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Relative Affinities of Divalent Polyamines and of Their N-Methylated Analogues for Helical DNA Determined by ^{23}Na NMR†

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ABSTRACT: Interactions of divalent polyamines with double-helical DNA in aqueous solution are investigated by monitoring the decrease in ^{23}Na NMR relaxation rates as NaDNA is titrated with $H_3N^+-(CH_2)_m-^+NH_3$, where m = 3, 4, 5, or 6. Analogous measurements are made for the same homologous series of methylated polyamines (methonium ions). The dependence of the ^{23}Na relaxation rates on the amount of added divalent cation (M^{2+}) is analyzed quantitatively in terms of a two-state model. The sodium ions are assumed to be in rapid exchange between a "bound" state, where they are close enough to DNA so that it affects their relaxation rate, and a "free" state in bulk solution, where their relaxation rate is the same as in solutions containing no DNA. The distribution of Na^+ and M^{2+} between these states is described quantitatively in terms of an ion-exchange parameter: $D_M \equiv (p_B^M)(1-p_B^{Na})^n/(p_B^{Na})^n(1-p_B^M)$, where p_B^{Na} and p_B^M are the fractions of Na^+ and M^{2+} that are close enough to DNA to be considered bound (by the NMR criterion), and n is the number of sodium ions displaced from DNA by the binding of one M^{2+} ion. For each of the polyamines and methonium ions investigated here, equations derived from this two-state model yield acceptable fittings of the titration curves if r_{Na}^0, the number of sodium ions bound per DNA phosphate when no competing cations are present, is assigned a value between 0.6 and 1.00. Within this range, changing the value assigned to r_{Na}^0 does change the best-fitted values of D_M determined for these polyamines (D_H) and for the methonium ions (D_{Me}) but does not alter the following conclusions about the trends in these parameters. (1) For polyamines and methonium ions of the same m, D_H exceeds D_{Me} by factors that are significantly larger for m = 3 and 4 than for m = 5 and 6. (2) D_H for m = 3 and 4 is larger than D_H for m = 5 and 6. (3) D_{Me} for m = 3 and 4 is smaller than D_{Me} for m = 5 and 6.

Polyamines interact with polyanionic nucleic acids and with membranes in vivo [cf. Tabor and Tabor (1985)]. To clarify in quantitative terms the biological significance of these interactions, their molecular and thermodynamic consequences have been extensively investigated in vitro. Thermodynamic and spectroscopic measurements have provided some information about competitive interactions of polyamines and inorganic cations with DNA in aqueous solution (Braunlin et al., 1982, 1986; Burton et al., 1981; Padmanabhan et al., 1988; Thomas & Bloomfield, 1984; Thomas et al., 1985; Thomas & Messner, 1988; Vertino et al., 1987). These studies indicate that the concentrations and types of polyamines and small inorganic cations (like Na^+) in a solution containing DNA have profound effects on its conformational stability and on the extent to which it forms complexes with proteins or other

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cationic ligands. Specifically, an increase in the concentration of a polyamine, while the concentrations of other cations are unchanged, is expected to increase the extent of dissociation of any protein–nucleic acid complex involving ionic interactions with DNA phosphates. In *Escherichia coli*, the cytoplasmic concentration of putrescine decreases approximately 10-fold (Munro et al., 1972), while the concentration of intracellular potassium increases, as part of the physiological adaptation to an increase in extracellular osmolarity (Richey et al., 1987; Cayley et al., 1989, 1991). The displacement by K^+ of divalent putrescine associated with nucleic acids in the cytoplasm is presumably part of the mechanism whereby the intracellular osmolarity is increased without perturbing the stability of protein–nucleic acid complexes.

In aqueous solutions containing NaDNA and NaCl where the total amount of sodium is not more than ~10 times the amount of DNA phosphate, measurements of ^{23}Na NMR relaxation rates are significantly enhanced over their values in the absence of DNA. On the basis of this effect, ^{23}Na NMR has been established as a sensitive method of determining the relative affinities for double helical B-DNA of univalent cations such as NH_4^+, quaternary ammonium ions, and the alkali metals (Anderson et al., 1978; Bleam et al., 1980; Burton et al., 1981; Delville et al., 1986; Padmanabhan et al., 1990). This approach has also been used to study the exchange of sodium ions near DNA with various higher valent competitors, including the divalent cations Mg^{2+} (Bleam et al., 1983; Braunlin et al., 1986, 1987), Ca^{2+} (Braunlin et al., 1987, 1989), and Hex^{2+} (Padmanabhan et al., 1988). A more complete survey is included in a recent review (Anderson & Record, 1990). During the course of a titration of NaDNA with some divalent cation, the relaxation rates of ^{23}Na are progressively reduced as sodium ions are displaced from the vicinity of DNA. Analysis of the concentration dependence of these relaxation rates provides quantitative information about the competitive association of the divalent cation and Na^+ with DNA.

Here we report measurements of ^{23}Na NMR relaxation rates observed during titrations of NaDNA with four different types of synthetic and naturally occurring polyamines, Pro^{2+}, Put^{2+}, Cad^{2+}, and Dhx^{2+}, and with three of the corresponding N-methylated analogues, Tri^{2+}, Tet^{2+}, and Pen^{2+}.[1] Comparable results for Hex^{2+} have been published previously (Padmanabhan et al., 1988). To quantify the affinity for helical DNA of each of these divalent cations relative to that of Na^+, the concentration dependence of its NMR relaxation rate is analyzed on the basis of a simple two-state model (Anderson et al., 1978). By comparing results obtained for these series of divalent polyamines and methonium ions, we have determined how their affinities for DNA (relative to Na^+) are affected by methylation of the terminal ammonium groups and by variations in the number of methylene groups linking the two charged centers. From this information, we assess the possible role of non-Coulombic factors in the association of polyamines with DNA.

Materials and Methods

DNA. Calf thymus DNA (Sigma) was extensively sonicated to obtain a sample with an average chain length of approximately 700 bp (as estimated by gel electrophoresis). The sample was purified by digestion with Pronase E from *Streptomyces griseus* (Sigma) followed by phenol extraction and ethanol precipitation. Stock solutions of NaDNA containing minimal amounts of added NaCl were obtained by extensive dialysis, first with a Tris-buffered NaCl solution containing EDTA, and then with a dilute unbuffered NaCl solution (Padmanabhan et al., 1988). The DNA-phosphate concentration ([P]) in the stock solution used in this study was 13.7 ± 0.4 mM, as determined by absorbance (A_{260}) with an extinction coefficient assumed to be 6.6×10^3 M^{-1} cm^{-1} (Nordenskiöld et al., 1984). For this sample, the hyperchromicity upon alkaline denaturation was 32%, with an absorbance ratio (A_{260}/A_{280}) of 1.95 ± 0.02. Therefore, the sonication and purification procedures employed here apparently did not denature or degrade the DNA to any significant extent. Sodium ion concentration in the stock solution of NaDNA was determined by neutron activation analysis to be 23.6 ± 1.0 mM ($[Na^+]/[P] = 1.72 \pm 0.09$).

Polyamines. The dihydrochloride salts of 1,3-diaminopropane and putrescine were obtained from Sigma; those of cadaverine and 1,6-diaminohexane were obtained from Aldrich. In all cases nominal purity was ≥98%. All of these salts were vacuum dried and used without further purification. Stock solutions of these salts with concentrations of 0.15–0.25 M were used in the titrations of NaDNA.

