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The binding of polyamines and of ethidium bromide to tRNA

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ABSTRACT

The binding of spermidine and ethidium bromide to mixed tRNA and phenylalanine tRNA has been studied under equilibrium conditions. The numbers and classes of binding sites obtained have been compared to those found in complexes isolated by gel filtration a low ionic strength. The latter complexes contain 10-11 moles of either spermidine or ethidium per mole of tRNA; either cation is completely displaceable by the other. In ethidium complexes, the first 2-3 moles are bound in fluorescent binding sites; the remaining 7-8 molecules bind in non-fluorescent form. At least one of the binding sites for spermidine appears similar to a binding site for fluorescent ethidium. Similar results are found with E. coli formylmethionine tRNA. Spermine, in excess of 18-20 moles per mole tRNA, causes precipitation of the complex. Putrescine does not form isolable complexes with yeast tRNA and displaces ethidium less readily from preformed ethidium-tRNA complexes.

Under equilibrium conditions, in the absence of Mg^{++}, there are 16-17 moles of spermidine bound per mole of tRNA as determined by equilibrium dialysis. Of these, 2-3 bind with a $K_a = 6.6 \times 10^4\ M^{-1}$; the rest bind with a $K_a = 7.6 \times 10^3\ M^{-1}$. In the presence of 9 mM Mg^{++}, the total number of binding sites is decreased slightly and there appears to be only one class of sites with a $K_a = 600\ M^{-1}$. Quantitatively similar results are obtained for the binding of spermidine to yeast phenylalanine tRNA. When the interaction between ethidium bromide and mixed tRNA is studied by equilibrium dialysis or spectrophotometric titration, two classes of binding sites are obtained: 2-3 molecules bind with an average $K_a = 6.6 \times 10^5\ M^{-1}$ and 14-15 molecules bind with an average $K_a = 4.1 \times 10^4\ M^{-1}$. Spermidine, spermine, and Mg^{++} compete effectively for both classes of ethidium sites and have the effect of reducing the apparent binding constants for ethidium. When the binding of ethidium is studied by fluorometry, there are 3-4 highly fluorescent sites per tRNA. These sites are also affected by spermidine, spermine and Mg^{++}. Putrescine has little effect on any of the classes of binding sites. These data are consistent with those found under non-equilibrium conditions. They suggest that polyamines bind to fairly specific regions of tRNA and may be involved in the maintenance of certain structural features of tRNA.

INTRODUCTION

Spermidine has been shown to modify the structure of both DNA and RNA and to stimulate many steps of transcription and translation[1]. The hypothesis of Tsuboi[2] placed spermidine in double-stranded helical regions of nucleic acid and suggested that this polyamine serves to stabilize such regions. The studies presented in this paper were undertaken to test the possibility that

spermidine might bind to tRNA to help stabilize regions of the macromolecule not believed to be as stable as other regions. Such an 'unstable' region was suggested, and most recently confirmed, to be in the dihydrouridine (hU) arm which, in all tRNAs sequenced to date, has been shown to contain only 3 or 4 complementary base pairs (see discussion). These hypotheses, plus the earlier finding that tRNA isolated at low ionic strength from bacterial and animal cells contains a limited number of molecules of spermidine or spermine per mole of nucleic acid [1,3,4], suggested the existence of specific sites for the binding of these organic cations.

Previous studies from this laboratory [5,6] showed that spermidine affects the conformation of tRNA in the region of the hU arm and that these effects differ from those induced by Mg^{++} in some respects and emulate Mg^{++} effects in others. The conformation of the hU arm, as monitored by the rate of formation of a photoproduct between 4-thiouridine (s^4U) in that region and a non-contiguous cytosine residue, is particularly sensitive to polyamines [5]. Other studies on the T_m of s^4U in tRNA, the accessibility of s^4U in tRNA to external reagents and the mobility of a spin-label attached to s^4U [6], indicate a similar sensitivity of the hU arm to restructuring by polyamines. Thus it appears that spermidine is particularly effective in organizing the tertiary structure of tRNA in the region of the hU arm. Other studies show polyamines to be important in the crystallization of tRNA [7], aminoacylation [8] and methylation [9,10] reactions.

We have studied the binding properties of spermidine with tRNA and, because our working hypothesis suggests a high affinity of spermidine for double-stranded helical regions of nucleic acid, we have compared these properties with those of ethidium bromide, which forms fluorescent complexes when bound to double-stranded regions [11-13], to determine if spermidine and ethidium might bind to the same region of tRNA. As shown in Figure 1, there is at least a

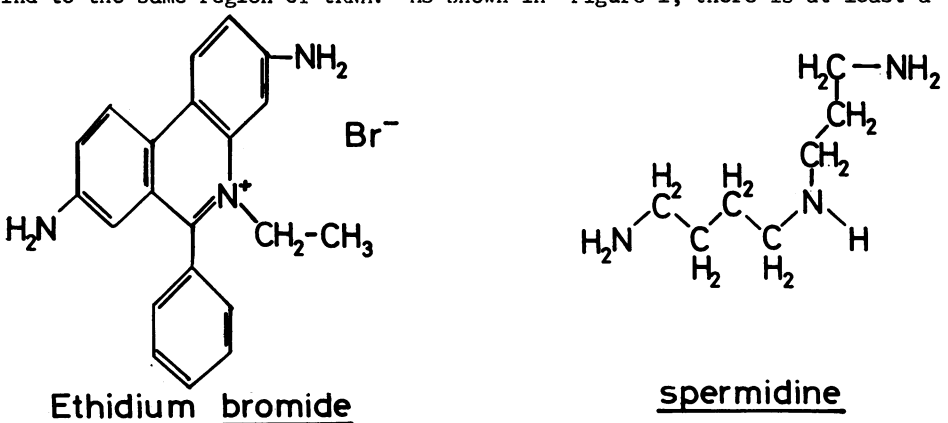

Figure 1. The structures of ethidium bromide (left) and spermidine (right). The latter is drawn to simulate bonds in ethidium.

superficial structural similarity between ethidium and spermidine, both bases containing two primary amino groups, each of which is separated similarly from the quaternary nitrogen in the former and from the secondary nitrogen in the latter. This paper describes experiments done to characterize and quantitate the binding sites of spermidine and ethidium bromide on tRNA.

