Competitive Interactions of $Co(NH_3)_6^{3+}$ and Na^+ with Helical B-DNA Probed by ^{59}Co and ^{23}Na NMR†

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ABSTRACT: ^{59}Co NMR is demonstrated to provide a useful probe of the interactions of $Co(NH_3)_6^{3+}$ with helical B-DNA. The association of $Co(NH_3)_6^{3+}$ with B-DNA produces relatively modest effects on the relaxation rate and chemical shift of ^{59}Co, which indicate that the octahedral coordination shell remains intact and that no significant number of long-lived "outer-sphere" complexes are formed at specific sites on the DNA surface. Under conditions where essentially all of the cobalt complex is associated with DNA, the chemical shift of ^{59}Co appears to depend on its binding density. This effect could be due to magnetic heterogeneity in the environments of $Co(NH_3)_6^{3+}$ adjacent to DNA. The local exchange reaction between $Co(NH_3)_6^{3+}$ and Na^+ in the vicinity of DNA has been investigated by measuring ^{59}Co chemical shifts and ^{23}Na line widths concurrently. The number of sodium ions displaced by the association of one $Co(NH_3)_6^{3+}$ with DNA cannot be uniquely determined, but the data indicate that this number remains constant over at least the initial stage of a titration of NaDNA with NaCl. ^{59}Co chemical shifts have been analyzed to construct binding isotherms for the association of cobalt hexaammine with DNA over a range of salt (NaCl) concentrations. The magnitudes of the resulting binding constants and their salt dependence are similar to those previously reported for the association of structurally diverse trivalent ligands, such as spermidine and trilysine, with helical nucleic acids. Therefore, these association equilibria appear to be governed primarily by electrostatic interactions. Analysis of the salt dependence of the $Co(NH_3)_6^{3+}$ association equilibrium constant determined by ^{59}Co NMR indicates that approximately 2.5–2.7 Na^+ ions are displaced per $Co(NH_3)_6^{3+}$ associated. This thermodynamic stoichiometric coefficient is predicted to be 2.6 by a simple model for purely electrostatic competitive interactions of trivalent and univalent counterions with B-DNA.

Cobalt hexaammine is a chemically inert trivalent cation, which in its interactions with DNA may serve as an inorganic analogue of the naturally occurring trivalent cation spermidine. Both $Co(NH_3)_6^{3+}$ and spermidine are highly effective as inducers of intramolecular collapse of double-helical B-DNA (Widom & Baldwin, 1980, 1983), of the B–Z transition in alternating dG-dC polynucleotide helices (Behe & Felsenfeld, 1981), and of cruciform formation by inverted-repeat sequences in supercoiled DNA molecules (Sullivan & Lilley, 1987). For a quantitative interpretation of the effects of $Co(NH_3)_6^{3+}$ on the kinetics and equilibria of intramolecular collapse, of the B–Z conformational transition, or of cruciform extrusion, it is necessary to quantify the association of $Co(NH_3)_6^{3+}$ with the various conformations of the highly charged DNA polyanion in solutions containing added salt. In such systems, the association of the multivalent cation with the polyion is an ion-exchange reaction, at both the local molecular and the bulk thermodynamic levels (Record et al., 1976, 1978, 1985). Consequently, the extent of association of $Co(NH_3)_6^{3+}$ with DNA is a sensitive function of the concentration of the univalent electrolyte. This salt dependence is an expression of the polyelectrolyte effect, which provides an entropic driving force that increases the extent of association of $Co(NH_3)_6^{3+}$ with DNA as the bulk salt concentration is reduced (Record & Richey, 1987; Record & Mossing, 1987).

