers, 1976; Bolton & Kearns, 1977a,b). The binding of divalent cations merely appears to stabilize an otherwise native tRNA structure existing at a high monovalent cation concentration (Cole et al., 1972; Römer & Hach, 1975; Bolton & Kearns, 1977b).

An often neglected factor in the interpretation of tRNA–ligand binding data is that of electrostatic interactions between multivalent cations and tRNA, although two groups of investigators (Walters et al., 1977; Leroy & Guéron, 1977) have demonstrated the importance of electrostatic interactions on cation binding to tRNA. Reinterpretation of several previous cation binding studies has shown that (a) the shapes of cation binding isotherms (Scatchard plots) can be accounted for quantitatively following corrections for the influence of electrostatic interactions and, where necessary, tRNA conformational changes and (b) one class (not two) of binding sites is present whereby all phosphate groups of the polyanion have an equal intrinsic binding affinity for divalent cations (Walters et al., 1977). The binding of divalent cations to tRNA is found to be strongly dependent on the monovalent cation concentration (Leroy & Guéron, 1977), suggesting the importance of electrostatic interactions.

It is apparent that several questions remain unanswered regarding the binding of small cations to tRNA, notably the following: (a) are there one or two classes of independent binding sites on tRNA, (b) does Mg^{2+} ion bind cooperatively to an interacting set of special binding sites, and (c) how do multivalent cations bind to tRNA and by what mechanism is this binding influenced by monovalent cations? In addressing ourselves to these questions, we have undertaken a detailed analysis of multivalent cation binding to tRNA by considering two important phenomena which must intervene in ion binding, namely, (a) the Donnan effect on the measurement of equilibrium binding and (υ) the electrostatic contribution to the interaction of small molecules with tRNA (Linderstrøm-Lang, 1953; Hill, 1955). Since these two factors have received only limited attention in the past (Sander & Ts'o,

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1971; Rialdi et al., 1972; Leroy & Guéron, 1977; Walters et al., 1977), we wanted to examine the possible effects on the interaction of monovalent and trivalent cations with tRNA by reinvestigating the binding of the trivalent polyamine spermidine to monomers and dimers of tRNA. The present paper is an account of these analyses. The results presented in this report indicate that, in fact, two classes of spermidine binding sites are evident on tRNA at low ionic strength in contrast to the observability of only one class of binding sites under high ionic strength. We also found that Na^+ ions and K^+ ions do not compete with spermidine for tRNA binding sites under the salt concentration range tested (0–0.5 M) but rather inhibit spermidine binding by forming a counterion atmosphere around the negatively charged macromolecule. As expected, Donnan effects are significant under equilibrium conditions at low ionic strength, but they can be corrected for in treatment of binding data.

Experimental Procedures

Transfer RNA. Yeast $tRNA^{Phe}$ and *Escherichia coli* $tRNA_2^{Glu}$ were purchased from Boehringer Mannheim; amino acid accepting activity, as determined by the manufacturer, was 1000 and 1200 pmol/A_{260} unit, respectively. Yeast $tRNA^{Phe}$ (10 mg) was dissolved in 2.5 mL of 0.01 M sodium cacodylate, pH 7.0, 0.002 M disodium ethylenediaminetetraacetate (Na_2EDTA), and 0.1 M NaCl and dialyzed for 2 h in $NaHCO_3$–EDTA-treated dialysis tubing against the same buffer. The tRNA was then extensively dialyzed against 0.01 M sodium cacodylate, pH 7.0, containing 0.02 M NaCl at 4 °C, divided into small aliquots, and stored at −80 °C until needed. Denatured and native conformers of Mg^{2+}-free $tRNA_2^{Glu}$ (*E. coli*) were prepared as described earlier (Eisinger & Gross, 1975). All solutions were made with glass-distilled water. Dialyzed tRNA preparations contained 0.1–1 Mg^{2+} ion per tRNA molecule, as determined by atomic absorption spectroscopy, and <0.25 spermidine molecule per tRNA molecule, as determined by the dansyl chloride method (Gray, 1967). The concentration of tRNA stock solutions was determined spectrophotometrically (260 nm) by using a molar extinction coefficient of 5×10^5 M^{-1} cm^{-1} for yeast $tRNA^{Phe}$ and 5.5×10^5 M^{-1} cm^{-1} for *E. coli* $tRNA_2^{Glu}$ (Eisinger & Gross, 1975).

Synthesis of Oligoribonucleotides. Tritium-labeled oligonucleotides were synthesized with primer-dependent polynucleotide phosphorylase (*Micrococcus luteus*) according to methods previously described (Pongs et al., 1973). Synthetic oligonucleotides were analyzed for purity and nucleotide composition by descending paper chromatography of alkaline hydrolysates and by high-performance liquid chromatography.

Equilibrium Dialysis Experiments. The buffer used in equilibrium dialysis experiments was 0.01 M sodium cacodylate (pH 7.0) containing fixed or varying concentrations of Na^+, K^+, and Mg^{2+} ions as indicated in the following section. Equilibrium dialyses were performed in Lucite cells each containing two compartments (50-μL capacity per compartment), separated by a $NaHCO_3$–EDTA-treated dialysis membrane (Spectropor). tRNA (20–80 μM) in 40 μL of dialysis buffer was injected into one compartment, opposite a 40 μL volume of dialysis buffer in the other compartment. [^{14}C]Spermidine (Amersham) was added to both compartments. Equilibration in the sealed dialysis cell was achieved within 48 h at 0 °C. Spermidine concentrations at equilibrium were assayed by spotting a 30-μL aliquot from each compartment on filter pads, drying, and liquid scintillation counting. tRNA was not detectable spectrophotometrically in the dialysis compartment opposite that containing the macromolecule. Recovery of spermidine was >94% at ligand concentrations >20 μM. Because it was observed in this study and in a previous study (Schreier & Schimmel, 1975) that polyamines at low concentrations (<10 μM) absorbed to surfaces in the presence of tRNA, recovery of spermidine was corrected to 100% when low ligand concentrations (<20 μM) were used. Experimental points in binding isotherms represent duplicate equilibrium dialyses.