MATERIALS AND METHODS

Chemicals. Ethidium bromide, yeast soluble RNA and the hydrochloride salts of the polyamines were obtained from Calbiochem. Ethidium bromide was also obtained from the Boots Pure Drug Co. Yeast phenylalanine tRNA was from Boehringer-Mannheim; E. coli formylmethionine tRNA was the gift of Dr. A. D. Kelmers of the Oak Ridge National Laboratory, Oak Ridge, Tennessee. [^{14}C]-Spermidine trihydrochloride (specific activity 9.8 μCi/μmole) was obtained from New England Nuclear. All other chemicals were the highest purity obtainable. All solutions were made with glass-distilled water.

The purity of ethidium bromide was checked by thin-layer chromatography on silica gel using a solvent system of n-butanol-acetic acid-H_2O (4:1:5,v/v)[14]. Polyamine samples were used which had been shown to be at least 98% pure by the dansyl method[3]. [^{14}C]-Spermidine was found to be greater than 97% pure by paper electrophoresis [15]. Samples of RNA which contained high molecular weight contaminants were purified by gel filtration as described previously [6]. Concentrations of tRNA were determined using a millimolar extinction coefficient of 570 $mM^{-1}cm^{-1}$ at 260 nm [16].

Spectrophotometric measurements were made on a Gilford Model 2000 or Zeiss PMQ II spectrophotometer. Fluorometric measurements were made on a Farrand Mark I spectrofluorometer.

For equilibrium studies, the standard buffer contained 0.05 M potassium cacodylate, pH 7.0 and 0.05 M KCl. Stock solutions of various ligands and/or salts were made up in this buffer and added as indicated. Dialysis membranes were washed with 50% ethanol to remove glycerol, boiled in an EDTA-sodium bicarbonate solution [17] and stored at 4°. All studies were done at 24°± 1°.

Isolation of complexes. Chromatographic separations of tRNA complexes with spermidine and ethidium bromide were effected on columns of Sephadex G-100 as previously described for the isolation of spermidine-tRNA complexes from bacterial cells [4]. Polyamines were analyzed by the dansyl method [3]. Ethidium concentrations were measured by the fluorescence produced in the presence of excess tRNA [18,19]; tRNA concentrations in the presence of ethidium were determined from the fluorescence obtained by the addition of excess ethidium [18].

Equilibrium gel filtration. The binding of spermidine to tRNA was measured by equilibrium gel filtration using the method of Hummel and Dreyer [20].

A column of Sephadex G-10 (0.5 x 30 cm) was equilibrated with the standard buffer containing various concentrations of [^{14}C]-spermidine with specific activities ranging from 3.1 x 10^4 to 6.2 x 10^5 cpm/μmole. The samples of tRNA (23.7 nmoles) in the equilibrating buffer were added to the column in a volume of 100 μl and the column was eluted with the buffer containing the ligand. Fractions of 100 μl were collected and each fraction was analyzed for tRNA and spermidine. Spermidine levels were determined by placing a 50 μl aliquot on a Whatman 3MM scintillation pad (2.3 cm), drying the pad and counting it in 6 ml of Liquifluor (New England Nuclear) in a Packard Model 3003 liquid scinitllation spectrometer. The amount of spermidine bound to tRNA was found by subtracting the equilibrium concentration of spermidine from the values obtained throughout the tRNA peak whose concentration was determined from the optical density at 260 nm.

Equilibrium dialysis studies. Studies on the binding of spermidine and ethidium bromide to tRNA were also done by equilibrium dialysis using a multicell apparatus (1.0 ml capacity per cell, CRC, Inc.) and cells with a 0.1 ml capacity (Drummond Scientific Co.). Both sizes of cell were found to give the same results. Generally, studies with mixed tRNAs were done with the larger cells and studies with $tRNA^{phe}$ with the smaller cells. The samples of tRNA (approximately 1 m$\underline{M}$ tRNA-phosphate), dissolved in the standard buffer, were placed in one side of the cell and allowed to equilibrate with various concentrations of [^{14}C]-spermidine in the standard buffer at room temperature for 14-16 hours. The concentrations of spermidine on each side of the membrane were determined by counting an aliquot as described above. No significant binding of spermidine to the membranes was noted under the conditions used.

For binding studies with ethidium bromide, similar procedures were followed. Ethidium bromide concentrations were determined spectrophotometrically at 510 nm, the isosbestic point for ethidium binding to tRNA[21], using an extinction coefficient of 4110 $\underline{M}^{-1}cm^{-1}$. In the absence of tRNA, about 6% of the dye at any dye concentration was found to bind to the membranes. This binding was not believed to affect the results since only that amount not bound to the membranes was measured in any experiment. Ethidium bromide was used from 0.06 to 0.30 m$\underline{M}$; tRNA concentration was 1.0 m$\underline{M}$ (tRNA-phosphate). Competition experiments with spermidine (0.06 to 0.24 m$\underline{M}$) were done under the same conditions. Spermidine was found not to affect the isosbestic point of ethidium-tRNA complexes.

Spectrophotometric and spectrofluorometric titrations. The binding of ethidium bromide to tRNA and the effects of various cations on this binding was determined as described by Waring[21] using a fixed total concentration of

ethidium bromide (0.123 mM) and varying concentrations of tRNA (0 to 8.0 mM tRNA-phosphate). Plots of the optical density at 460 nm of dye solutions containing varying concentrations of tRNA as a function of tRNA concentration gave saturation curves which were analyzed by the method of Peacocke and Skerrett[22]. In experiments done in the presence of added polyamines or Mg^{++}, the level of saturation was assumed to be the same as that in the absence of added cation. The fluorescence of each solution was also measured, using an excitation wavelength of 520 nm and analyzing the emitted light at 590 nm. The fluorescence data were analyzed similarly[22] to estimate free dye and bound fluorescent dye.