The effects of electrolyte ions on the association of charged ligands with polyelectrolytes result primarily from the long-range Coulombic interactions of electrolyte ions with polyions. Theoretical descriptions of these interactions and their molecular and thermodynamic consequences have been based on the counterion–condensation (CC) hypothesis (Manning, 1978) or on the Poisson–Boltzmann (PB) equation (Katchalsky, 1970). Various aspects of these different theoretical approaches, including their applicability to the interactions of ions with DNA, have been compared by Anderson and Record (1982). The key parameter in both theories is the axial charge density of the polyion, which is a known quantity for the B-DNA double helix. Both the CC hypothesis and the PB equation give theoretical predictions for r (the fractional extent of neutralization of polyion charge by closely associated counterions), for n (the number of univalent counterions displaced from the local vicinity of a polyion by the association of a Z-valent positively charged ligand), and for the thermodynamic counterparts of these two quantities, which can be calculated from the electrolyte–polyion preferential interaction coefficient (Anderson & Record 1982; Record et al., 1985; Mills et al., 1986).

To gain experimental information about r and n, a sensitive and highly localized probe of the interactions of small ions with a polyion is required. For this purpose the NMR relaxation rates of quadrupolar cations (in particular, $^{23}Na^+$) have been measured in a number of systems. In solutions of B-DNA, $^{23}Na^+$ NMR studies have supported the CC hypothesis insofar as r^0, the fraction of DNA phosphate charge neutralized by association of univalent cations in the absence of multivalent competitor ligands, remains constant over a wide range of solution conditions (Anderson et al., 1978; Bleam et al., 1980, 1983). Analogous results recently have been found for $^{39}K^+$ (Braunlin & Nordenskiöld, 1984) and for $^{87}Rb^+$ (Chang, 1983). For B-DNA in the presence of excess uni-univalent salt, the CC hypothesis predicts that in the limit of infinite dilution $r^0 = 0.76$ and $n = Z$ for a Z-valent competitor (Manning, 1978). These predictions somewhat overestimate

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the values of r and n inferred from ^{23}Na NMR. Titrations of NaDNA with $MgCl_2$ (Bleam et al., 1983) and with other multivalent cations (Braunlin et al., 1986) permit the evaluation of the quotient r^0/n from a two-state analysis of changes in the ^{23}Na NMR relaxation rates as the binding density of Mg^{2+} on DNA is increased. Plausible bounds on n for Mg^{2+} ($1 \leq n \leq 2$) imply that $0.2 \leq r^0 \leq 0.6$ (Bleam et al., 1983). Titrations with various multivalent cations, monitored by $^{23}Na^+$ line-width measurements, indicate that n may depend not simply on the valence but also on the chemical nature of the competing ion (Braunlin et al., 1986). Nevertheless, the CC hypothesis appears to provide a description of local ion-exchange reactions that is both qualitatively satisfactory and computationally much simpler than that based on the PB equation. Therefore, the NMR results presented in this paper are analyzed in terms of simple models that incorporate various consequences of the CC hypothesis (Anderson et al., 1978; Record et al., 1976, 1978).

To investigate the local ion exchange between Na^+ and $Co(NH_3)_6^{3+}$ in the vicinity of B-DNA, both ^{23}Na NMR measurements of the type reported previously (Braunlin et al., 1986) and ^{59}Co NMR measurements have been performed over a wide range of conditions. Of the multivalent ligands whose interactions with DNA are of biological interest, $Co(NH_3)_6^{3+}$ has particularly favorable properties from the standpoint of NMR spectroscopy. Like ^{23}Na, ^{59}Co has a high inherent NMR sensitivity, and its natural abundance is 100%. The general utility of ^{59}Co NMR line-width measurements in studying the interactions of small ions with proteins has been demonstrated (Raj et al., 1980). The chemical shift of the ^{59}Co nucleus is exquisitely sensitive to slight variations in its electronic environment (Kidd & Goodfellow, 1979; Laszlo, 1983), and the ^{59}Co relaxation mechanism in the diamagnetic hexaammine complex has been well characterized (Rose & Bryant, 1979). We have measured these ^{59}Co NMR properties as functions of temperature and concentration in order to investigate the nature and extent of the association of $Co(NH_3)_6^{3+}$ with B-DNA.