Control experiments to monitor the formation of yeast $tRNA^{Phe}$·*E. coli* $tRNA_2^{Glu}$ dimeric complexes were done by performing equilibrium dialyses of the tRNA with ^{3}H-labeled tri- or tetranucleotides in a manner similar to that described above. Oligonucleotides complementary to the anticodon loop of one or the other tRNAs were used. Serving as an internal control, the same complementary oligonucleotides were allowed to bind to $tRNA^{Phe}$ or $tRNA_2^{Glu}$ alone under equilibrium conditions.

Results and Discussion

Spermidine Binding to tRNA at Low Ionic Strength. At low ionic strength (≃0.013 M Na^+ ion), titration of a fixed concentration of the denatured conformer of *E. coli* $tRNA_2^{Glu}$ shows strong binding of spermidine to the polynucleotide (Figure 1). Virtually all the spermidine had diffused to the polynucleotide compartment at the low spermidine concentrations tested. The experimental points for spermidine binding follow a curve that is biphasic as evidenced by a local change in curvature at a [spermidine]/[tRNA] ratio of approximately 10/1.

When equilibrium dialysis experiments are conducted at low ionic strength, consideration must be given to the effect of nondiffusible ions on the distribution of diffusible ions at equilibrium. This Donnan effect arises from the fact that the net charge of the nondiffusible macromolecule (tRNA) present in one dialysis cell compartment must be compensated for in the same compartment in part by reduced concentrations of diffusible ions of like charge, namely, chloride and cacodylate ions, and in part by increased concentrations of diffusible ions of opposite charge, namely, sodium and spermidine ions (Tanford, 1961). Thus the Donnan effect results in an apparent "spermidine binding" to tRNA, and equilibrium dialysis data must be corrected for this extraneous factor. We analyzed the binding data by developing an expression for the free spermidine concentration as a function of the diffusible ions and the nondiffusible polynucleotide under equilibrium conditions.

The tRNA (t), present in the right-hand compartment of the dialysis cell, can bind spermidine to form a tRNA–ligand complex. The term x, defined as the molarity of spermidine bound at binding sites on tRNA, is not governed by the Donnan equilibrium. We can define the dissociation constant (K_d) of bound spermidine as

$$K_d = \frac{(n_0 t_0 - x)S_r}{x} \tag{1}$$

where n_0 is the number of independent and equivalent spermidine binding sites on tRNA and S_r is the free spermidine concentration in the right compartment. For equal-sized compartments we also have the following stoichiometric relationships:

$$2N_0 = N_l + N_r$$

$$2D_0 = D_l + D_r$$

$$2C_0 = C_l + C_r$$

$$2S_0 = S_l + S_r + x = S_l + S_r + \frac{n_0 t_0 S_r}{K_d + S_r}$$

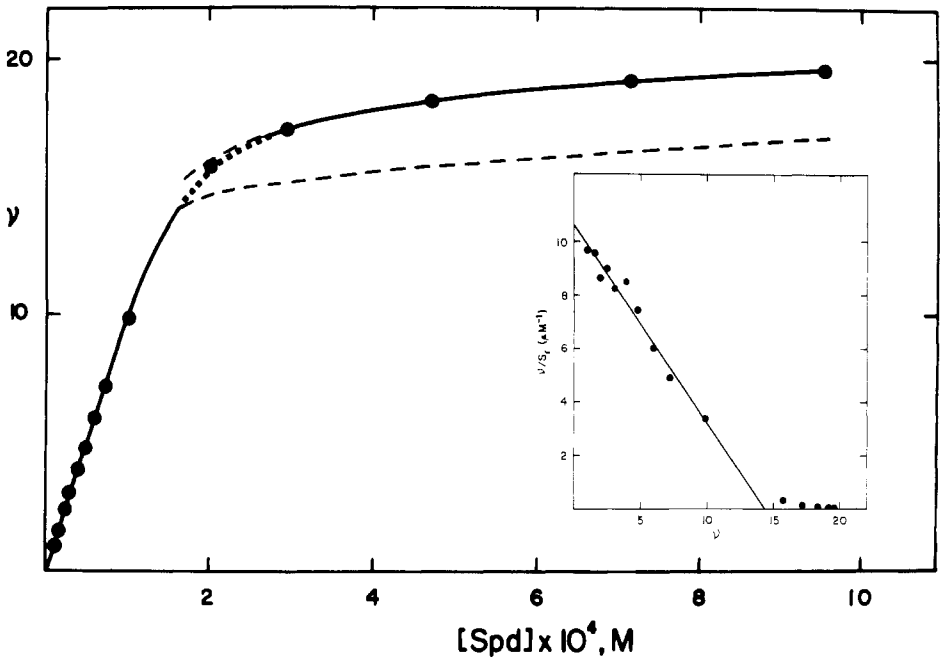

FIGURE 1: Binding of spermidine to *E. coli* $tRNA_2^{Glu}$ as a function of spermidine concentration in 0.01 M sodium cacodylate, pH 7.0, and 0.003 M Na^+ (NaCl and Na salt of tRNA) at 0 °C. [tRNA] = 2×10^{-5} M. The binding curve, theoretically fit by eq 4–6, is designated as follows: (i) lower dashed–solid curve represents strong spermidine binding; (ii) upper dashed–solid curve represents weak spermidine binding. (Inset) Scatchard plot for equilibrium spermidine binding to *E. coli* $tRNA_2^{Glu}$. The linear plot represents a least-squares fit of experimental data for $\nu < 12$. ν is moles of spermidine bound per mole of tRNA; [Spd] is spermidine concentration; S_r is the free spermidine concentration in the right compartment of the equilibrium dialysis cell at equilibrium.

where N_0, D_0, C_0, and S_0 are the initial concentrations of sodium, cacodylate, chloride, and spermidine ions, respectively, while the corresponding terms with subscripts l and r represent the ion concentrations in the left and right compartments, respectively, at equilibrium.

On the other hand, the chemical potentials governing the Donnan equilibrium yield

$$N_l C_l = N_r C_r$$
$$N_l D_l = N_r D_r$$
$$S_l C_l^3 = S_r C_r^3$$

Finally, the requirement for electrical neutrality is expressed by

$$N_r + 3S_r = C_r + 75t - 3x + D_r$$

if we assume that the tRNA molecule has a net negative charge of 75.