Analysis of binding data. The binding of a ligand to a macromolecule can be described by the equation

$$\bar{\nu} = \sum_{i=1}^{m} n_i \frac{K_i c}{1 + K_i c} \qquad (1)$$

where $\bar{\nu}$ is the mean number of moles of ligand bound per mole of macromolecule, m is the number of classes of binding sites, n_i is the number of binding sites in the i^{th} class, K_i is the average association constant for that class and c is the concentration of free ligand [23,24].

When there is only one class of independent, non-interacting binding sites, a plot of $\bar{\nu}/c$ vs. $\bar{\nu}$ gives a straight line with the slope equal to $-K_a$ and the intercept on the abscissa is equal to the total number of binding sites [23]. However, when two or more classes of sites are present, such a plot yields a curve and the binding parameters usually cannot be determined by the usual extrapolation of the straight line portions of the plot. Klotz and Hunston have analyzed such plots and have derived a series of simultaneous equations based on the limiting values of the slopes and intercepts of the Scatchard plots [24]. When such equations are solved algebraically or by computer fit, the parameters may be fairly easily determined. In the Figures, the curves drawn are the theoretical lines generated by equation (1) when the binding constants and numbers of binding sites obtained by the method of Klotz and Hunston are substituted. Binding data are given in terms of association constants (K_a) and values of n (as moles of cation per mole of tRNA-phosphate). In most cases, values of n were also converted to moles of cation per mole of tRNA, assuming that an average tRNA molecule contains 80 nucleotides.

In the cases in which a single class of binding site was found, the values of n and K_a are accurate to ± 15-20%. For two classes of binding sites, the total number of sites ($\bar{\nu}$) and K_2 and n_2, the association constant and number of sites for the looser class of sites are accurate to ± 10%. The values of n_1 and K_1 for the tighter class, because of their small values were

often round to have 50% deviation.

RESULTS

Polyamine-tRNA complexes. Spermidine and yeast mixed tRNA were mixed in a molar ratio of 40:1 and chromatographed on Sephadex G-100 in 0.01 M potassium acetate, pH 7.0 (Fig. 2). The first peak of spermidine elutes with tRNA; the

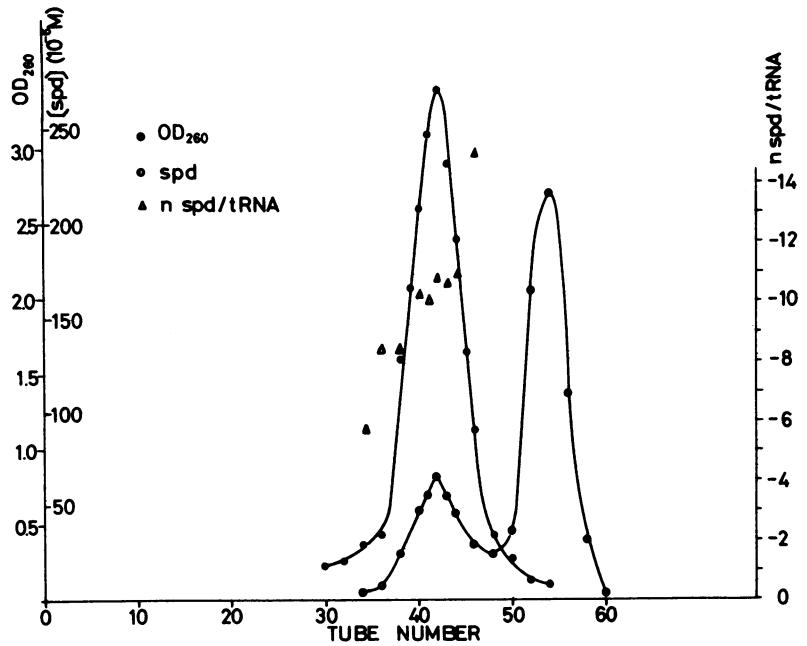

Figure 2. The chromatographic separation of a mixture of spermidine and yeast tRNA (40:1). A 2.5 ml sample (3.2×10^{-5} M tRNA + 1.28×10^{-3} M spermidine) in 0.01 M potassium acetate, pH 7.0 was added to a Sephadex G-100 column (90 x 1.5 cm) and eluted with the same buffer. Optical density at 260 nm (●); spermidine (O); spermidine/tRNA (Δ).

second peak is that of free spermidine. In the central portion of the tRNA peak, there are approximately 10-11 molecules of spermidine per molecule of tRNA. At the leading edge of the tRNA peak there are 6-8 molecules per tRNA; at the trailing edge the ratio increases to about 15 molecules per tRNA. These results indicated significant dissociation of spermidine from the complex. When a 2:1 mixture of spermidine and tRNA was chromatographed, no peak of free spermidine was detected. Under these conditions, little difference in the ratio of spermidine to tRNA was observed between the leading and trailing edges of the tRNA peak, indicating little dissociation of spermidine from the complex.

On the addition of spermine to yeast tRNA under the same conditions,

precipitation is observed at about 18-20 moles of spermine per mole tRNA, i.e., when neutralization approached completion. No peak of free spermine is observed when a 12:1 mixture of spermine and tRNA is chromatographed, indicating no dissociation of spermine from the complex. Under the same conditions, putrescine forms no detectable complexes with mixed tRNA. Quantitatively similar results are obtained for the binding of these cations in complexes with E. coli formylmethionine tRNA. Limited complex formation was detected with putrescine and the latter tRNA.