Experimental Procedures

Calf-thymus DNA was purchased from Worthington and prepared as previously described (Bleam et al., 1980) except that after the final dialysis the DNA was lyophilized and redissolved in 99.8% D_2O (Aldrich Chemical Co.) a total of 3 times. $Co(NH_3)_6Cl_3$ was purchased from Alpha Inorganics, recrystallized twice (Lindholm, 1978), and dissolved in 99.8% D_2O. The sample was dried under vacuum and redissolved in fresh D_2O a total of 3 times. (Since deuteriation can have no effect on the properties of cobalt hexaammine that are of interest in this paper, the conventional symbol for the fully protonated species will be retained.)

The DNA concentration was determined from the absorbance at 260 nm with an extinction coefficient of 6.98 mM^{-1} cm^{-1}. The sodium concentrations in stock DNA solutions were determined by neutron activation analysis at the Nuclear Reactor Laboratory of the University of Wisconsin. On the basis of duplicate determinations, the uncertainty in these measurements is estimated to be ±5%.

Titrations were carried out by adding microliter amounts of concentrated stock solutions of $Co(NH_3)_6Cl_3$ (~0.15 M) or NaCl (~3 M) to 1.5-mL volumes of NaDNA solutions in 8-mm NMR tubes. The small extent of precipitation that occurred upon adding $Co(NH_3)_6Cl_3$ was easily dissolved by vortexing the sample. Titrations of DNA with $Co(NH_3)_6Cl_3$ were continued up to the point where the precipitate could no longer be redissolved.

^{23}Na and ^{59}Co NMR measurements were carried out in ^2H_2O on a JEOL FX-200 spectrometer equipped with a tunable broad-band probe at resonance frequencies of 53.7 MHz for ^{23}Na and 47.7 MHz for ^{59}Co. The deuterium NMR signal provides a useful internal lock, which promotes the accuracy of the spectroscopic measurements made on ^{59}Co and ^{23}Na. In preparing samples for the spectrometer, protons were substituted by deuterium to the fullest extent feasible in order to minimize complications arising from the isotope shift effect on the ^{59}Co signal (Bendall & Dodrell, 1978). For all experiments the temperature was monitored by a thermocouple inserted into an 8-mm NMR tube placed in the probe. Unless otherwise stated, the sample temperature was maintained at 21.4 ± 0.2 °C. The chemical shift, δ, of the ^{59}Co resonance for a stock sample of 0.1 M $Co(NH_3)_6$ at 21.4 °C was set equal to zero; all chemical shifts reported here are given relative to this standard. Shifts in the downfield direction (decreased shielding) are considered to be positive. Unless otherwise stated, reported uncertainties in δ are 95% confidence limits obtained from application of the t-test for normally distributed experimental uncertainties.

Results and Discussion

(A) Characteristics of ^{59}Co NMR in Cobalt Hexaammine Solutions. (1) Line Width. For a standard solution containing only 0.1 M $Co(NH_3)_6Cl_3$ in D_2O at 21.4 °C, comparison of the ^{59}Co absorption spectrum with a computer simulation demonstrates that the line shape is Lorentzian, with a line width at half-height of 144 ± 7 Hz. The corresponding value of the ^{59}Co transverse relaxation rate is 452 ± 22 s^{-1}. This value is about 15% smaller than that reported previously for $Co(NH_3)_6Cl_3$ in H_2O (Rose & Bryant, 1979). The apparent discrepancy is probably due to the differences in the temperature and viscosity of the $Co(NH_3)_6^{3+}$ samples in the two studies. The relaxation mechanism of ^{59}Co in highly symmetric complexes gives rise to an unusual dependence of the ^{59}Co line width on temperature. Although ^{59}Co has a large electric quadrupole moment, the octahedral symmetry of the inert hexaammine complex produces a very small mean square electric field gradient at the central nucleus. Consequently, the quadrupolar mechanism is relatively inefficient, and the transverse relaxation process of ^{59}Co in $Co(NH_3)_6^{3+}$ is dominated by the scalar interaction of the second kind that results from ^{59}Co–^{14}N coupling (Rose & Bryant, 1979). This mechanism accounts for the observed increase in the ^{59}Co line width with increasing temperature in aqueous solutions. Under these conditions the relaxation of ^{59}Co is in the "extreme narrowing" limit. Therefore, the line shape is Lorentzian with a line width at half-height that is directly proportional to the correlation time characteristic of the molecular process that modulates the relaxation. The relevant correlation time for the scalar interaction is the longitudinal NMR relaxation time of ^{14}N. This relaxation time *increases* with increasing temperature, because it is inversely proportional to the correlation time that characterizes the diffusional motions of the cobalt complex. [The longitudinal relaxation of ^{59}Co in solutions of $Co(NH_3)_6^{3+}$ has been attributed alternatively to a spin rotation interaction (Jordan, 1980), which also could account for the unusual temperature dependence of the ^{59}Co line width.]