Preparation and Characterization of Methonium Ions. Pentamethonium bromide (obtained from K&K Laboratories) was recrystallized from ethanol. Tetramethonium and trimethonium bromides were synthesized from trimethylamine and the appropriate 1,ω-dibromoalkane as described by Fuoss and Chu (1951). The methonium salts were recrystallized from ethanol and subsequently vacuum dried to constant weight. Compositions were checked by quantitative elemental analysis (Galbraith Laboratories, Tennessee).

The ^1H NMR spectra of each of the recrystallized methonium salts in aqueous solution agree with those reported earlier (Dufourcq et al., 1972). A Redfield 2–1–4 pulse was used to suppress the water ^1H signal (Redfield et al., 1975). For Tri^{2+}, the ^1H-decoupled ^{13}C spectrum consisted of three peaks corresponding to N-$^{\alpha}CH_2$, N-CH_3, and β-CH_2, with relative intensities in the ratio 2:6:2. For Tet^{2+}, three ^{13}C peaks corresponding to N-$^{\alpha}CH_2$, N-CH_3, and β-CH_2, with intensity ratio 1:3:1, were observed. For Pen^{2+}, four ^{13}C peaks corresponding to N-$^{\alpha}CH_2$, N-CH_3, γ-CH_2, and β-CH_2 were observed with intensity ratio 2:6:1:2. The N-$^{\alpha}CH_2$ and the N-CH_3 ^{13}C resonances of all the methonium ions exhibit characteristic (partially resolved) triplets due to coupling with the (relatively small) nuclear dipole of ^{14}N.

Sodium NMR Experiments. All measurements of ^{23}Na NMR relaxation rates reported here were carried out on a Bruker AM-360 spectrometer at a field corresponding to a ^{23}Na Larmor frequency of 95.263 MHz. Solutions of NaDNA to be titrated (2.7–3.0 mL in a 10-mm NMR tube) contained 10% D_2O to provide a lock signal. Titrations were carried out by adding to the DNA sample microliter quantities of concentrated stock solutions containing the chloride (or bromide) salt of the divalent cation. After each addition, the sample was thermally equilibrated before NMR measurements were performed. The temperature of all samples (297 ± 1.0 K) was controlled with air precooled by an ice water bath. Earlier work (Padmanabhan et al., 1988) indicated that the addition of Hex^{2+} to a solution containing DNA at a concentration comparable to those investigated here does not cause detectable aggregation, precipitation, or denaturation. The inability of

[1] Abbreviations: Pro^{2+}, 1,3-diaminopropane $[H_3N^+-(CH_2)_3-{}^+NH_3]$; Put^{2+}, putrescine $[H_3N^+-(CH_2)_4-{}^+NH_3]$; Cad^{2+}, cadaverine $[H_3N^+-(CH_2)_5-{}^+NH_3]$; Dhx^{2+}, 1,6-diaminohexane $[H_3N^+-(CH_2)_6-{}^+NH_3]$; Tri^{2+}, trimethonium $[(H_3C)_3N^+-(CH_2)_3-{}^+N(CH_3)_3]$; Tet^{2+}, tetramethonium $[(H_3C)_3N^+-(CH_2)_4-{}^+N(CH_3)_3]$; Pen^{2+}, pentamethonium $[(H_3C)_3N^+-(CH_2)_5-{}^+N(CH_3)_3]$; Hex^{2+}, hexamethonium $[(H_3C)_3N^+-(CH_2)_6-{}^+N(CH_3)_3]$; Tris, tris(hydroxymethyl)aminomethane ($C_4H_{11}NO_3$); bp, base pairs.

divalent cations to cause aggregation of DNA (of the type called "condensation") at the concentrations examined in this study has been independently documented (Wilson & Bloomfield, 1979).

Longitudinal ^{23}Na NMR relaxation rates (R_1^{Na}) were obtained by using the standard (180–τ–90) inversion–recovery method. Under all conditions of interest here for solutions either with or without DNA, the decay of the longitudinal magnetization by the quadrupolar relaxation process is well described by a single exponential. At the field strength applied here, the departures from "extreme narrowing" are not large enough to permit separate detection of the fast and slow components of the longitudinal relaxation process of ^{23}Na (Nordenskiöld et al., 1984; Delville et al., 1986; van Dijk et al., 1987).

At the field strength used in this study (where the sodium Larmor frequency ω_{Na} = 95.263 MHz), the transverse NMR relaxation of ^{23}Na in solutions containing DNA exhibits significant departures from the single-exponential decay characteristic of the limit of extreme narrowing (but remains well within the "Redfield" or "motional narrowing" limit). Consequently, the FT NMR spectrum of ^{23}Na is a nearly symmetric superposition of two Lorentzian peaks, whose integrated intensities are in the ratio 2:3 and whose line widths are proportional, respectively, to the "slow" and "fast" transverse relaxation rate constants, R_{2s}^{Na} and R_{2f}^{Na}, characteristic of the NMR relaxation of a spin $^3/_2$ nucleus by the quadrupolar mechanism (Hubbard, 1970). The absence of any detectable dynamic shift between these peaks under the conditions of our experiments is in accord with recent ^{23}Na NMR measurements in an ion-exchange resin (Tromp et al., 1990).

To evaluate R_{2s}^{Na} and R_{2f}^{Na} (after application of the correction described below) each ^{23}Na NMR line shape was deconvoluted into two Lorentzian components. For this purpose, the line widths measured at five different peak heights (one-eighth, one-sixth, one-fourth, one-half, and three-fourths) were used as input into equations analogous to those introduced by Delville et al. (1979). These equations were then solved for the relaxation rates of the fast and slow components by applying a nonlinear least-squares algorithm of the Marquardt type (Bevington, 1969; Padmanabhan et al., 1988). The accuracy of our deconvolution procedure has been checked by comparisons with computer generated bi-Lorentzian spectra (B. Richey, unpublished results).

In solutions containing only NaCl at ~20 °C, the motions modulating the quadrupolar relaxation of ^{23}Na are sufficiently rapid so that the condition of extreme narrowing is fulfilled at the field strength applied here. The FT NMR line shape therefore should be a single Lorentzian with a line width of half-height, $\Delta\nu_{1/2}$, proportional to the ^{23}Na transverse NMR relaxation rate (R_2^{Na}), which also is equal to the corresponding ^{23}Na longitudinal relaxation rate $R_2^{Na} = R_1^{Na} = 18.5 \pm 1.0\ s^{-1}$ (Eisenstadt & Friedman, 1967). However, under the conditions of the present study, it was not possible to eliminate all of the field inhomogeneity by the standard procedures of shimming and sample spinning. Consequently, even in the absence of DNA, the ^{23}Na NMR line shape exhibited some (generally) symmetric departures from the true Lorentzian form. Therefore, before deconvoluting each non-Lorentzian ^{23}Na FT NMR spectrum for a solution containing DNA and a given amount of M^{2+}, the observed ^{23}Na line widths at the five selected peak heights were corrected by the following procedure.

For a sample containing only 0.1 M NaCl in 10% D_2O, we measured the longitudinal relaxation rate (R_1^{Na}) and the five ^{23}Na NMR line widths at the fractional peak heights chosen for the purpose of the deconvolution analysis. If there were *no* broadening due to residual magnetic field inhomogeneity, each FT NMR spectrum would be a single Lorentzian peak whose line width at half-height would be equal to the longitudinal relaxation rate actually measured ($\Delta\nu_{1/2} = R_1^{Na}/\pi$). From this theoretical value of $\Delta\nu_{1/2}$, the theoretical line widths at each of the other four peak heights were calculated by standard expressions pertaining to the mathematical form of the Lorentzian line shape. Then we determined the differences between these Lorentzian line widths and the corresponding line widths actually measured at the five peak heights selected for the purpose of deconvolution. These differences, pertaining to ^{23}Na spectra for samples containing no DNA, were used to correct the corresponding five line widths for each ^{23}Na spectrum acquired during each titration of NaDNA. In no case did the correction exceed 10% of the line width.