Ethidium-tRNA complexes. Chromatography of a 40:1 mixture of ethidium and yeast tRNA gave the separation shown in Figure 3. The first peak of

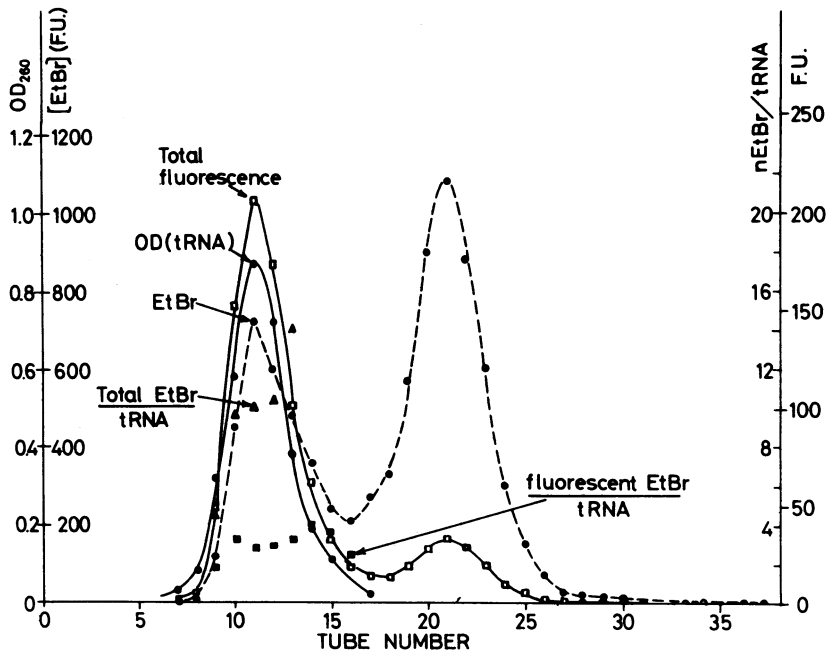

Figure 3. The chromatographic separation of a mixture of ethidium bromide and yeast tRNA (40:1). A 2.0 ml sample (6.4×10^{-6} M tRNA + 2.56×10^{-4} M ethidium bromide) in 0.01 M potassium acetate, pH 7.0 was added to a Sephadex G-100 column (30 x 1.5 cm). Optical density at 260 nm (tRNA) (●); total fluorescence (□); total ethidium bromide/tRNA (■).

ethidium is strongly fluorescent and coincides with tRNA; the second is that of free dye. Approximately 10 moles of total ethidium were bound per mole of tRNA in the peak. The peaks of fluorescence and tRNA are almost congruent and there are the equivalent of 3 moles of fluorescent ethidium per mole of tRNA. Again, there is some asymmetry in the elution profile, suggesting some dissociation of the complex, particularly of the non-fluorescent bound ethidium.

The chromatography of an equimolar mixture of ethidium and tRNA gives only a single peak and nearly all of the ethidium is bound to fluorescent sites. When a mixture of 2 molecules of ethidium per tRNA was chromatographed, no detectable levels of free ethidium were found; however, a significant portion (about 20-25%) of the ethidium is now present in a non-fluorescent form, suggesting that the affinity of ethidium for a relatively non-fluorescent site approaches that for a second fluorescent site in tRNA. Identical results are obtained in comparable studies with E. coli formylmethionine tRNA.

Titrations of complexes. Complexes of tRNA with either spermidine or ethidium were isolated at varying ratios of cation to tRNA. The ability of polyamines to displace ethidium from ethidium-tRNA complexes and the ability of ethidium to bind to spermidine-tRNA complexes was measured by the fluorescence of ethidium in the complexes.

The addition of ethidium equimolar to spermidine in a 1:1 spermidine-tRNA complex resulted in a level of fluorescence 20% lower than that obtainable in the absence of spermidine. This suggests that a single molecule of spermidine can occupy a primary fluorescent binding site for ethidium. It is of interest that a 1:1 spermidine-tRNA complex isolated from E. coli [3] exhibited the same behavior towards ethidium as did the artificial 1:1 yeast tRNA-spermidine complex. A 10:1 spermidine-tRNA complex requires the addition of 50 moles of ethidium per mole of complex before full fluorescence is attained, suggesting that spermidine can be replaced at all sites by a sufficient excess of ethidium. Similarly, spermidine and spermine in excess can displace fluorescent ethidium from ethidium-tRNA complexes. Putrescine was less effective than spermidine in displacing ethidium from fluorescent sites. Complexes containing E. coli $tRNA_f^{Met}$ behaved similarly to the complexes with yeast tRNA.

A mixed complex was isolated from a solution initially containing spermidine, ethidium and tRNA in the ratio of 11:10:1. The resulting fluorescent complex contained spermidine and ethidium in the ratio of 11:2 per tRNA; almost all of the bound ethidium was fluorescent. This suggests that possibly only one of the three fluorescent ethidium sites is the same as a tight binding site for spermidine.

Equilibrium gel filtration. Studies were begun on the binding of spermidine to tRNA by using the equilibrium gel filtration method of Hummel and Dreyer[20]. Whereas experiments at concentrations of spermidine greater than approximately 0.05 mM gave the same results as found by equilibrium dialysis (below), studies at low concentrations (below 0.05 mM), i.e., low $\bar{\nu}$ values in the Scatchard plot, gave results which were interpreted as artifacts of binding to the carbohydrate matrix of the Sephadex beads. Control experiments

in which small amounts of [^{14}C]-spermidine were eluted from the column showed that spermidine did not elute as a discrete peak but was spread throughout several void volumes. Uracil, with the approximate molecular weight of spermidine, elutes as a sharp peak at a volume expected for its molecular weight. This indicates that spermidine is interacting, probably electrostatically, with the gel matrix.

In addition, although this method relies on the separation of macromolecule and free ligand, it was found that the deficit of spermidine, in the buffer used, eluted very near the void volume and overlapped with the tRNA peak. To overcome this, excess spermidine had to be added to the tRNA to just eliminate the deficit and give a symmetric elution profile of tRNA and spermidine. Thus, although the initial experiments using this modification of the Hummel-Dreyer method gave results which agreed well with the data obtained later by equilibrium dialysis, the problems described suggested that it would be necessary to use another method; equilibrium dialysis was chosen. Although the Hummel-Dreyer method is applicable in many cases, any studies done using this method should bear in mind the types of artifacts described in this paper.