(2) Chemical Shift. In the standard solution of 0.1 M $Co(NH_3)_6Cl_3$ in D_2O, the chemical shift of ^{59}Co over the range 16–30 °C has a significant, approximately linear temperature dependence of 1.7 (±0.1) ppm/°C in the downfield direction. This temperature coefficient to the chemical shift in D_2O solution is comparable to the reported value of 1.42 ppm/°C for a solution of $Co(NH_3)_6Cl_3$ in H_2O (Benedek & Englman,

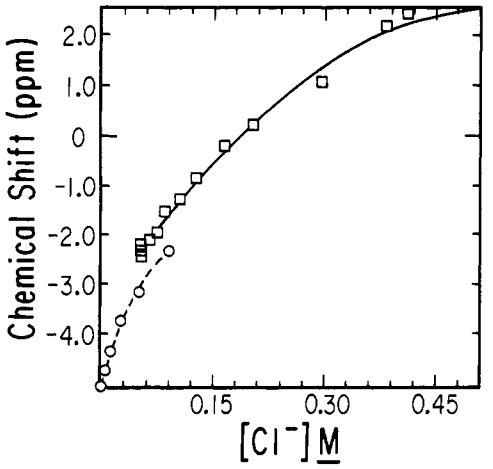

FIGURE 1: Dependence of the ^{59}Co chemical shift on $[Cl^-]$ in solutions of $Co(NH_3)_6Cl_3$ and excess NaCl (□) or without added NaCl (○). For the excess NaCl curve, 0.015 M $Co(NH_3)_6^{3+}$ was titrated with NaCl. The uncertainty in δ is ±0.3 ppm.

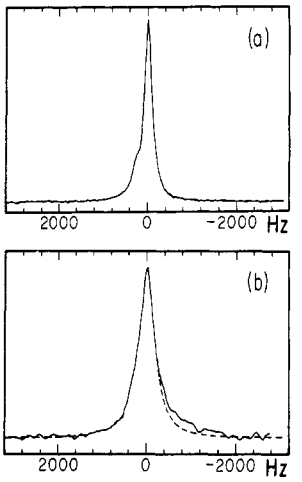

FIGURE 2: Two representative ^{59}Co spectra for $Co(NH_3)_6^{3+}$ in the presence of NaDNA (solid curves). Also shown are the computer simulations (dashed curves) assuming that 0.08% of the cobalt hexaammine is in the form $Co(ND_3)_5NHD_2^{3+}$, for which the isotope shift relative to $Co(ND_3)_6^{3+}$ is 5.7 ppm (Bendall & Dodrell, 1978). Spectrum a ($\Delta\nu_{1/2}$ = 200 Hz) was obtained under conditions where the majority of the $Co(NH_3)_6^{3+}$ is free in solution ($[Na^+]$ = 210 mM, [P] = 8.8 mM, $[Co(NH_3)_6^{3+}]$ = 2.7 mM). For the conditions of spectrum b ($\Delta\nu_{1/2}$ = 430 Hz) the majority of the $Co(NH_3)_6^{3+}$ is associated with DNA ($[Na^+]$ = 110 mM, [P] = 18 mM, $[Co(NH_3)_6]$ = 1.0 mM).