Theoretical Background

Two-State Model of Cation NMR Relaxation Rates. In a solution containing NaDNA and some added salt(s), each observable ^{23}Na NMR relaxation rate R_{qo}^{Na} can be expressed, according to the two-state model (Anderson et al., 1978), as

$$R_{qo}^{Na} = p_F^{Na} R_F^{Na} + p_B^{Na} R_{qB}^{Na} \quad (1)$$

Here q (= 1, 2s, or 2f) specifies one of the three quadrupolar NMR relaxation rates of ^{23}Na defined in the preceding section. For each case, R_{qB}^{Na} designates the (average) relaxation rate characterizing the fraction of Na^+ ions (p_B^{Na}) that are near enough to DNA to be considered bound, in the sense that their quadrupolar coupling constants and/or correlation times are significantly enhanced by short-range interactions with the polyion (Anderson & Record, 1990; Reddy et al., 1987). As a result of the long-range Coulombic attraction between small mobile cations and the phosphate charges on the nucleic acid backbone, "bound" nuclei are radially localized in the volume surrounding DNA but are not necessarily fixed at certain sites. The relaxation rate R_F^{Na}, characterizing the fraction of "free" sodium ions (p_F^{Na}), is assumed to equal the relaxation rate observed for a comparable concentration of ^{23}Na nuclei in the absence of DNA (Anderson et al., 1978; Bleam et al., 1980). The applicability of eq 1 is predicated on the assumption that the exchange lifetime of ^{23}Na in the bound state is short compared to R_{qB}^{Na}. The validity of this fast exchange condition for sodium in DNA solutions has been verified by an analysis of the temperature dependence of the ^{23}Na NMR line widths under conditions comparable to those investigated here (Bleam et al., 1983).

In a DNA solution where Na^+ is the only type of counterion, the fraction of sodium nuclei that are bound to DNA (by the NMR criterion) can be expressed in terms of r_{Na}^o, the fraction of DNA phosphates that are, in effect, neutralized by the presence of nearby sodium ions:

$$p_B^{Na} \equiv r_{Na}^o\ [P]/[Na^+] \quad (2)$$

where $[Na^+]$ and [P] are the total concentrations of Na^+ and DNA phosphates, respectively. From eq 2, it should be clear that r_{Na}^o can also be interpreted as the number of sodium ions bound per DNA phosphate. Since there is no evidence that the association of sodium with DNA can cause this polyion to undergo charge reversal, r_{Na}^o cannot exceed unity. The superscript "o" emphasizes that this extent of counterion association pertains to a solution that contains no other types of cations capable of displacing Na^+ from DNA. For such a solution, various ^{23}Na NMR studies (Anderson et al., 1978;

Bleam et al., 1980; Padmanabhan et al., 1988) indicate that r^{o}_{Na} does not vary with the amount of NaCl during a titration of NaDNA.

In DNA solutions containing more than one type of cation, the local concentration of each near DNA varies with the overall composition of the system. This variation can be monitored directly by measuring NMR relaxation rate(s) characteristic of either (or both) cation(s). To analyze such measurements quantitatively, ion exchange in the vicinity of DNA can be described in terms of D_M and n. The relative distribution of M^{2+} and Na^+ between the bound and free states is characterized by an ion-exchange parameter D_M, defined by analogy to the D parameter introduced by us to describe the displacement of univalent cations by Na^+ (Anderson et al., 1978).

$$D_M \equiv (p_B^M)(p_F^{Na})^n/(p_F^M)(p_B^{Na})^n \quad (3)$$

Here p_B^M is the fraction of M^{2+} ions that are associated with DNA, in the sense that they have displaced sodium nuclei whose NMR relaxation rates are influenced by the presence of DNA; $p_F^M = 1 - p_B^M$. The parameter n in eq 3 is equated to the cumulative displacement of Na^+ by M^{2+} at any point in the titration:

$$n \equiv (r^{o}_{Na}[P] - p_B^{Na}[Na^+])/p_B^M[M^{2+}] \quad (4)$$

If $n < 2$, the total amount of cationic charge near DNA increases during a titration of NaDNA with M^{2+}. Nevertheless, in eq 4 r^{o}_{Na} is constant by definition. Both D_M and n are *assumed* to be invariant during a titration of NaDNA with M^{2+}.

The average NMR relaxation rates of rapidly exchanging sodium nuclei depend directly on local ion distributions, rather than thermodynamic activities. Therefore, measurements of R_{qo}^{Na} as the composition of the solution is varied during a titration yield microscopic, rather than macroscopic, information about ionic interactions with DNA. For two different divalent cations, M and M′, the ratio $D_M/D_{M'}$ provides a measure of their relative affinities for DNA, as reflected by their capacity to displace Na^+ from its vicinity. Since D_M is not a thermodynamic equilibrium constant, there is no fundamental guarantee that it cannot vary as NaDNA is titrated with M^{2+}. However, theoretical calculations based on the Poisson–Boltzmann equation, as well as Monte Carlo simulations, predict at most a slight variation in D_M (Paulsen et al., 1988). The same calculations also predict that n, as defined in eq 4, does change significantly during the titration of NaDNA with M^{2+}. However, this prediction does not appear to be borne out by comparisons of ^{23}Na and ^{14}N NMR rates measured during titrations of NaDNA with various methonium ions (Padmanabhan, 1988).

In a titration of NaDNA with M^{z+}, the effect of ion exchange on any of the three ^{23}Na NMR relaxation rates can be expressed by combining eqs 1, 2, and 4:

$$R_{qo}^{Na} = R_F^{Na} + (r^{o}_{Na} - np_B^M[M^{z+}]/[P])(R_{qB}^{Na} - R_F^{Na})[P]/[Na^+] \quad (5)$$

where the fraction of bound divalent cations p_B^M is determined uniquely by the parameters D_M and n, as specified in eqs 3 and 4 (Anderson et al., 1978; Bleam et al., 1983). Equation 5 is useful as a basis for quantifying D_M and n only if, in addition to these parameters, R_{qB}^{Na} itself does not vary with the extent of titration. [This requirement may not be fulfilled under certain conditions that have been discussed in detail previously (Bleam et al., 1983).]

Results

In each of the four panels of Figures 1 and 2, the titration curve for one of the polyamines is compared with the corresponding curve for the methonium ion having the same number of methylene groups. The first three panels in each figure show the dependence on $[M^{2+}]/[P]$ of one of the relaxation rates R_{qo}^{Na} (q = 2s in Figure 1 and q = 1 in Figure 2). The corresponding concentration dependences of R_{2f}^{Na} are not shown, for reasons explained in the Appendix. Data shown in panels A–C of each figure were obtained during titrations of samples taken from the same stock solution of NaDNA (characterized under Materials and Methods). The same stock solution also was used for the titration with Dhx^{2+}, but a different stock solution was used for the titration of NaDNA with Hex^{2+}. [The R_1^{Na} data for this titration are analyzed here for the first time; an analysis of the R_{2s}^{Na} data was published previously (Padmanabhan et al., 1988). It should be noted that the values of D reported in this earlier paper are, within uncertainties in the fittings, the reciprocals of the values of D_M reported here, which were calculated according to a different definition of the ion-exchange parameter (eq 3).] The concentration dependence of R_{qo}^{Na} observed during a titration of NaDNA with Dhx^{2+} can be compared meaningfully with the corresponding data for Hex^{2+} only if due allowance is made for differences in the ratio $[Na^+]/[P]$ in the two stock solutions. For this purpose, the excess relaxation rate determined at each point in a titration with Dhx^{2+} or Hex^{2+} is normalized as follows:

$$(R_{qo}^{Na} - R_F^{Na})[Na^+]/[P]r^{o}_{Na}(R_{qB}^{Na} - R_F^{Na}) = 1 - np_B^M[M^{2+}]/r^{o}_{Na}[P] \quad (6)$$

The values of the parameters appearing on the left side of this equation were either fixed (r^{o}_{Na} and R_F^{Na}) or fitted (R_{qB}^{Na}, as described in the Appendix) in order to calculate the ordinate for each data point in panel D of each figure.