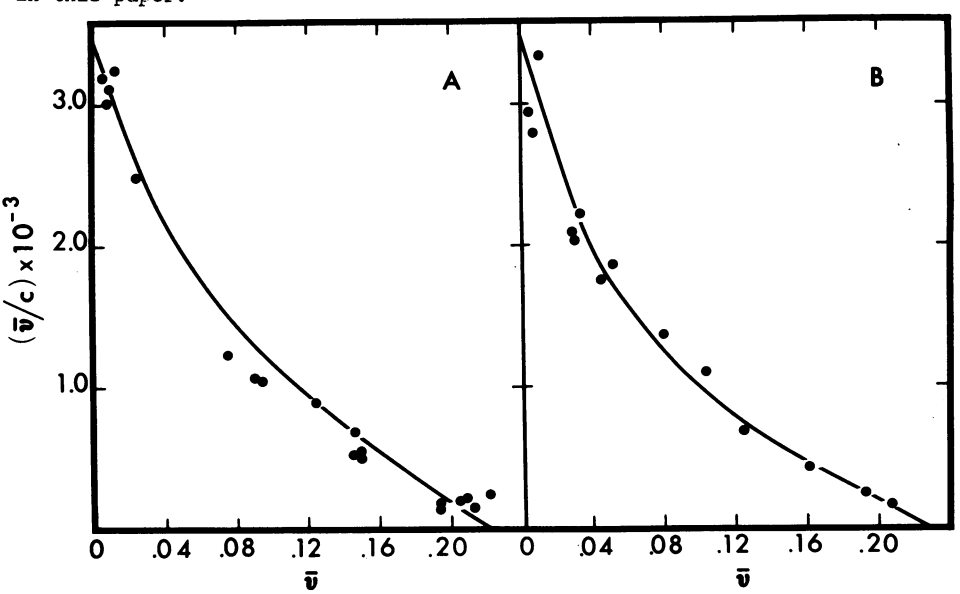

Figure 4. Scatchard plot for the binding of spermidine to yeast mixed tRNA (A) and phenylalanine tRNA (B) in 0.05 M potassium cacodylate (pH 7.0)-0.05 M KCl, obtained by equilibrium dialysis. Binding parameters are calculated from the experimental points using the method of Klotz and Hunston[18]. The curves in this and subsequent figures are the theoretical curves generated by substitution of the binding parameters into equation (1).

Spermidine binding to tRNA. In Figure 4A is shown the Scatchard plot for the binding of spermidine to yeast mixed to tRNA. The data show that there are two classes of binding sites for spermidine. An amount of spermidine equivalent to 16-17 moles can be bound per mole of tRNA (n =0.221), assuming approximately 80 nucleotides per molecule of tRNA. The higher affinity site, corresponding to 2-3 molecules of spermidine per tRNA molecule (n = 0.031), has a K_a = 6.6 x 10^4 $\underline{M}^{-1}$. The other class (n = 0.190) shows a K_a = 7.6 x 10^3 $\underline{M}^{-1}$. Very similar results are seen with yeast phenylalanine tRNA (Fig. 4B). Again, there are two classes of binding sites; the higher affinity type of site has a K_a = 6.1 x 10^4 $\underline{M}^{-1}$ and binds the equivalent of about 3 moles of spermidine per mole of tRNA (n = 0.041). The other type of site, which binds about 14-15 moles per mole of tRNA (n = 0.184), has a K_a = 5.4 x 10^3 $\underline{M}^{-1}$. A comparison of the values is shown in Table 1.

In the presence of 9 m$\underline{M}$ Mg^{++}, both mixed tRNA and phenylalanine tRNA (Fig. 5) show a single class of binding sites for spermidine. There appear to

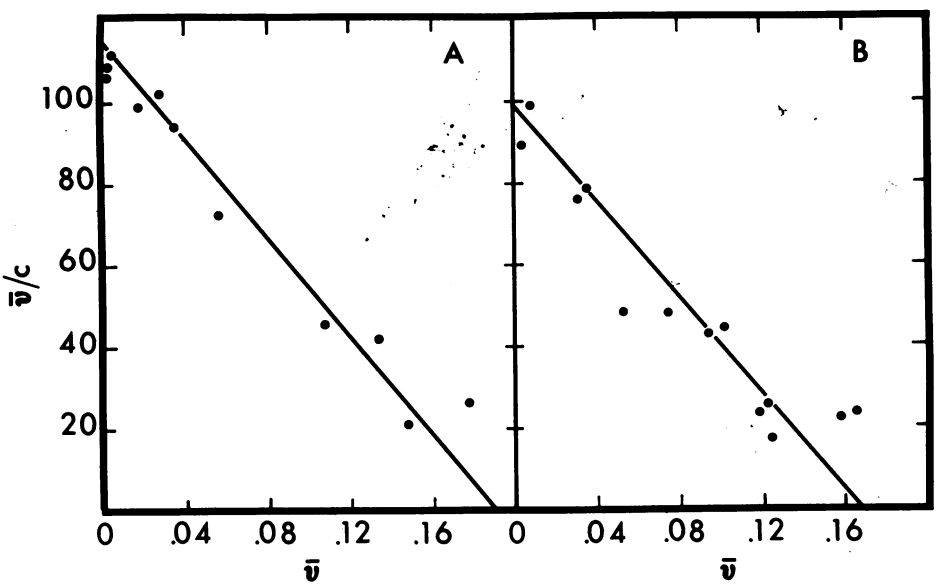

Figure 5. Scatchard plot for the binding of spermidine to yeast mixed tRNA (A) and phenylalanine tRNA (B) in 0.05 $\underline{M}$ potassium cacodylate (pH 7.0)-0.05 $\underline{M}$ KCl-9.0 m$\underline{M}$ $MgCl_2$, obtained by equilibrium dialysis.

be about 15-16 molecules of spermidine bound in mixed tRNA (n=0.190) with an average K_a = 6.0 x 10^2 $\underline{M}^{-1}$ (Fig. 5A). In phenylalanine tRNA (Fig. 5B) there are about 13 moles of spermidine bound per mole of tRNA (n = 0.170) with an average K_a = 5.9 x 10^2 $\underline{M}^{-1}$ (see Table 1).