So that these equations can be related to the experimental values, namely, the total spermidine concentrations in the left compartment (E_l) and right compartment (E_r), the experimental variables are defined as

$$E_r = S_r + x$$

and

$$E_l = S_l$$

Therefore, S_r can be expressed in terms of experimentally determined quantities as

$$S_r = E_l\left(\frac{2C_0 + 2D_0 + 75t - 3E_r}{2N_0 - 75t + 3E_r}\right)^3 \qquad (2)$$

The initial concentrations of the diffusible ions used in the experiments at low ionic strength are 0.0126 M Na^+ (sodium ion contribution from tRNA counterions, sodium cacodylate buffer, and NaCl), $(0.0011 + 3S_0)$ M Cl^- (chloride ion contribution from NaCl and spermidine trihydrochloride), and 0.0106 M cacodylate ion. At pH 7.0, cacodylic acid is only about 84% ionized, but a correction for this incomplete ionization did not significantly affect the binding parameters calculated with respect to those obtained by using the above initial concentrations.

Since $x = E_r - S_r = \nu$ (moles of sperimidine bound per mole tRNA), eq 1 can be transformed to the linear form

$$\frac{1}{E_r - S_r} = \left(\frac{K_d}{n_0 t_0}\right)\left(\frac{1}{S_r}\right) + \frac{1}{n_0 t_0} \qquad (3)$$

In a more conventional way, the data corrected for Donnan effect can be plotted according to Scatchard, i.e., ν/S_r vs. ν (see inset to Figure 1). Again, the plot is biphasic, suggesting the presence of more than one class of spermidine binding sites on $tRNA_2^{Glu}$. However, a treatment of binding data from the initial portion of the sperimidine binding isotherm (Figure 1) by eq 3 does produce a linear plot (Figure 2a). The binding parameters calculated from the slope and y intercept of this straight line are $K_d = 1.4 \times 10^{-6}$ M and $n_0 = 14.8 \pm 1.6$ spermidine molecules bound per $tRNA_2^{Glu}$ molecule (Table I). Linearity of the reciprocal plot is consistent with one class of spermidine binding sites which are independent and equivalent.

At low ionic conditions, even at the lowest [spermidine]/[tRNA] ratios (≤2), we found no evidence to suggest that the first spermidine molecules would bind in a cooperative fashion to $tRNA_2^{Glu}$. All experimental points are within close proximity to the least-squares fit line, thus indicating that all strong spermidine binding sites have the same intrinsic binding constant. These findings are in contrast to several previous studies on yeast $tRNA^{Phe}$ (Cohn et al., 1969; Sander & Ts'o, 1971; Danchin, 1972; Schreier & Schimmel, 1974, 1975) and the denatured conformer of *E. coli* $tRNA_2^{Glu}$ (Bina-Stein & Stein, 1976) which were interpreted as indicating a cooperative binding of the first few di- or trivalent cations to a class of interacting binding sites on tRNA. We feel that in the early phases of binding the Donnan effect is the most pronounced and the quasi-quantitative binding of the ligand renders the

Table I: Binding Parameters for Spermidine–tRNA Interactions at 0 °C

ionic conditions	tRNA species	strong binding n_0	strong binding K_d (M)	weak binding n_0	weak binding K_d (M)
low salt[a]	yeast $tRNA^{Phe}$	13.9 ± 2.3	1.39×10^{-6}	ND[d]	ND
	E. coli $tRNA_2^{Glu}$	14.8 ± 1.6	1.40×10^{-6}	4.0 ± 0.1	1.23×10^{-4}
high salt[b]	yeast $tRNA^{Phe}$	12.8 ± 6.9	3.4×10^{-3}[c]		
	yeast $tRNA^{Phe}$·*E. coli* $tRNA_2^{Glu}$	10.4 ± 1.2	2.0×10^{-3}[c]		

[a] 0.01 M sodium cacodylate, pH 7.0 ($\Sigma Na^+ \simeq 0.013$ M). [b] 0.01 M sodium cacodylate, pH 7.0, 0.01 M KCl, and 0.01 M $MgCl_2$ [Σ(monovalent cation) ≃ 0.12 M]. [c] Apparent dissociation constant for tRNA-bound spermidine. [d] Not determined.

experimental data the most susceptible to small experimental error.

The binding parameters for strong spermidine binding, together with the correction for the Donnan effect (eq 2), allow us to fit a theoretical curve to the experimental points at low spermidine concentrations (Figure 1). A rearrangement of eq 2 results in the form

$$S_r = \{-[3K_d(1 - f^{1/3}) + 3n_0t_0 - G] + [[3K_d(1 - f^{1/3}) + 3n_0t_0 - G]^2 + 4[3(1 - f^{1/3})](GK_d)]^{1/2}\}/[2[3(1 - f^{1/3})]] \quad (4)$$

We defined in this equation that

$$f = \frac{E_l}{S_r}$$

and

$$G = f^{1/3}(2C_{00} + 2D_0 + 75t) + 75t - 2N_0 = 3S_r\left(1 - f^{1/3} + \frac{n_0t_0}{K_d + S_r}\right)$$

where $C_0 = C_{00} + 3S_0$ (S_0 = initial spermidine concentration) to account for the Cl^- ion contribution from spermidine trihydrochloride. In addition, the initial spermidine concentration (S_0) and the bound spermidine (ν) are found to be a function of f according to

$$S_0 = \frac{S_r}{2}\left(1 + f + \frac{n_0t_0}{K_d + S_r}\right) \quad (5)$$

and

$$\nu = \frac{2(S_0 - fS_r)}{t_0} \quad (6)$$

Knowing that f is ≤1, theoretical values for f are substituted into eq 4–6 to obtain corresponding S_0 and ν values. The theoretical curve for strong binding, fit according to calculated theoretical values, fits the experimental values for strong binding at low spermidine concentrations within experimental error and without any apparent trend in the residuals (Figure 1). Since the above calculations included a correction for the Donnan effect, good agreement between theoretical and observed values is consistent with the hypothesis that the Donnan effect is the only factor influencing the experimental data for strong binding at the ionic strength (≃0.013 M Na^+ ion) used in these experiments.