The theoretical curves shown in Figures 1 and 2 were calculated by fitting the data to the equations characteristic of the two-state model (1–5) using the numerical procedure outlined in the Appendix. Before the results of these fittings are summarized, some general features of the titration curves can be explained in terms of the two-state model. According to eq 5 (or 6) the decrease in R_{qo}^{Na} with the addition of M^{2+} is due to the progressive displacement of sodium nuclei from an environment where their (average) relaxation rate is relatively high (near DNA) to an environment where their relaxation rate is relatively low (the bulk solution). So long as (virtually) very divalent cation introduced by titration becomes associated with DNA, p_B^M remains negligibly different from unity, and the plot of R_{qo}^{Na} vs $[M^{2+}]/[P]$ is linear, with slope $-n(R_{qB}^{Na} - R_F^{Na})[P]/[Na^+]$ and intercept (at $[M^{2+}] = 0$) of $R_F^{Na} + r^{o}_{Na}(R_{qB}^{Na} - R_F^{Na})[P]/[Na^+]$. For all of the titration curves shown in Figures 1 and 2, the initial linearity persists over the range $0 \leq [M^{2+}]/[P] \lesssim 0.2$.

Since R_{qB}^{Na} for sodium nuclei associated with DNA in solution cannot be measured independently (or predicted theoretically with sufficient reliability), the linear portion of a titration curve cannot provide separate estimates of n and r^{o}_{Na} but does permit estimation of their ratio (as indicated by eq 6). In previous ^{23}Na studies of the interactions of multivalent cations with DNA, values of r^{o}_{Na}/n have been reported for polyamines and for various inorganic cations (Bleam et al., 1983; Braunlin et al., 1986). As explained in the Appendix, knowledge of the initial slopes of the titration curves shown in Figures 1 and 2 does prove useful in establishing the ranges of values of D_M that are consistent with our NMR data, as analyzed in terms of the two-state model.

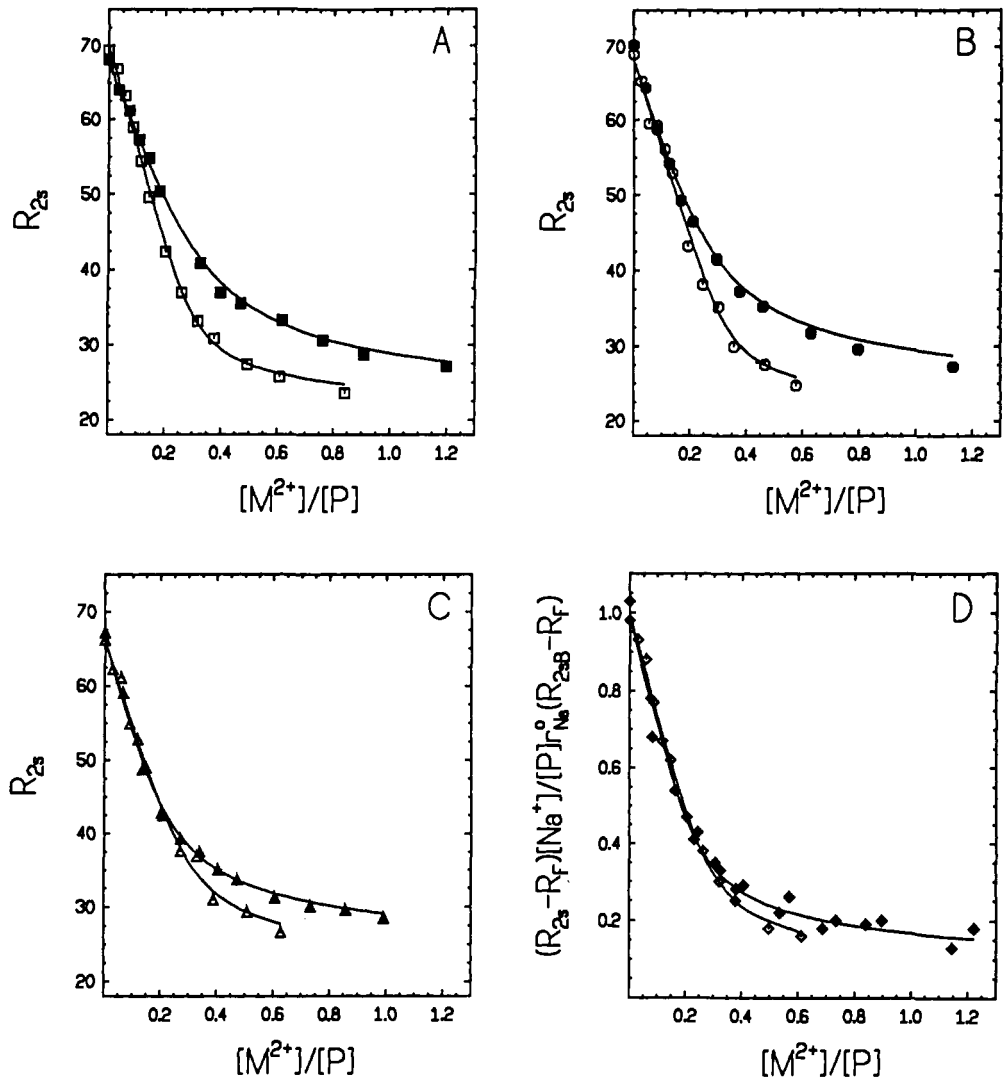

FIGURE 1: Dependence on $[M^{2+}]/[P]$ of the ^{23}Na slow transverse relaxation rate R_{2s}^{Na} (panels A–C) and of the normalized excess rate (panel D) for titrations of NaDNA with divalent polyamines (open symbols) or methonium cations (closed symbols): (A) Pro^{2+} (□) and Tri^{2+} (■); (B) Put^{2+} (○) and Tet^{2+} (●); (C) Cad^{2+} (△) and Pen^{2+} (▲); (D) Dhx^{2+} (◇) and Hex^{2+} (◆). For the titration with Hex^{2+}, [P] = 13.4 mM and $[Na^+]$ = 17.8 mM; in all other cases [P] = 12.4 mM and $[Na^+]$ = 21.3 mM. Each solid line represents the best theoretical fitting of R_{2s}^{Na} and R_1^{Na} to the two-state model (eqs 1–5) at r^o_{Na} = 0.85. (For each curve, the best-fitted values of D_M and of n are given in Table I and Table AIII, respectively.)

Table I: Best-Fitted Values of D_M ($\times 10^{-2}$) for Various Fixed Values of r^o_{Na}

	polyamines[a]				methonium ions[a]			
r^o_{Na}	Pro (3)[a]	Put (4)[b]	Cad (5)[c]	Dhx (6)[d]	Tri (3)[a]	Tet (4)[a]	Pent (5)[a]	Hex (6)[c]
1.00	6.4	5.6	1.9	3.2	0.36	0.42	0.67	0.76
0.85	4.4	3.8	1.5	2.5	0.32	0.39	0.65	0.74
0.77	3.4	3.0	1.3	2.0	0.30	0.38	0.62	0.71
0.60	1.9	1.7	0.83	1.3	0.23	0.33	0.52	0.60

[a] The values in these columns have computed relative uncertainties of (a) 8–10%, (b) 20%, and (c) 12–15%.