Table 1

Comparison of the Binding Parameters for the Binding of Spermidine to Yeast Mixed tRNA and Phenylalanine tRNA in the Presence and Absence of Mg^{++}.

	$\bar{\nu}$	n_1	$K_1(\underline{M}^{-1})x10^{-4}$	n_2	$K_2(\underline{M}^{-1})x10^{-4}$
Yeast Mixed tRNA	0.221	0.031	6.6	0.190	0.76
Phenylalanine tRNA	0.225	0.041	6.1	0.184	0.54
Yeast mixed tRNA + 9 m$\underline{M}$ Mg^{++}				0.190	0.060
Phenylalanine tRNA + 9 m$\underline{M}$ Mg^{++}				0.170	0.059

Table 2

Comparison of the Binding Parameters for the Binding of Ethidium Bromide to Yeast Mixed tRNA as Determined by Equilibrium dialysis and Spectrophotometric Titration.

Conditions	$\bar{\nu}$	n_1	K_1 ($\underline{M}^{-1}$) x 10^{-5}	n_2	K_2 ($\underline{M}^{-1}$) x 10^{-5}
Equilibrium dialysis					
Ethidium bromide	0.206	0.037	4.16	0.169	0.426
plus 0.06 m$\underline{M}$	0.220	0.020	3.81	0.200	0.274
0.12 m$\underline{M}$	0.235	0.030	1.82	0.205	0.177
0.24 m$\underline{M}$	0.250	0.021	1.15	0.229	0.122
spermidine	assuming single class			0.220	0.170
Spectrophotometric titration					
Ethidium bromide	0.237	0.023	9.03	0.214	0.403
plus 0.06 m$\underline{M}$	0.235	0.032	2.38	0.203	0.250
0.12 m$\underline{M}$	0.217	0.021	2.08	0.201	0.242
0.24 m$\underline{M}$	0.228	0.022	0.89	0.228	0.138
spermidine	assuming single class			0.190	0.140

The number of binding sites is given as values of n in terms of moles of ethidium per mole of tRNA-phosphate. The values subscripted 1 are the values for the tight binding sites, those subscripted 2 are for the looser sites.

Ethidium bromide binding and competition by spermidine. In Figure 6 are shown the Scatchard plots for the binding of ethidium bromide to yeast mixed tRNA by equilibrium dialysis in the presence of varying concentrations of spermidine. The binding curves are biphasic in each case. In the absence of

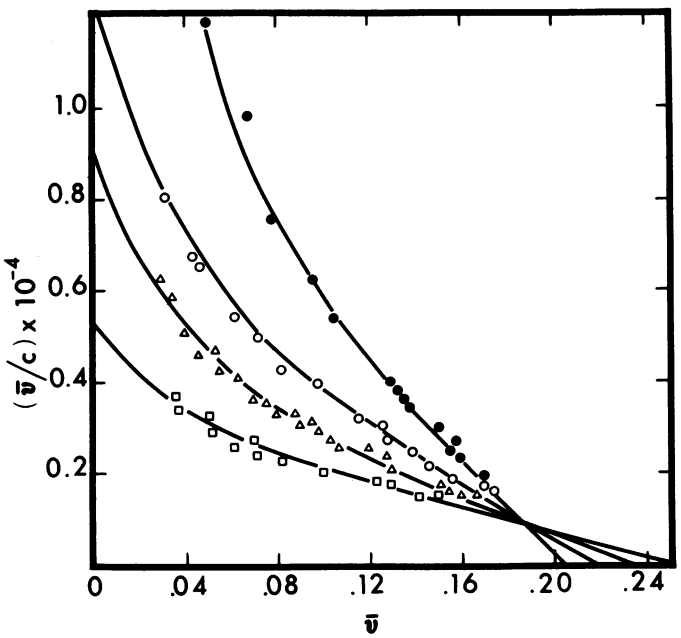

Figure 6. Scatchard plots for the binding of ethidium bromide to yeast mixed tRNA by equilibrium dialysis in 0.05 M potassium cacodylate (pH 7.0)-0.05 M KCl: no additions (●); plus 0.06 (○), 0.12 (△), and 0.24 mM (□) spermidine.

spermidine there are about 17 total sites for ethidium binding to tRNA (n = 0.206), approximately 3 of which (n = 0.037) have a $K_a = 4.2 \times 10^5\ M^{-1}$. With increasing spermidine concentrations, the affinity constants for both classes of sites decreases. The number of tight binding sites for ethidium appears to decrease to about 2 and the number of loose binding sites increases as apparently does the total number of sites. The Scatchard plot of ethidium at 0.24 mM spermidine (Fig. 6) may also be fit by eq. (1), assuming a single class of binding sites, as was found with the binding of spermidine to tRNA in the presence of Mg^{++} (Fig. 5). The inability to obtain data at low $\bar{\nu}$ values makes the plot difficult to interpret; but if a single class of sites for ethidium binding is assumed, there are 17-18 sites (n = 0.22) with a $K_a = 1.7 \times 10^4\ M^{-1}$.

Slightly different results are obtained when the experiments are repeated using the spectrophotometric method (Fig. 7). In the absence of spermidine there appear to be about 19 total sites, two of which (n = 0.023) are high affinity sites with a $K_a = 9.0 \times 10^5\ M^{-1}$. Again, spermidine decreases the affinity of ethidium for both classes of sites, but has essentially no effect on the number of sites of either class. A comparison of the data from the

two methods is given in Table 2. The slight differences in the Scatchard plots obtained by the two methods have been obtained reproducibly; however, the reasons for the discrepancies between the results from the two methods is not known.