Figure 1 shows that the theoretical curve for strong binding approaches a saturation plateau at high spermidine concentrations (1.5×10^{-4}–1×10^{-3} M) but at a level significantly below experimental ν values. This suggests that the binding behavior of spermidine to $tRNA_2^{Glu}$ in this region of the binding curve is distinct from characteristics of strong spermidine binding. Therefore, an analysis of binding characteristics at high [spermidine]/[tRNA] ratios was done by merely subtracting the binding contribution of strong-binding spermidine from total spermidine binding. The double-reciprocal plot (derived from eq 3) of these corrected values is linear at the high spermidine concentrations (Figure 2b). A determination of binding parameters from the linear plot shows that $K_d = 1.23 \times 10^{-4}$ M with $n_0 = 4.0 \pm 0.1$ spermidine molecules bound per tRNA molecule (Table I). Clearly, a second class of spermidine binding sites exists in $tRNA_2^{Glu}$ with reduced binding affinity and number of binding sites.

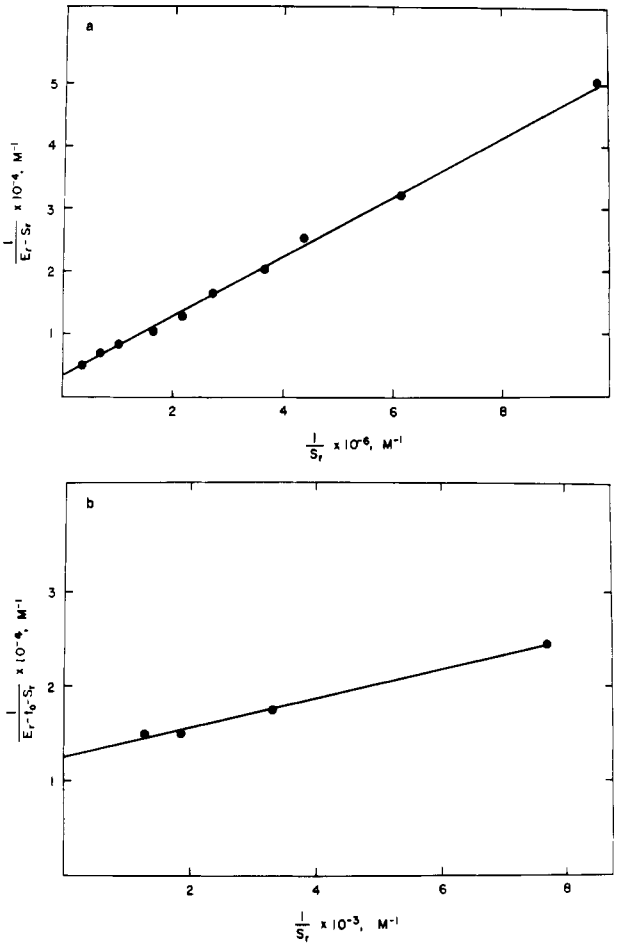

FIGURE 2: Reciprocal plots for spermidine binding to *E. coli* $tRNA_2^{Glu}$ under experimental conditions described in the legend to Figure 1. (a) Strong spermidine binding; (b) weak spermidine binding. The linear plots are least-squares fit to the experimental points. E_r and S_r are the experimentally determined, total spermidine concentration and the free spermidine concentration, respectively, in the right compartment of the equilibrium dialysis cell; $t_0 = n_0$[tRNA] where n_0 is the number of strong spermidine binding sites on $tRNA_2^{Glu}$.

A theoretical curve (Figure 1) for weak binding is fit to experimental values by inserting the parameters for weak binding into eq 4–6. The net negative charge of $tRNA_2^{Glu}$ is adjusted to 30.6 to account for the charge neutralization of phosphate groups by the strong binding of 14.8 trivalent cations per tRNA molecule. In addition, the concentration of strong-binding spermidine is subtracted from S_0 while n_0 = 4. The theoretical curve for weak spermidine binding is

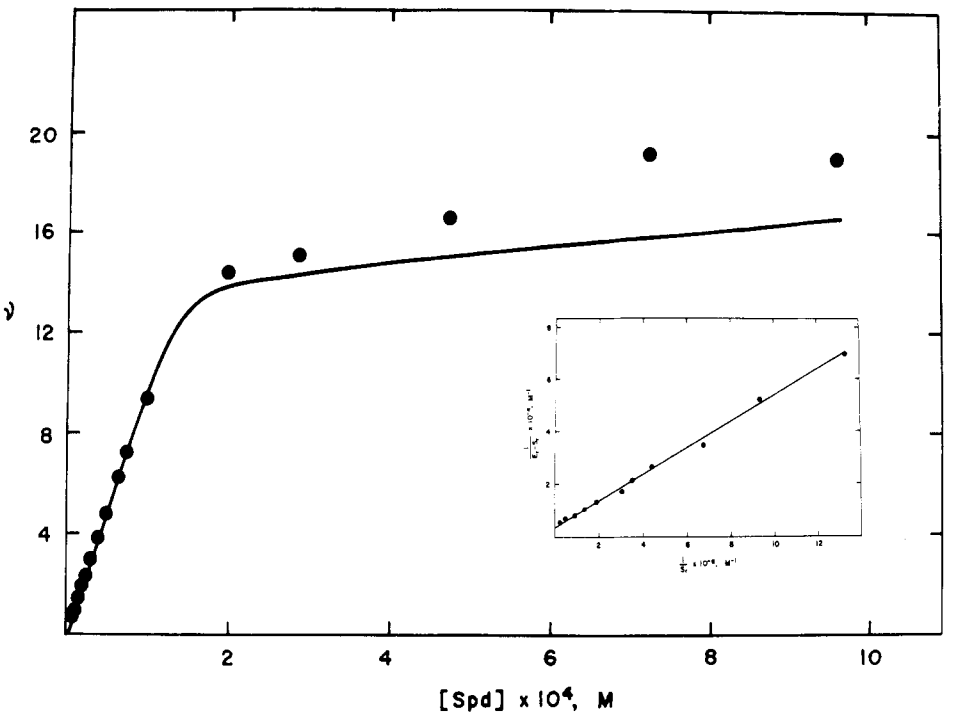

FIGURE 3: Binding of spermidine to yeast $tRNA^{Phe}$ as a function of spermidine concentration under experimental conditions described in the legend to Figure 1. The curve, fit theoretically by eq 4–6, represents strong spermidine binding to $tRNA^{Phe}$. (Inset) Reciprocal plot for the strong binding of spermidine to yeast $tRNA^{Phe}$. The linear plot is least-squares fit to the experimental points. ν and [Spd] are defined in the legend to Figure 1; E_r and S_r are defined in the legend to Figure 2.

coincident with experimental values in this region of the binding curve. Therefore, we can account for the totality of spermidine binding (19 ± 1.5 sites) to *E. coli* $tRNA_2^{Glu}$ based on evidence presented for strong and weak spermidine binding and a correction for Donnan effect at low ionic strength.