After the addition of a sufficient amount of the titrating cation ($[M^{2+}]/[P] \gtrsim 0.3$), the titration curves in Figures 1 and 2 begin to exhibit monotonic upward departures from linearity that reflect the decrease of p_B^M from its maximal value of unity, according to eq 5 or 6. The relative affinities of two different divalent cations for DNA can be judged qualitatively by comparing the ^{23}Na relaxation rates determined at the same point ($[M^{2+}]/[P]$) in the titration (provided that both DNA solutions have the same ratio of $[Na^+]/[P]$ and that R_{qB}^{Na} does not change with the identity of the competing cation). The lower the value of R_{qo}^{Na}, the more effective is the divalent cation at displacing Na^+ from DNA. Comparisons of the titration curves represented in each panel of Figures 1 and 2 clearly show that each of the polyamines has a higher affinity for DNA (relative to Na^+) than does the methonium ion of the same m. The effectiveness with which methylation reduces affinity of a given polyamine for DNA is, however, significantly greater for the smaller (3 and 4) than for the larger (5 and 6) values of m.

To quantify the effect of methylation and the effect of varying m on the relative affinities of these diamines for DNA, values of D_M were determined by the fitting procedure outlined in the Appendix. To attain acceptable levels of uncertainty in the best-fitted values of D_M, r^o_{Na} was fixed (in addition to R_F^{Na}). As described in the Appendix, the range of choices of r^o_{Na} consistent with all the data acquired in the present study

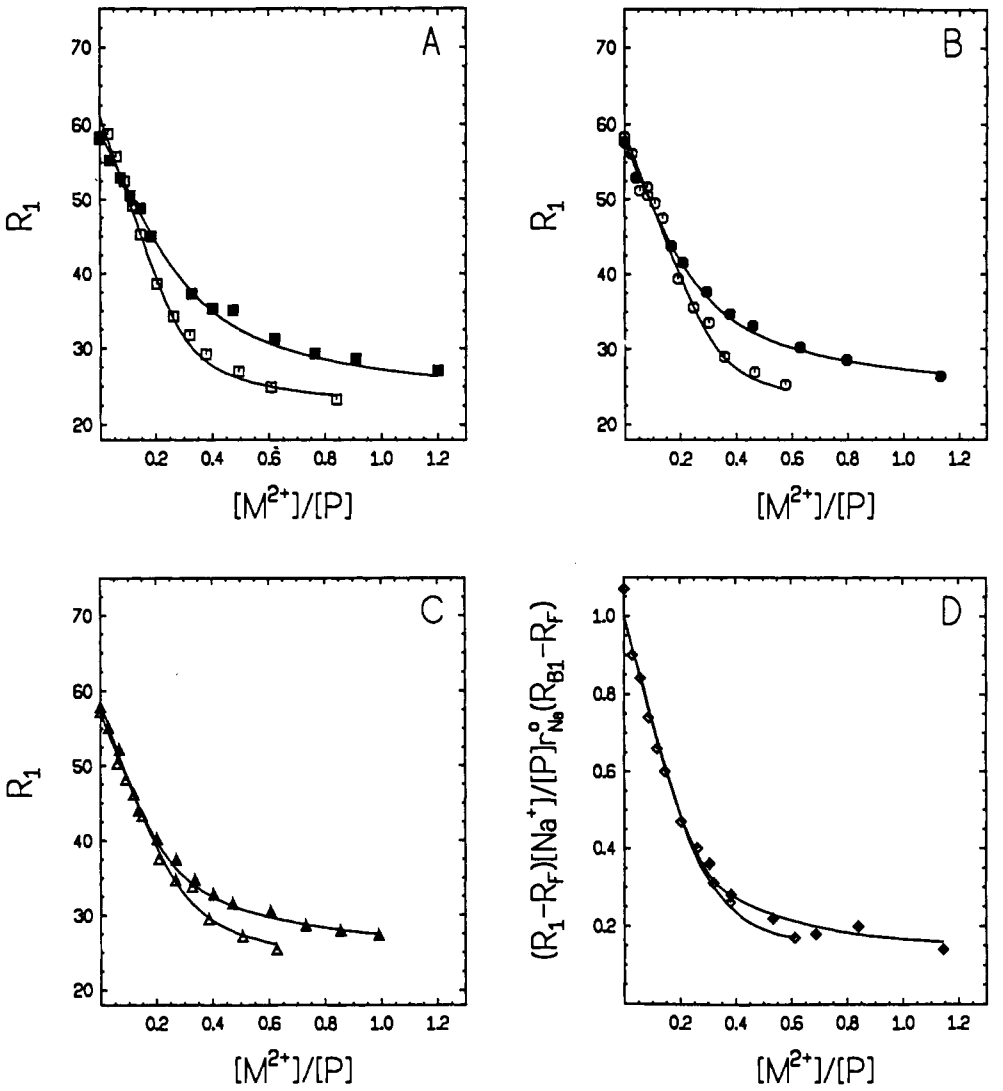

FIGURE 2: Dependence on $[M^{2+}]/[P]$ of the ^{23}Na longitudinal relaxation rate R_1^{Na} (panels A–C) and of the normalized excess rate (panel D) for titrations of NaDNA with divalent polyamines (open symbols) or methonium cations (closed symbols). The NaDNA concentrations and the figure symbols are the same as in Figure 1. Each solid line represents the best theoretical fitting of R_{2s}^{Na} and R_1^{Na} with eqs 1–5 at r^o_{Na} = 0.85. (For each curve, the best-fitted values of D_M and of n are given in Table I and Table AIII, respectively.)

was determined to be 0.6–1.00. For several values of r^o_{Na} in this range, the best-fitted values of D_M are given in Table I. (The corresponding best-fitted values of the other parameters specified by the two-state model are given in Tables AII and AIII.) The theoretical curves shown in Figures 1 and 2 were calculated for r^o_{Na} = 0.85. For any choice of r^o_{Na} in the range 0.6–1.00, the calculated titration curves provide fittings of the NMR data that are of similar quality (as reflected by the SDF statistic, explained in the Appendix).

The best-fitted values of D_M in Table I provide a quantitative basis for comparing the affinities for DNA of each of the diamines (relative to Na^+). Methylation of the terminal ammonium groups reduces D_M by a factor ranging from at least 8 (for m = 3) to as little as 1.5 (for m = 5). For each series of diamines there are some smaller, but significant, correlations (outside of uncertainties in the fittings) between the magnitude of D_M and the number of methylene groups. For the shorter polyamines (m = 3 and 4) the values of D_M, probably not distinguishable from each other, are approximately twice as large as D_M for Cad^{2+} (m = 5). The relative affinity of Dhx^{2+} appears to be intermediate between that of Cad^{2+} and those of Pro^{2+} and Put^{2+}. The opposite trend is manifested by the methonium ions. For m = 3 and 4, the values of D_M are approximately half the values determined for m = 5 and 6. The relative affinities of Tri^{2+} and Tet^{2+}, like those of Pen^{2+} and Hex^{2+}, cannot be distinguished outside of uncertainties. For these methonium ions, however, there is no indication of a nonmonotonic variation in D_M with m, as may be exhibited by the larger polyamines (m = 5 and 6). The results in Table I clearly indicate that methylation of the terminal amines is more significant than the number of intervening methylene groups as a determinant of the relative affinities for DNA of these divalent cations. The differential effect of methylation on the magnitude of D_M is most significant at the lower values of m. Some physical implications of these trends in D_M are considered under Discussion.