1963). The sensitivity of the ^{59}Co chemical shift of $Co(NH_3)_6Cl_3$ to variations in the concentration of chloride ion is illustrated in Figure 1. This effect is the result of ion pairing between $Co(NH_3)_6^{3+}$ and Cl^-, which at low salt concentrations has been studied by other techniques and analyzed according to Bjerrum's theory (Jenkins & Monk, 1951). However, over the salt concentration range investigated by us, Bjerrum's theory is not expected to provide a useful description of the interaction. Consequently, the fitting of the data in Figure 1 is empirical. The chloride dependence of the ^{59}Co chemical shift characteristic of cobalt hexaammine in the absence of DNA is required in section E for the purpose of analyzing the concentration dependence of ^{59}Co chemical shifts observed in solutions where not all of the cobalt complexes are associated with DNA.

(B) Characteristics of ^{59}Co NMR in Cobalt Hexaammine Solutions Containing DNA. (1) Line Width. Figure 2 shows two representative ^{59}Co spectra for cobalt hexaammine in solutions containing DNA. Slight asymmetries in the peaks are due to the isotope shift effect arising from the presence of small residual amounts of ^1H in the samples. Under the conditions of the present study, chemical exchange of D, or H, from the (nonlabile) ammonia molecules complexed to cobalt is slow on the NMR time scale. Thus, separate ^{59}Co resonances are given by the species $Co(ND_3)_6^{3+}$ and $Co(ND_3)_5NHD_2^{3+}$, for which the isotope shift is about 5.6 ppm (Bendall & Dodrell, 1978). This shift is quite small compared to the ^{59}Co line width, and the proportion of $Co(ND_3)_5NHD_2^{3+}$ contributing to the ^{59}Co resonance is <1%. After correction for the contribution of the protonated species is made, computer simulations indicate that all of the ^{59}Co spectra analyzed here appear Lorentzian. However, small deviations from the Lorentzian form would be difficult to detect because long delay times (~250 μs) were employed before pulse acquisition to avoid artifacts arising from acoustic ringing. During these delay times any rapidly decaying components of the transverse relaxation process, which in general cause non-Lorentzian line shapes outside the extreme narrowing limit, could effectively vanish.

In the DNA solutions investigated by us, the line width of the ^{59}Co resonance is at most 4 times broader than that observed in the absence of DNA. Thus, association with DNA enhances the relaxation rate of ^{59}Co by no more than a factor of 4 (at 21.4 °C). This level of enhancement is not large enough to indicate any significant displacement of ammonia from the inner coordination shell of $Co(NH_3)_6^{3+}$. In asymmetric complexes of the type $Co(NH_3)_5X^{2+}$, the ^{59}Co resonance is often too broad to be observed by high-resolution NMR spectroscopy (Laszlo, 1983). The relaxation processes of cobalt in intact hexaammine complexes near DNA can be investigated further by determining the temperature dependence of the ^{59}Co line width.

For a solution containing 9.6 mM DNA, 1.7 mM $Co(NH_3)_6^{3+}$, and 22.4 mM Na^+, the ^{59}Co line width, which at 21.4 °C is about 4 times that observed in the absence of DNA, is found to decrease with increasing temperature. Under these conditions nearly all of the cobalt hexaammine is localized in the near vicinity of DNA. (This claim is supported in following sections.) Association with DNA causes a reversal in the sign of the temperature dependence of the ^{59}Co line width from that observed in the absence of DNA. This dramatic effect can be attributed to a change in the mechanism that governs the relaxation of ^{59}Co. If the transverse relaxation of cobalt in complexes associated with DNA remains in the extreme narrowing limit but is governed by the quadrupolar, rather than the scalar, mechanism, then the observed ^{59}Co line width would be directly proportional to the correlation time that characterizes the modulation of the quadrupolar interaction. Since this correlation time is due to diffusional molecular processes, it would decrease with increasing temperature and so would the line width.