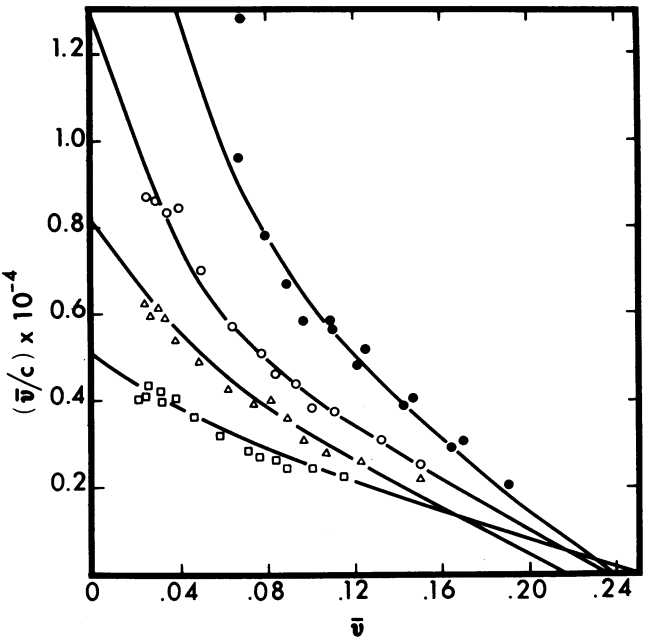

Figure 7. Same experiment in Figure 3, except the data were obtained by spectrophotometric titration of 0.123 mM ethidium bromide with varying concentrations of tRNA.

Fluorometric analysis of ethidium binding in the presence of spermidine gives the data shown in Figure 8. Because of the scatter, the data are difficult to interpret; however, there appear to be 3-4 sites per tRNA (n ≈ 0.04-0.05) for the binding of fluorescent ethidium. These correspond fairly closely with the numbers of tight binding sites obtained by equilibrium dialysis. Addition of increasing amounts of spermidine decreases the affinity of fluorescent ethidium slightly with very little effect on the number of binding sites.

Effects of other polyamines and Mg^{++} on ethidium binding. The addition of putrescine (0.12 mM) has little or no effect on the binding of ethidium to yeast tRNA as measured by spectrophotometric titration. Spermine and Mg^{++} (0.12 mM) both appear to eliminate the loose class of sites, although the data are difficult to quantitate. The binding constant for ethidium, in the presence of either spermine or Mg^{++}, assuming a single class of sites (n ≈ 0.04) is of the order of 5×10^5 M^{-1}.

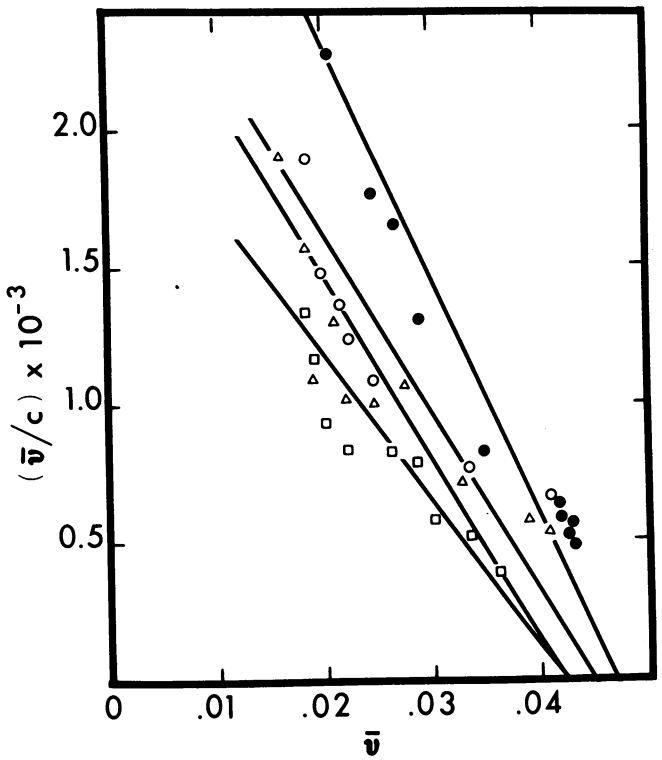

Figure 8. Scatchard plots for the binding of fluorescent ethidium to tRNA in the experiments described in Figure 3.

If these solutions are simultaneously analyzed for fluorescent ethidium binding sites, putrescine is found to have little effect on the binding of fluorescent ethidium. Spermine (0.12 mM) decreases the number of fluorescent sites from 3 or 4 ($n \simeq 0.04$) to 1 or 2 ($n \simeq 0.02$) per tRNA. At Mg^{++} concentrations of 1 or 3 mM, the number of fluorescent sites remains unchanged, but the affinity of ethidium for tRNA is decreased. At 9 mM Mg^{++}, the number of fluorescent sites decreases to about 1 per molecule of tRNA ($n \simeq 0.01$-0.02). Again, the data are difficult to obtain more precisely.

DISCUSSION

The studies described in this paper have been aimed at determining the binding characteristics of spermidine and ethidium bromide to tRNA. Because of the well-defined structure of tRNA--primary, secondary and tertiary--it was hoped that the data obtained could be interpreted in terms of such defined structures. As described earlier [3], tRNA from bacteria can be isolated containing 2 moles of spermidine per mole of tRNA. Complexes isolated from arti-

ficial mixtures could contain up to 10-11 moles of spermidine per mole of tRNA. Similarly, tRNA could bind 3 fluorescent ethidium molecules, and complexes with tRNA could be formed which contained 10-11 moles of dye per mole of tRNA. The limited number of spermidine and fluorescent ethidium molecules bound per tRNA suggested that these ligands might bind to specific regions of the tRNA molecule and perhaps hold together regions of the molecule which would otherwise become unfolded. However, because the studies with the complexes were done under non-equilibrium conditions, there were some questions about the nature of the binding of the ligands; in particular, it could not be ruled out that binding or mixing artifacts caused by the low ionic strength and non-equilibrium conditions were occurring.To eliminate such possibilities, equilibrium studies were undertaken. It must be emphasized that the values of the binding constants in this study were obtained by the method of Klotz and Hunston[24]. The accuracy of this method depends on reliable points at either extreme of the Scatchard plot, i.e., at very low and very high ratios of ligand to macromolecule. In the case of ethidium binding to tRNA, data obtained at low values of $\bar{\nu}$ were not reliable and, at times, not obtainable because of the limits of sensitivity of determining ethidium concentrations. Thus, although the calculated values of the number of binding sites and association constants are consistent with the data, in several experiments, such as the studies of the fluorescence of ethidium in the presence of spermine and Mg^{++}, the possibility of significant error must be borne in mind.