The binding of spermidine to yeast $tRNA^{Phe}$ (Figure 3) at low ionic strength (0.013 M Na^+ ion) is similar to that observed for *E. coli* $tRNA_2^{Glu}$. A double-reciprocal plot according to eq 3 was again biphasic (data not shown), suggestive of the presence of two classes of binding sites. A fit of the experimental points results in a straight line for the region of strong binding (inset to Figure 3). Parameters for strong binding, determined from the slope and y intercept of this plot, are $K_d = 1.39 \times 10^{-6}$ M and $n_0 = 13.9 \pm 2.3$ molecules of spermidine per molecule of $tRNA^{Phe}$ (Table I). A good fit of the theoretical binding curve for strong spermidine binding (Figure 3) again indicates that by correcting for the Donnan effect the results are consistent with a simple equilibrium binding of spermidine to $tRNA^{Phe}$.

Our experimental values at the high spermidine concentrations (Figure 3) had too much experimental error for allowing quantitative analysis of the weak binding to $tRNA^{Phe}$. However, we can say with confidence that a weak binding of spermidine to yeast $tRNA^{Phe}$ does occur, since experimental values at high [spermidine]/[$tRNA^{Phe}$] ratios are consistently above the theoretical curve for strong binding and, furthermore, weak binding of spermidine seems to reach half-saturation at about 0.0005 M spermidine, similar to weak binding on *E. coli* $tRNA_2^{Glu}$ (Figure 1).

The total number of spermidine binding sites is similar for both tRNAs examined in this study, but this number is slightly higher than the number of spermidine binding sites on $tRNA^{Phe}$ in 0.1 M triethanolamine buffer (pH 8.35) (Schreier & Schimmel, 1975) and in approximately 0.1 M K^+ ion (Sakai et al., 1975). This discrepancy in total binding sites is perhaps a reflection of differences in ionic concentrations used in equilibrium dialysis experiments.

Spermidine Binding to tRNA at High Ionic Strength plus Mg^{2+}. The effect of high ionic strength (0.115 M monovalent cation) and 0.01 M Mg^{2+} ion on spermidine binding to yeast $tRNA^{Phe}$ results in a hyperbolic plot as spermidine is titrating a fixed concentration of tRNA (Figure 4). The curvature throughout the binding plot indicates that saturation binding of spermidine to $tRNA^{Phe}$ is being approached.

The initial attempts to analyze spermidine binding data for $tRNA^{Phe}$ under high ionic strength and Mg^{2+} were conducted under the assumptions that both Mg^{2+} and spermidine could bind to $tRNA^{Phe}$ (possibly competitively for identical sites), that monovalent cations interact with the macromolecule, and that a correction for Donnan effect on equilibrium is made. However, we found that under our experimental conditions it was not necessary to include all these interactions for the analysis of the data. We could simplify the treatment of the binding data by assuming that the Donnan effect is suppressed under these high salt and Mg^{2+} conditions. Parameters for spermidine binding could then be evaluated by the simplified expression

$$\frac{1}{\upsilon} = \frac{1}{n_0} + \left[\frac{K_d\left(1 + \frac{M_0}{K_m}\right)}{n_0}\right]\left(\frac{1}{S_0 - \frac{t\upsilon}{2}}\right) \quad (7)$$

where υ is the moles of spermidine bound per mole of tRNA, K_m is the apparent dissociation constant for Mg^{2+}–tRNA complex, K_d is the dissociation constant for spermidine, and M_0 is the initial Mg^{2+} concentration. The sperimidine binding scheme of eq 7 is based on competitive binding of Mg^{2+} ions with spermidine for binding sites on tRNA.

The inset to Figure 4 shows that the spermidine binding curve, plotted according to eq 7, is linear, which indicates that only one class of spermidine binding sites is apparent at high salt and Mg^{2+} conditions. Binding parameters obtained from the linear plot revealed an apparent $K_d = 3.4 \times 10^{-3}$ M

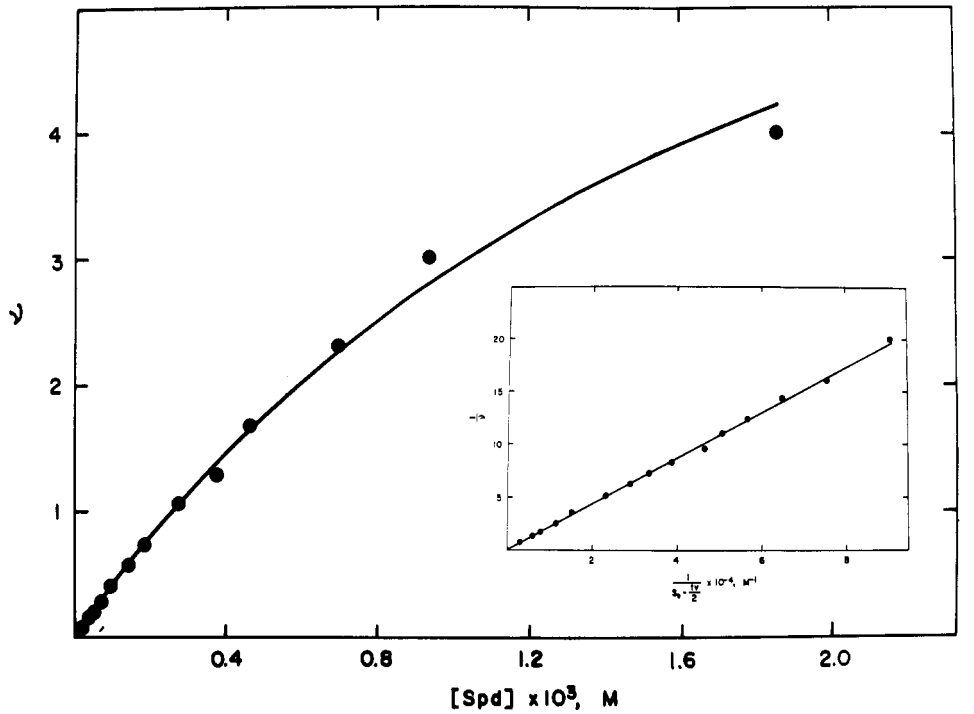

FIGURE 4: Binding of spermidine to yeast $tRNA^{Phe}$ as a function of spermidine concentration in 0.01 M sodium cacodylate, pH 7.0, 0.1 M KCl, and 0.01 M $MgCl_2$ at 0 °C. [tRNA] = 4×10^{-5} M. The theoretical curve is fit to the experimental points as described in the text. (Inset) Reciprocal plot for spermidine binding to yeast $tRNA^{Phe}$. The linear plot is a least-squares fit to the experimental points. ν (v) and [Spd] are defined in the legend to Figure 1; S_0 is the initial spermidine concentration at equilibrium; t = [tRNA].