The predominance of the quadrupolar over the scalar mechanism could result simply from a lengthening of the correlation time that characterizes the diffusional motions of $Co(NH_3)_6^{3+}$ associated with DNA. The change in this correlation time that would be required to account for the fourfold increase observed in the ^{59}Co relaxation rate of complexes associated with DNA can be estimated from knowledge of the independent contributions of the scalar and quadrupolar mechanisms to the ^{59}Co transverse relaxation rate characteristic of $Co(NH_3)_6^{3+}$ in the absence of DNA. (The following comparison neglects any small correction arising from the difference in sample temperatures.) In aqueous solutions of $Co(NH_3)_6Cl_3$ at 35 °C, the scalar contribution to T_2^{-1} for ^{59}Co is approximately 500 s^{-1} and the quadrupolar contribution

approximately 20 s^{-1} (Rose & Bryant, 1979). These numbers would be changed to 5 and 2000 s^{-1}, respectively, if the correlation time governing the diffusional motions of $Co(NH_3)_6^{3+}$ were lengthened by a factor of 100 (without violating the limit of extreme narrowing). Then the total transverse relaxation rate would be enhanced by the observed factor of 4, and its temperature dependence would be determined by that of the quadrupolar correlation time.

The foregoing analysis is strictly valid only if the relaxation of ^{59}Co remains in the limit of extreme narrowing when $Co(NH_3)_6^{3+}$ is associated with DNA. In aqueous solutions containing only cobalt hexaammine, the relaxation process of ^{59}Co is modulated by the rotational diffusion of the octahedral complex. For this molecular motion the characteristic correlation time is approximately 10 ps (Hartmann & Silescu, 1964). According to the preceding line of reasoning, this time is lengthened by a factor of 100 when a cobalt complex is associated with DNA. At the field strength applied in the present study, a correlation time on the order of nanoseconds would cause a relatively small departure from the limit of extreme narrowing for ^{59}Co. Any resulting deviations in the line shapes from the Lorentzian form would be correspondingly slight. As noted above, the long delay times employed before pulse acquisition could effectively obscure minor non-Lorentzian features of the ^{59}Co line shape.

In summary, the relatively modest enhancement in the ^{59}Co line width characteristic of $Co(NH_3)_6^{3+}$ associated with DNA could be entirely dynamic in origin. A much greater enhancement would be expected if the octahedral symmetry of the electric field gradient acting on the cobalt quadrupole were significantly disrupted in the vicinity of DNA. Correlation times on the order of nanoseconds frequently have been found to characterize the NMR relaxation of quadrupolar nuclei associated with polyelectrolytes. In particular, correlation times in the range of 3–5 ns have been estimated for ^{23}Na (Nordenskiöld et al., 1984; Van Dijk et al., 1987), for ^{39}K (Braunlin & Nordenskiöld, 1984), and for ^{87}Rb (Chang, 1983). Although the nature of the molecular motions that modulate the quadrupolar interactions of univalent counterions associated with DNA has not yet been determined, the available evidence implies that these ions remain hydrated and mobile within a small volume surrounding DNA. Analogous inferences can be drawn for $Co(NH_3)_6^{3+}$. On the basis of our ^{59}Co line-width measurements, it appears that the correlation time characteristic of ^{59}Co in complexes associated with DNA cannot much exceed those of associated univalent counterions. Thus, there is no indication that $Co(NH_3)_6^{3+}$ forms a significant number of long-lived outer-sphere complexes at specific sites on B-DNA.