Discussion

Our earlier work (Padmanabhan et al., 1988) demonstrated the efficacy of quadrupolar NMR relaxation rate measurements, analyzed in terms of a simple two-state model, for the purpose of characterizing quantitatively the exchange of uni- and divalent cations (Na^+ and Hex^{2+}) associated with helical DNA. The present study does provide some additional evidence for the utility of the parameterization specified by eqs 1–5. A thorough quantitative application of this two-state model to analyze the competitive association of Na^+ and polyamines, or methonium ions, with DNA would have re-

quired a much more exhaustive set of measurements for each of these divalent cations. Instead, our objective here was to obtain sufficient data to discern any trends in the magnitude of D_M that could indicate how affinity for DNA is affected by methylation of the terminal ammonium groups and by varying m, the number of intervening methylene groups. These trends are clearly evident in Table I, irrespective of the value assigned to r^o_{Na}.

An analogous study using ^{23}Na NMR to determine the relative affinities of the alkali metal ions for DNA indicates that increasing hydrated ion size (decreasing charge density) is correlated (except for Li^+) with decreasing affinity for DNA (Bleam et al., 1980). Although K^+ and NH_4^+ are generally considered to be of comparable size in aqueous solution, the relative affinity of the latter for DNA (as determined by ^{23}Na NMR) is significantly greater, possibly as a result of hydrogen-bonding interactions. Replacing the ammonium protons with ethyl groups reduces the relative affinity of tetraethylammonium by approximately a factor of 10 (Anderson et al., 1978) in comparison to that of NH_4^+ (Bleam et al., 1980). Similarly, the values of D_M shown in our Table I for the methonium ions are 2- to 10-fold smaller than the values for the corresonding polyamines.

Methylation of the terminal amine groups increases the size of a given diamine, thereby lowering its effective charge density. This effect may account (at least qualitatively) for the observation that $D_H > D_{Me}$ for each pair of diamines in the series m = 3–6. Replacing amine protons with methyl groups also precludes the possibility of hydrogen bonding with phosphates (or other moieties) on DNA. However, various kinds of NMR measurements (Wemmer et al., 1985; Besley et al., 1990) give no indication of any hydrogen-bonding between polyamines and sites on nucleic acids in solution.

The relative amount by which methylation augments the size of a diamine diminishes as the number of methylene groups increases. Thus, the observed decrease in D_H/D_{Me} with increasing m is in accord with the hypothesis that the sizes of these cations are inversely correlated with their DNA affinities. The apparent increase in D_H for Dhx^{2+} in comparison to Cad^{2+}, though possibly not significant in comparison to statistical uncertainties, and the (relatively small) increase in D_{Me} with increasing m may indicate that these relative affinities are affected by factors other than cation size. However, the length of the methylene chain (in the all-trans conformation) may not be correlated simply with the *effective* size of a divalent amine in solution near a nucleic acid. As m increases, so does the nonpolar character of the cation, which may therefore show an increasing tendency to adopt more compact conformations in order to minimize surfacial contact with water. With increasing m, the Coulombic repulsions between terminal amine groups may become progressively less important, from the standpoint of free energy, in determining the average size of the cations, particularly if the amine charges are shielded by methyl groups.

Accurate theoretical predictions of the trends in D_M for the various polyamines and methonium ions investigated here probably will require considering the structural flexibility of these cations and possibly an explicit treatment of solvent interactions. Such calculations do not appear to be feasible at present. The order of magnitude of the values of D_M reported here is consistent with the results of some Poisson–Boltzmann and Monte Carlo simulations of the distributions of uni- and divalent hard spheres surrounding a hard cylinder of uniform charge representing DNA (Paulsen et al., 1988; Padmanabhan et al., 1990). Thus, there appears to be no need

Table AI: Best-Fitted Values of Parameters Computed by Fitting the Concentration Dependences of R_1^{Na} and R_{2s}^{Na}

polyamines	n	r^o_{Na}	D_M ($\times 10^{-2}$)
Pro (3)[a]	1.81 ± 0.32	0.64 ± 0.14	2.2 ± 1.2
Put (4)[a]	2.13 ± 0.90	0.86 ± 0.41	4.0 ± 4.6
Cad (5)[a]	1.34 ± 0.46	0.46 ± 0.23	0.52 ± 0.47
Dhx (6)[a]	1.41 ± 0.40	0.43 ± 0.18	0.66 ± 0.51
methonium ions	n	r^o_{Na}	D_M ($\times 10^{-2}$)
Tri (3)[a]	1.90 ± 0.26	0.92 ± 0.19	0.34 ± 0.06
Tet (4)[a]	1.47 ± 0.22	0.44 ± 0.15	0.27 ± 0.09
Pent (5)[a]	2.08 ± 0.26	0.68 ± 0.15	0.57 ± 0.11
Hex (6)[b]	1.86 ± 0.27	0.48 ± 0.14	0.49 ± 0.14

[a] Parameters obtained by nonlinear least-squares fitting of eqs 2–5 with four floated parameters and R_F^{Na} = 19.2 s^{-1}. [b] Parameters obtained by fitting previously published data (Padmanabhan et al., 1988).

to invoke non-Coulombic interactions in order to account for the magnitude of the ion-exchange parameters that characterize the affinity for DNA (relative to Na^+) of the divalent polyamines and methonium ions.

Various other types of experimental studies have been interpreted to imply that interactions of polyamines with DNA are primarily nonspecific. A detailed quantitative equilibrium dialysis study (Braunlin et al., 1982) showed that the apparent polyamine–DNA binding constant is temperature independent between 4–37 °C (within experimental uncertainty)—a characteristic of equilibria governed by Coulombic forces. An earlier equilibrium dialysis study indicated that there is no DNA base composition dependence of the interactions of the tetravalent polyamine spermine with DNA (Hirschmann et al., 1967). These results provide indirect evidence that the interactions of polyamines with DNA in solution do not entail site binding.

Although the association of divalent polyamines and DNA appears to be dominated by Coulombic attraction, differences in the structural characteristics of a series of polyamines having the same charge cause detectably different effects on equilibria involving nucleic acids. A number of sensitive experimental approaches have been used to determine the consequences of varying the number of methylene groups between the amine charges and of varying the number and chain-length of alkyl substituents on the amines [cf. Bloomfield et al. (1974) and references therein]. In particular, there have been extensive studies of the effects of divalent polyamines on the stability of double helical DNA (Tabor, 1962; Mahler & Mehrotra, 1963; Thomas & Bloomfield, 1984) and on the stability of double- and triple-stranded RNA (Gabbay, 1966; 1967). The latter study reports the increases in melting temperature of helical RNA caused by the presence of various polyamines (with m = 2–5) and methonium ions (with m = 3–6). These results indicate that, for a given m, the affinity of the polyamine for RNA is greater than that of the corresponding methonium ion and that in both series the cation having m = 3 exhibits the greatest affinity for helical DNA. When Pro^{2+} (or Tri^{2+}) is in the maximally extended (all-trans) conformation, the distance between the terminal ammonium charges is ~7 Å, which is nearly identical with the separation between adjacent phosphate groups along a given nucleic acid chain (in either DNA or RNA). On the basis of this structural consideration, Gabbay rationalized his observation that the maximum increment in RNA melting temperature occurs in the presence of Pro^{2+}. However, in an analogous earlier study Cad^{2+} was found to have the largest effect on the melting temperature of DNA (Mahler & Mehrotra, 1963). No structural explanation appears to have been proposed for the finding that divalent polyamines of different m appear to

Table AII: Best-Fitted Values of n for Various Fixed Values of r^{o}_{Na}

	polyamines[a]				methonium ions[a]			
r^{o}_{Na}	Pro (3)	Put (4)	Cad (5)	Dhx (6)	Tri (3)	Tet (4)	Pent (5)	Hex (6)
1.00	2.61	2.44	2.43	2.64	2.00	2.25	2.61	2.71
0.85	2.27	2.11	2.13	2.31	1.80	2.03	2.37	2.48
0.77	2.09	1.93	1.97	2.13	1.70	1.92	2.23	2.35
0.60	1.71	1.56	1.63	1.77	1.47	1.69	1.95	2.07

[a] The values in these columns have computed relative uncertainties ranging from 2 to 4%.