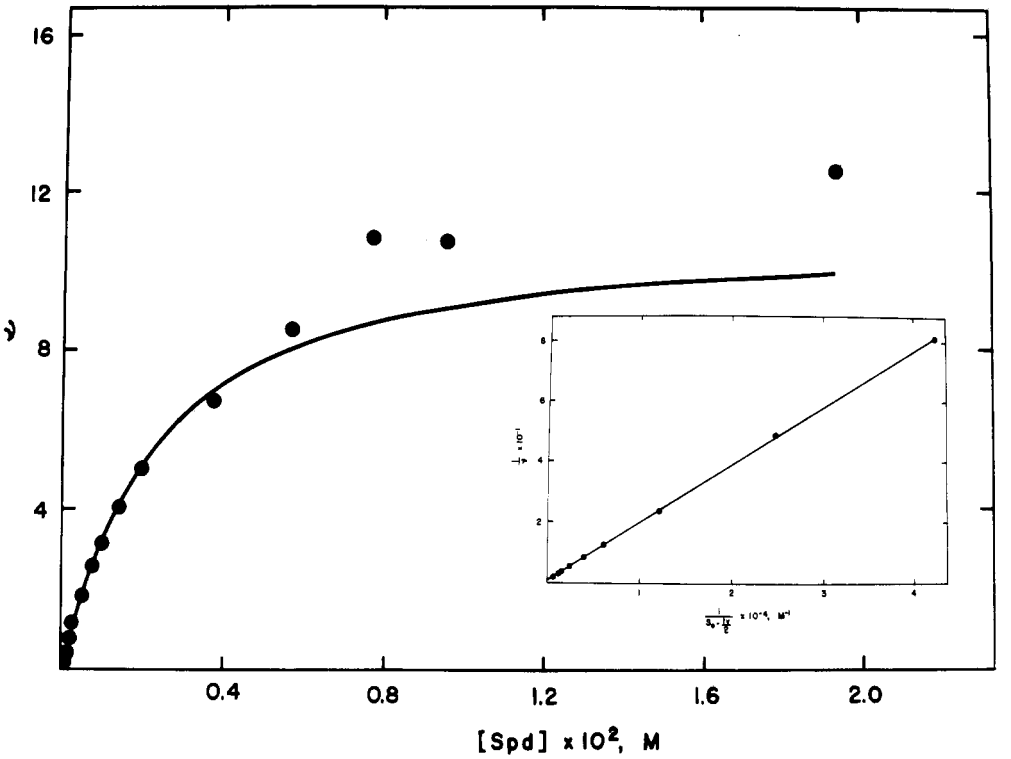

FIGURE 5: Binding of spermidine to yeast $tRNA^{Phe}$·*E. coli* $tRNA_2^{Glu}$ dimeric complex as a function of spermidine concentration under experimental conditions described in the legend to Figure 4 with the exception that [$tRNA^{Phe}$ + $tRNA_2^{Glu}$] is 8×10^{-5} M. (Inset) Reciprocal plot for spermidine binding to yeast $tRNA^{Phe}$·*E. coli* $tRNA_2^{Glu}$ dimeric complex. The linear plot is a least-squares fit to the experimental points. ν (v) and [Spd] are defined in the legend to Figure 1; S_0 and t are defined in the legend to Figure 4.

whereby n_0 = 12.8 ± 6.9 mol of spermidine bound per mol of $tRNA^{Phe}$ (Table I). These parameters are in good agreement with previous results (Sakai et al., 1975) obtained by studying spermidine binding to yeast $tRNA^{Phe}$ under similar ionic conditions.

A dimeric complex between yeast $tRNA^{Phe}$ and *E. coli* $tRNA_2^{Glu}$ is formed at equimolar concentrations of the two tRNAs because of anticodon complementarity (Grosjean et al., 1976). In our study, tRNA complex formation, as monitored by reduced binding of ^{3}H-labeled oligonucleotides complementary to the anticodons, does not significantly alter the levels of spermidine binding. The curve at low [spermidine]/[tRNA] ratios again represents an approach to saturation binding (Figure 5), and analysis at low ligand con-