In general, the results are consistent with the data obtained under non-equilibrium conditions. The data support the initial hypotheses made when these studies were initiated: (1) there are fairly specific binding sites for spermidine in tRNA; (2) there is a second, looser class of sites for binding spermidine; (3) ethidium may be formally analogous to spermidine and may occupy the same or similar sites as spermidine; and (4) the data, when taken with other data, are consistent with a role for spermidine in which it plays a particular role in organizing tRNA structure.

At equilibrium in the absence of Mg^{++}, there are two classes of binding sites for spermidine in mixed tRNA or $tRNA^{phe}$. The lower affinity class has an average number of 14-15 sites per tRNA with an apparent association constant of 7.6×10^3 $\underline{M}^{-1}$. The second class has a 10-fold greater affinity ($K_a = 6.6 \times 10^4$ $\underline{M}^{-1}$) and an average number of 2-3 sites per tRNA molecule. The number of tight binding sites corresponds well to the 2 moles of spermidine per tRNA found in tRNA isolated from cells. However, the total number of sites for spermidine observed under non-equilibrium conditions is lower than than found under equilibrium conditions. This may be explained by the loss of

spermidine under the conditions which were used for the isolation of the complexes. It is interesting to note that in the presence of 9 m$\underline{M}$ Mg^{++} all sites for spermidine apparently become equivalent with a K_a of approximately 7 x 10^2 $\underline{M}^{-1}$. Under these conditions, no sites are lost, suggesting at least some equivalence or overlap of Mg^{++} and spermidine sites.

When ethidium is used as a probe for the binding of spermidine, the hypothesis that the dye is binding in a manner similar to spermidine is borne out by the data on the apparent competition between dye and spermidine, suggesting that ethidium may indeed be thought of as an analog of spermidine. In the absence of spermidine and Mg^{++}, there are two classes of sites for ethidium binding, the association constants of which are 2- to 5- fold greater than the corresponding values for spermidine, although the number of sites for each class are similar. The difference in binding constants may reflect the hydrophobic interactions of ethidium with tRNA postulated in intercalation, in addition to simple electrostatic bonds. The numbers agree fairly well with those found by Urbanke $\underline{et\ al.}$[25], for the binding of ethidium to the folded state of tRNA. In the presence of the higher concentrations of spermidine used in our studies, it appears that the two classes of sites for the binding of ethidium may become equivalent. As stated above, the lack of data at low $\bar{\nu}$ values makes the interpretation difficult; however, the results from the mixed complex of spermidine, ethidium and tRNA suggests that the tight binding sites for ethidium are retained in the presence of spermidine.

The intercalative binding of ethidium bromide to nucleic acids, which gives what we have called "fluorescent sites", is believed to involve at least two steps: (1) electrostatic interaction between the positively charged dye molecule and the phosphate backbone of the nucleic acid, and (2) insertion of the electrostatically positioned dye molecule between two adjacent base pairs. Tritton and Mohr[14,26] have found 3 steps for the interaction of ethidium with tRNA. The data indicate that increasing amounts of polyamine have the effect of decreasing the apparent affinity of ethidium for tRNA. Because spermidine presumably does not intercalate, it is likely that the competition between the polyamine and dye involves the first step. In these cases the number of high affinity sites remains fairly constant, which is consistent with the idea that the polyamine and ethidium sites may be equivalent. Also, it is noted that in the presence of spermine or high concentrations of Mg^{++}, there are only one or two sites for binding fluorescent ethidium. This site or sites may correspond to the first fluorescent ethidium site observed in the isolated complexes. The competitive effects of spermidine, spermine and Mg^{++} for ethidium sites suggests that all of these cations are binding to similar areas. An alter-

native possibility, as suggested by Urbanke et al.[25], is that the structure of the tRNA molecule is altered by the binding of other cations and this limits the ability of ethidium to bind to tRNA.

It is interesting to note that the 16-17 moles of spermidine bound per mole of tRNA can neutralize approximately 50 phosphate groups of the tRNA backbone. The latter number correlates well with the number of nucleotides involved in complementary base pairing in an average tRNA molecule, suggesting the possibility that these cations are binding to phosphate groups in double-stranded regions of the nucleic acid. Similarly, the number of total ethidium sites may be related to the number of base pairs in helical regions; however, it appears that the intercalative binding of ethidium is limited to 2 or 3, perhaps by the folding of the tRNA molecule.

The relative ineffectiveness of putrescine in displacing ethidium from tRNA correlates well with the inability of putrescine to form any isolable complexes with tRNA. The fairly good correlation between the equilibrium dialysis studies and the titration experiments indicates that mixing artifacts are not present in the experiments.

The recent x-ray crystallographic studies by Rich and co-workers[27] and Klug et al.[28] on the structure of yeast phenylalanine tRNA indicate the presence of tertiary interactions not apparent in the secondary structure. Several recent studies on the thermal denaturation of pure tRNA species have shown that the first interactions to be broken are the tertiary bonding interactions. This breakdown, in many cases, is accompanied by, or directly precedes, the melting of the dihydrouridine arm[29-31]. Our earlier studies[5,6] suggested that polyamines help stabilize the dihydrouridine arm. Another study on the effect of the diamine, cadaverine, at a relatively high concentration on the methylation of tRNA shows that the amine has a specific effect on tRNA structure[32]. These studies, however, provide only indirect evidence for the binding of polyamines in the region of the dihydrouridine arm itself. Recent reports indicate that some multivalent rare earth ions bind directly to the dihydrouridine arm at low ion to tRNA ratios[33-35], suggesting that this arm may in fact be a general binding site for many different cations.

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