Table AIII: Comparison of Values of $n(R^{Na}_{qB} - R^{Na}_{F})[P]/[Na]$ Calculated by Alternative Data Analyses

	polyamines				methonium ions			
	Pro (3)	Put (4)	Cad (5)	Dhx (6)	Tri (3)	Tet (4)	Pent (5)	Hex (6)
slope[a]	139 ± 7	125 ± 9	115 ± 5	125 ± 6	95 ± 3	124 ± 4	124 ± 8	128 ± 9
$r^{o}_{Na} = 1.00$[b]	135 ± 4	123 ± 5	113 ± 6	124 ± 4	99 ± 3	119 ± 4	132 ± 5	137 ± 7
$r^{o}_{Na} = 0.85$[b]	138 ± 3	125 ± 5	117 ± 6	127 ± 4	105 ± 2	126 ± 4	140 ± 5	145 ± 7
$r^{o}_{Na} = 0.77$[b]	140 ± 4	127 ± 6	120 ± 6	130 ± 4	109 ± 3	131 ± 4	146 ± 5	151 ± 7
$r^{o}_{Na} = 0.60$[b]	148 ± 4	131 ± 3	127 ± 7	137 ± 5	121 ± 4	147 ± 3	164 ± 5	169 ± 8
slope[a]	96 ± 12	91 ± 9	96 ± 4	114 ± 8	70 ± 3	80 ± 6	88 ± 8	
$r^{o}_{Na} = 1.00$[b]	103 ± 4	92 ± 5	92 ± 5	117 ± 7	75 ± 3	82 ± 2	93 ± 4	
$r^{o}_{Na} = 0.85$[b]	106 ± 5	94 ± 5	96 ± 5	122 ± 7	81 ± 3	87 ± 2	100 ± 4	
$r^{o}_{Na} = 0.77$[b]	108 ± 5	95 ± 5	97 ± 5	125 ± 7	84 ± 3	92 ± 3	104 ± 4	
$r^{o}_{Na} = 0.60$[b]	113 ± 6	100 ● 6	105 ± 6	134 ± 7	95 ± 5	104 ± 3	118 ± 5	

[a] Calculated as the magnitude of the best-fitted slope of the initial linear portion of a plot of R^{Na}_{2s} (upper) or R^{Na}_{1} (lower) vs $[M^{2+}]/[P]$. [b] Calculated with the best-fitted values of n and R^{Na}_{qB} obtained from nonlinear least-squares fitting of *entire* plots of R^{Na}_{2s} (upper) or R^{Na}_{1} (lower) vs $[M^{2+}]/[P]$.

exhibit maximal affinity for DNA and RNA. An unambiguous interpretation of differential polyamine effects on the melting temperature of a nucleic acid is difficult to achieve, because these measurements reflect only *overall differences* in polyamine interactions with the native and denatured conformations of the nucleic acid.

A complete description of the thermodynamic effects of polyamine interactions with DNA on its conformational equilibria would require some method, such as equilibrium dialysis, capable of determining the thermodynamic extents of association (Braunlin et al., 1982; Record et al., 1990) of polyamines with each conformation of DNA. These thermodynamic extents of association are determined by the properties of the entire radial distribution functions of both the cations and the anions and hence cannot be directly compared with the results of spectroscopic measurements (such as cation NMR relaxation rates) that are sensitive only to the features of the cation radial distribution adjacent to DNA. Such information nevertheless can advance the development of comprehensive theories of cation–polyion interactions (Anderson & Record, 1990), and NMR measurements of various kinds also can provide useful qualitative indications concerning the nature of the bound state.

Measurements of ^{1}H nuclear Overhauser enhancements (NOE) of protons on spermine in solutions containing a short-chain nucleic acid of specified sequence (Wemmer et al., 1985) indicate that when this tetravalent polyamine is near an oligomer, there is no significant slowing of the motions that affect the NMR spectral densities pertaining to the NOE of protons on spermine. Similar inferences for putrescine and other polyamines of higher charge type in solutions of DNA are drawn from ^{1}H NMR measurements by Besley et al. (1990). Our ^{14}N NMR study of Hex^{2+} (Padmanabhan et al., 1988) and analogous results (to be presented elsewhere) for the methonium ions with $m = 3$–5 demonstrate that the retardation in the motions that govern the relaxation of ^{14}N in the near vicinity of DNA is comparable to the effect on Na^{+}. These results, as well as some stochastic dynamics simulations of small ion motions in DNA solution (Reddy et al., 1987), are consistent with the inference that the Coulombic interactions responsible for the high local concentrations of small ions close to DNA do not cause any significant extent of immobilization at specific sites.

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Appendix

Use of a Nonlinear Least-Squares Fitting Routine To Determine the Parameters of the Two-State Model. For each titration of NaDNA with one of the polyamines or methonium ions investigated here, the dependence of each of the three ^{23}Na relaxation rates (R^{Na}_{1}, R^{Na}_{2s}, and R^{Na}_{2f}) on $[M^{2+}]/[P]$ was analyzed in terms of eqs 1–5. Since n is an exponent in eq 3, R^{Na}_{qo} cannot be expressed as an explicit function of $[M^{2+}]/[P]$ and the other parameters of the two-state model *unless* n has an integral value (2 or 1). Simultaneous solution of eqs 3 and 5 to determine the best-fitted values of the parameters was accomplished by an iterative application of a nonlinear least-squares fitting routine. The "nonlinear" least-squares program used in fitting our data is based on one of the standard adaptations of the Marquardt algorithm (Bevington, 1969). Input for this program consists of one or more sets of relaxation rates (R^{Na}_{1}, R^{Na}_{2s}, R^{Na}_{2f}). The value assigned to R^{Na}_{F} is 19.2 s^{-1}, determined as the extrapolated intercept of a plot of R^{Na}_{qo} vs $[P]/[Na^{+}]$ for a titration of NaDNA with NaCl. As output the program yields the best-fitted values of the parameters characteristic of the two-state model and their symmetrized standard deviations. This program also computes an overall statistic called the standard deviation of the fitting (SDF), whose magnitude reflects both the scatter in a particular set of data and the overall accuracy of the fitting (Bevington, 1969).

At the field strength (and temperature) where our titrations were performed, the departure from extreme narrowing of the quadrupolar relaxation process causes the three observable relaxation rates to differ in the order $R^{Na}_{2f} > R^{Na}_{2s} > R^{Na}_{1}$. However, as long as eq 1 is valid and each of the R^{Na}_{qB} is constant, each of the three observable relaxation rates must exhibit the same concentration dependence. In principle, therefore, they should provide equivalent information for the

purpose of determining the ion-exchange parameters of interest here. In fact, however, differences in the magnitudes of the R_{qB}^{Na} and in the accuracy with which they can be determined from the raw measurements can affect significantly the uncertainties in the best-fitted values of the parameters of interest.