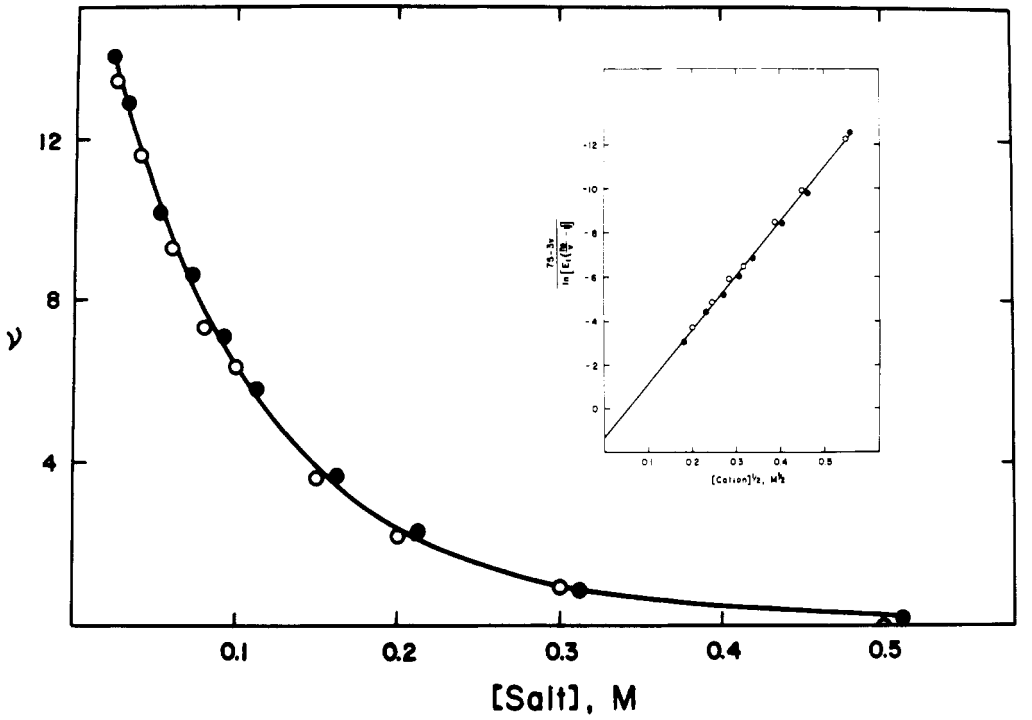

FIGURE 6: Binding of spermidine to yeast $tRNA^{Phe}$ as a function of NaCl (○) or KCl (●) concentration in 0.01 M sodium cacodylate, pH 7.0. The curve is a theoretical fit by eq 10. [Spd] = 2×10^{-4} M; [tRNA] = 2×10^{-5} M. (Inset) The effect of monovalent cations on spermidine binding to yeast $tRNA^{Phe}$ as a function of the square root of Na^+ (○) or K^+ (●) ion concentrations. The data points, calculated from eq 10, are fit by least squares. ν is moles of spermidine bound per mole of tRNA; [Salt] is NaCl or KCl concentration; n_0 is the number of spermidine binding sites on $tRNA^{Phe}$; E_l is the experimentally determined total spermidine concentration in the left compartment of the equilibrium cell at equilibrium; [Cation] is the ion concentration of Na^+ or K^+.

centrations by eq 7 produces a linear plot (inset to Figure 5) at which n_0 = 10.4 ± 1.2 mol of spermidine bound per mol of tRNA at an apparent $K_d = 2.0 \times 10^{-3}$ M (Table I).

It is noted, however, that at the higher concentrations of ligand the experimental ν values are above the theoretical binding curve (Figure 5). We feel that an explanation for this behavior is a competition of strong-binding spermidine with bound Mg^{2+} ions for identical binding sites on tRNA with a gradual increase in the [spermidine]/[Mg^{2+}] ratio. One might expect a similar result for the $tRNA^{Phe}$ monomer at higher spermidine concentrations (Figure 4).

Effect of Monovalent Cations on Spermidine Binding to $tRNA^{Phe}$. In order to understand the mechanism by which high ionic strength influences spermidine binding to tRNA, we examined the effect of Na^+ and K^+ ions on spermidine binding. In these experiments, spermidine and tRNA concentrations were kept constant while monovalent cation concentration was varied. An increase in either Na^+ or K^+ ion concentrations resulted in a decrease in spermidine binding to $tRNA^{Phe}$, and ν approached zero asymptotically (Figure 6).

It has been proposed that monovalent cations influence the binding of multivalent cations to tRNA by either a reduction in electrostatic potential due to a counterion atmosphere around the macromolecule or a binding of monovalent cations to tRNA with a concomitant decrease in tRNA net charge and number of free binding sites (Leroy & Guéron, 1977). It is important to ask if Na^+ or K^+ ions bind competitively to spermidine binding sites on the polyanion. If one assumes that (i) Donnan equilibrium is negligible at low ionic strength, (ii) if binding occurs, Na^+ or K^+ ions bind to equivalent sites, and (iii) the Debye–Hückel effect would be insignificant, analysis of the data showed that reduction in spermidine binding could not be accounted for by competitive inhibition by Na^+ or K^+ ions. This finding is consistent with earlier studies (Walters & Van Os, 1971; Krakauer, 1974; Stein & Crothers, 1976) stating that monovalent cations do not compete effectively with divalent cations for binding sites on polynucleotides.

On the other hand, the Debye–Hückel effect proved to be the dominant factor and amenable to analysis by the Linderstrøm-Lang theory (Steinhardt & Beychok, 1964). In using this theory one assumes that the only role of Na^+ and K^+ ions is to decrease the electrostatic interaction between highly negatively charged tRNA and the positively charged spermidine. These analyses were made possible by the fact that the Donnan effect is negligible at >0.04 M monovalent cation and that only strong spermidine binding (n_0 = 13.9; Table I) to $tRNA^{Phe}$ occurs. Therefore, the dissociation constant for bound spermidine is expressed in the form

$$K_d = E_l\left(\frac{n_0}{\nu} - 1\right)$$

The Gibbs free energy is related to K_d by the expression

$$\Delta G = -RT \ln K_d$$

where ΔG is the standard free energy change of complexation, R is the universal gas constant, and T is the absolute temperature.

A determination of electrostatic free energy of the macromolecule requires one to calculate the work (ω_{el}) necessary to bring a trivalent cation (e.g., spermidine) from infinity to the surface of the macromolecule of radius b. In the Linderstrøm-Lang approximation, this electrostatic work can be expressed as

$$\omega_{el} = \frac{3\bar{z}^2\epsilon^2}{2D}\left(\frac{1}{b} - \frac{\kappa}{1 + \kappa b}\right)$$

where ϵ is the charge on one electron, D is the dielectric constant of the solvent, $\bar{z}$ is the mean net charge of the

macromolecule, κ is the Debye–Hückel parameter, and b is the radius of the macromolecule. Therefore, one can calculate the apparent free energy of $tRNA^{Phe}$–spermidine complex by transforming the above equation into

$$\ln\left[E_1\left(\frac{n_0}{v}-1\right)\right] = -\frac{\Delta G_{int}}{RT} - \frac{3\epsilon^2\bar{z}}{DkT}\left(\frac{1}{b}-\frac{\kappa}{1+\kappa b}\right) \quad (8)$$

where ΔG_{int} is the change in intrinsic free energy of the macromolecule upon complexation without electrostatic effects, k is the Boltzmann constant, and

$$\kappa = \left(\frac{8\pi\epsilon^2 N_A}{1000DkT}\right)^{1/2} K_0^{1/2}$$

where N_A is Avogadro's number.

Under high ionic conditions, binding of spermidine to $tRNA^{Phe}$ is virtually nonexistent (Figure 6), suggesting that the mean net charge of the macromolecule, $\bar{z}$, is constant and is approximately 75. With this simplifying condition, eq 8 can be transformed into

$$\ln K_d = -\frac{\Delta G_{int}}{RT} - \frac{3\epsilon^2(75)}{DkTb}\left(\frac{1}{1+\kappa b}\right) \quad (9)$$

Therefore, at these low ν values, one can determine whether the intrinsic free energy $[\Delta G_{int}/(RT)]$ is significant with respect to electrostatic interactions of spermidine with $tRNA^{Phe}$. For this purpose the approximate radius of the $tRNA^{Phe}$ molecule (35 Å), based on X-ray crystallographic studies (Kim, 1978), was substituted for b in eq 9. At high monovalent cation concentrations (0.15–0.3 M), i.e., $\bar{z} = 75$, a plot of $\ln K_d$ vs. $1/(1+\kappa b)$ was obtained with a slope of 38.9 and y intercept $[-\Delta G_{int}/(RT)]$ of 0.53 ± 0.5. Thus, $\Delta G_{int}/(RT)$ is at least 10-fold less than any of our experimental values of $-\ln K_d$. In other words, the intrinsic free energy happens to be close to zero and, therefore, negligible with respect to the effects of electrostatic interactions. A check on the validity of the macromolecular radius of 35 Å can be made since the slope of the plot according to eq 9 yields an independent value for b. The b value calculated from the slope is 40 Å, in excellent agreement with the assigned b value.

Therefore, since $\Delta G_{int}/(RT)$ is negligible, eq 8 can be transformed into the equation

$$\frac{75-3v}{-\ln\left[E_1\left(\frac{n_0}{v}-1\right)\right]} = \frac{bDkT}{3\epsilon^2} + \frac{b^2BDkT}{3\epsilon^2}K_0^{1/2} \quad (10)$$

where $B = \kappa/K_0^{1/2}$.

The total charge of the polyelectrolyte, $\bar{z}$, is 75 less the negative charges neutralized by bound trivalent cations. Therefore, a plot of $(75-3v)/\{-\ln[E_1(n_0/v-1)]\}$ as a function of $(Na_0)^{1/2}$ or $K_0^{1/2}$ should yield a straight line of positive slope and positive intercept. Treatment of binding data by eq 10 gives a linear plot (inset to Figure 6). Moreover, the plot is the same for both Na^+ and K^+ ions, showing that both cations have an identical effect on the binding of spermidine to $tRNA^{Phe}$. The slope and y intercept of the straight line in Figure 6 allow one to obtain a theoretical fit to the experimental values of the titration experiments (Figure 6) since eq 10 can be solved explicitly for K_0. The theoretical binding curve appears to fit experimental points for both Na^+ ion and K^+ ion titration experiments.

On the basis of the preceding analyses, we feel confident that the interaction of small cations with tRNA is predominantly electrostatic in nature. This conclusion appears compatible with the reinterpretation (Walters et al., 1977) of previous divalent cation studies, indicating that Mg^{2+} and Mn^{2+} ions seem to interact with tRNA by neutralizing negative charges on the polyelectrolyte. When electrostatic interactions were taken into account, previous binding studies could be explained within a simple model of only one class of equal and independent binding sites for divalent cations (Walters et al., 1977).

In an interpretation of equilibrium binding data obtained from the interaction of small cations with tRNA, it must be borne in mind that the small molecule may be held in the neighborhood of the macromolecule by any one of three ways, namely, (i) Donnan effect, (ii) an electrostatic buildup of a counterion atmosphere, and (iii) a specific binding or group interaction. Specific binding between a ligand and a macromolecule is the only interacting factor that actually represents a thermodynamic equilibrium, whereby an ion pair complex is formed. Results presented in this report indicate that the strong binding of spermidine to tRNA represents a thermodynamic equilibrium originating primarily from electrostatic interactions between properly spaced charges on the cation and charges on the polyanion. It is hypothesized that all strong spermidine binding sites remain on the tRNA regardless of ionic conditions but that the affinity of spermidine for tRNA is decreased significantly with increase in ionic conditions (Table I). It is tempting to speculate that the presence of a class of weak spermidine binding to tRNA at low ionic conditions represents an interaction of only two of the three possible positive charges on spermidine with two phosphate groups on the tRNA.

Since spermidine bears three positive charges, clearly the free energy of electrostatic interactions should be high, resulting in strong binding at about micromolar K_d. In turn, spermine, with four positive charges, should bind even stronger to tRNA, as documented earlier for $tRNA^{Phe}$ (Schreier & Schimmel, 1975). Since a monovalent cation can interact with only one negative charge on the macromolecule, its binding is expected to be extremely weak and undetectable even up to 1 M salt. Although a quantitative analysis of magnesium binding was not possible under experimental conditions described in this report, we propose that divalent cations have electrostatic interactions which can be analyzed by the equations presented here.

Under the experimental conditions described, we can not infer that a structural change of tRNA occurs. All data analyses account for the experimental observations without being forced to invoke a change in tRNA conformation under the influence of multivalent cations. Although the possibility exists that the binding of the first spermidine to tRNA results in cooperative binding of additional spermidine molecules under experimental conditions used in previous binding studies (Cohn et al., 1969; Sander & Ts'o, 1971; Danchin, 1972; Schreier & Schimmel, 1974, 1975; Bina-Stein & Stein, 1976), we feel that interpretation of cooperative ligand binding at low spermidine concentrations is in error based primarily upon the following considerations. First, at very low spermidine concentrations under equilibrium conditions, virtually all spermidine is complexed with the tRNA. Therefore, experimental data obtained at low spermidine concentrations are highly susceptible to experimental error in calculated free spermidine concentration. An over- or undercorrection for nonspecific binding of spermidine to surfaces at low polyamine concentrations could have a significant effect on the shape of a binding curve in Scatchard or double-reciprocal plot analyses. Second, even a small effect, such as the Donnan effect, could

have a large effect on calculated bound spermidine to tRNA at low ligand concentrations if ionic conditions are not sufficiently high to completely suppress the Donnan effect.

The data clearly indicate that equilibrium binding studies at low ionic strength should consider the Donnan effect on equilibrium since, at certain trivalent cation concentrations, the Donnan effect contributes to an apparent spermidine binding which is significantly above that of true ligand–tRNA complex concentration.

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