Since R_{2f}^{Na} is the largest of the three relaxation rates, it provides the widest window for analyzing the approach of R_{2f}^{Na} to R_F^{Na} as $[M^{2+}]$ is added to the solution. However, for each type of divalent cation, the titration curve representing the dependence of R_{2f}^{Na} of $[M^{2+}]$ exhibits considerably more scatter than do the curves that show the concentration dependence of R_{2s}^{Na} and R_1^{Na}. The accuracy with which R_{2f}^{Na} can be evaluated by the deconvolution procedure may be particularly sensitive to uncertainties in the line widths closest to the baseline, where random noise fluctuations are most pronounced. These uncertainties may be compounded by the corrections for inhomogeneity (as described under Materials and Methods) that tend to be larger for line widths close to the baseline. In every case the value of SDF calculated by fitting the R_{2f}^{Na} data is substantially higher (by at least a factor of 2) than the SDF for either of the corresponding sets of R_1^{Na} or R_{2s}^{Na} data. Consequently, only the latter sets of data were used to determine best-fitted values of the parameters of interest. Any uncertainties arising from the corrections for inhomogeneity are thereby minimized, since these corrections have little effect on the determination of values of R_{2s}^{Na} by deconvolution.

After performing nonlinear least-squares fittings of the individual sets of R_1^{Na} and R_{2s}^{Na} for each divalent cation, a "composite" fitting was obtained by analyzing simultaneously both sets of data. In these composite fittings each value of a given type of relaxation rate (R_1^{Na}, R_{2s}^{Na}) is assigned the same weighting factor, equal to the SDF determined by fitting only relaxation rates of the same type. (In no case did the weighting factors for the two types of relaxation rates differ by more than 50%, and generally they were within 20%.) For each type of divalent competitor, Table AI collects the results of fitting composite sets of R_{2s}^{Na} and R_1^{Na} data with all parameters floated. Clearly, the uncertainties in these best-fitted values are unacceptably high, so additional information must be built into the analysis in order to obtain useful estimates of D_M.

According to the definition of r_{Na}^{o} (see eq 2), this parameter must have the same value in all DNA samples taken from the same stock solution of DNA. Overlapping uncertainties of the best-fitted values of r_{Na}^{o} shown in Table A1 indicate that the unique value of this parameter lies somewhere between 0.6 and 1.00. At four representative values in this range (0.60, 0.77, 0.85, and 1.00) r_{Na}^{o} was fixed and the remaining parameters were floated in order to generate titration curves for each of the polyanions and methonium ions. Best-fitted values and uncertainties for the parameters determined by these fittings are summarized in Table I (D_M), Table AII (n), and Table AIII [$n(R_{qB}^{Na} - R_F^{Na})$].

Since it is unlikely that n exceeds the "Coulombic limit" of 2, the entries in Table AII indicate that r_{Na}^{o} is closer to 0.60 than to 1.00. On the other hand, the comparisons between values of $n(R_{qB}^{Na} - R_F^{Na})$ shown in Table AIII indicate that r_{Na}^{o} is more like 1.00. (If n and the other parameters do not vary during the titration, then the slope of the initial linear region of the titration curve must agree with the value of $n(R_{qB}^{Na} - R_F^{Na})$ determined by fitting the entire curve.) To accommodate the indications of both Table AII and Table AIII, r_{Na}^{o} was fixed at the intermediate value of 0.85 in order to generate the titration curves represented in Figures 1 and 2. Alternative choices of r_{Na}^{o} in the range 0.6–1.0 have no significant effect on the quality of the fittings as judged by comparing the magnitudes of the SDF, but the results in Tables AII and AIII do suggest that r_{Na}^{o} most probably is near the middle of this range.

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In Vitro and in Vivo DNA Bonding by the CC-1065 Analogue U-73975

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ABSTRACT: CC-1065, a cyclopropylpyrroloindole (CPI), is a highly potent antitumor DNA-alkylating agent. We have devised a simple method to detect CPI bonding sites on double-stranded DNA (dsDNA). The technique utilizes a modified form of bacteriophage T7 polymerase, Sequenase, to synthesize a radiolabeled nascent strand from dsDNA that has been reacted in vitro with the CC-1065 analogue U-73975 (adozelesin). The reaction products were electrophoresed on sequencing gels containing 8 M urea and visualized by autoradiography. The transit of this DNA polymerase is inhibited at the sites where CPIs are bound to the template strand. Thus, the enzyme stalls or stops at the nucleotide immediately adjacent to the modified base, resulting in the accumulation of DNA strands at these sites and in diminished read-through beyond these sites in a set of CPI-treated DNA molecules. The precise positions of polymerase inhibition can be determined by comparison of CPI-treated and unreacted DNA reactions. This modified dideoxynucleotide sequencing technique has been used to establish the sequence selectivity of U-73975. Approximately 1 kilobase of dsDNA has been analyzed to derive a consensus canonical bonding sequence, 5′(T/A)-T/A-T-A*-(C/G)-(G), where A* is the site of U-73975 alkylation and parentheses denote deoxynucleotide preferences. Noncanonical sites were also found at poly(A) sites. This technique yielded a consensus sequence for U-73975 bonding that is similar to, but not identical with, the published consensus obtained for CC-1065 by a modified Maxam and Gilbert sequencing technique. We have also examined the bonding of [^{3}H]U-73975 to the DNA of viable cultured mammalian cells, using gel electrophoresis and autoradiographic techniques. [^{3}H]U-73975 does appear to be covalently bound to nuclear DNA in vivo (intracellular) and to a subset of human highly repetitive sequence elements. U-73975 is bound predominantly to nuclear DNA at sites other than telomeric repeat sequences, which contain the potential CPI bonding sequence $(TTAGGG)_n$.

The interaction of cyclopropylpyrroloindole (CPI)[1] compounds with DNA has been established (Reynolds et al., 1986). CC-1065 is an extremely potent cytotoxic CPI compound produced by *Streptomyces zelensis* (Reynolds et al., 1986). Structurally, this unique compound consists of three repeating pyrroloindole subunits, one of which contains a potentially reactive cyclopropane ring (Hurley et al., 1990). The chemistry of CC-1065 and its biological properties have been documented (Reynolds et al., 1986). The intracellular target for CC-1065 is double-stranded DNA (dsDNA), with little or no affinity for protein, RNA, or single-stranded DNA (Reynolds et al., 1986). This agent binds in the minor groove of duplex B-DNA, where the cyclopropyl moiety reacts with the N3 of adenine to form a covalent adduct (Reynolds et al., 1985, 1986). It is this irreversible covalent association with DNA that is believed to be responsible for its extraordinary potent antitumor activity (Hurley, 1990).

A modified Maxam and Gilbert sequencing technique has demonstrated CC-1065 alkylation of dsDNA to be highly sequence-specific (Reynolds et al., 1985). Initial analysis of CC-1065 binding/bonding sites revealed that 5′PuNTTA* and

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[1] Abbreviations: bp, base pair; cDNA, complementary DNA; CPI, cyclopropylpyrroloindole; DNA, deoxyribonucleic acid; dNTP, deoxynucleoside triphosphate; EDTA, ethylenediaminetetraacetic acid; EtBr, ethidium bromide; kb, kilobase (1000 bp); NaOAc, sodium acetate; NaOH, sodium hydroxide; PBS, phosphate-buffered saline; RNA, ribonucleic acid; SDS, sodium dodecyl sulfate; dsDNA, double-stranded deoxyribonucleic acid; DMA, *N,N*-dimethyacetamide; DMF, dimethylformamide.

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