(*2*) *Chemical Shift.* In the presence of DNA at low salt concentrations, the ^{59}Co resonance is shifted upfield with respect to the standard 0.1 M $Co(NH_3)_6Cl_3$ solution. For a solution of 2.3 mM $Co(NH_3)_6Cl_3$, 9.5 mM DNA phosphate, and 0.02 M Na^+ at 21.4 °C, $\delta = -20$ ppm. This relatively small effect on the ^{59}Co chemical shift of $Co(NH_3)_6^{3+}$ in solutions of DNA is comparable to the shifts caused by the transient outer-sphere interactions of intact octahedral Co(III) complexes (Kidd & Goodfellow, 1979; Laszlo, 1983). For inner-sphere complexes of ^{59}Co the observed chemical shifts span more than 18 000 ppm (Laszlo, 1983). Even such a minor chemical change as the substitution of deuterons for protons in $Co(NH_3)_6Cl_3$ produces a shift in the ^{59}Co resonance of 5.6 ppm per deuteron substituted (Laszlo, 1983). The temperature coefficient of the ^{59}Co shift for $Co(NH_3)_6^{3+}$ associated with DNA is very close to that of the standard solution of 0.1M $Co(NH_3)_6Cl_3$. Thus, it appears that B-DNA causes little perturbation of the electron shell surrounding ^{59}Co and hence that the inner coordination shell remains intact when the $Co(NH_3)_6^{3+}$ complex is in the vicinity of DNA. This inference corroborates that drawn above from the modest magnitude of the enhancement in the ^{59}Co line widths caused by the association of $Co(NH_3)_6^{3+}$ with B-DNA.

Under conditions such that not all of the cobalt complexes are associated with DNA, the most straightforward analysis of the concentration dependence of the ^{59}Co chemical shift is based on the assumption that $Co(NH_3)_6^{3+}$ occupies either of two distinct magnetic environments in a DNA solution. According to this two-state model, $Co(NH_3)_6^{3+}$ ions that are radially localized near DNA are characterized by an average "bound" ^{59}Co chemical shift, δ_B. The rest of the $Co(NH_3)_6^{3+}$ ions are characterized by an average free chemical shift, δ_F. If the rate of exchange of $Co(NH_3)_6^{3+}$ ions between the vicinity of the DNA and the bulk solution is fast compared to the difference between δ_B and δ_F (approximately 10^3 s^{-1}), then the observed chemical shift is the weighted average of the free and bound shifts

$$\delta = p_F\delta_F + p_B\delta_B \quad (1)$$

where p_F and p_B are the fractions of free and bound $Co(NH_3)_6^{3+}$, respectively. With r_{Co} defined as the number of associated $Co(NH_3)_6^{3+}$ per DNA phosphate, eq 1 can be rewritten

$$\delta = r_{Co}(\delta_B - \delta_F)([P]/[Co]) + \delta_F \quad (2)$$

where [P] is the concentration of DNA phosphate and [Co] is the total concentration of $Co(NH_3)_6^{3+}$.

According to eq 2, the observed ^{59}Co chemical shift is a linear function of $r_{Co}[P]/[Co]$. If the two-state model were strictly valid, then δ_B and δ_F both would be invariant as the populations of associated and free $Co(NH_3)_6^{3+}$ change. In fact, ion pairing between chloride and cobalt hexaammine causes δ_F to vary during the course of a titration, and it appears that δ_B varies with the binding density of $Co(NH_3)_6^{3+}$ on DNA. Nevertheless, it is demonstrated in section E that ^{59}Co chemical shift measurements can provide direct quantitative information about the extent to which $Co(NH_3)_6^{3+}$ associates with DNA. An alternative, indirect means of investigating this equilibrium is provided by ^{23}Na NMR.

(*C*) *Use of ^{23}Na NMR Line Widths To Analyze the Competition between Na^+ and $Co(NH_3)_6^{3+}$ in DNA Solutions.* In simple salt solutions the nuclear magnetic relaxation of ^{23}Na has been well characterized (Eisenstadt & Friedman, 1967). This relaxation is due to the interaction of the ^{23}Na nuclear quadrupole with local electric field gradients. For sodium ions in the vicinity of DNA the quadrupolar relaxation rate of ^{23}Na is enhanced by at least a factor of 10 (Anderson et al., 1978; Bleam et al., 1983). At sufficiently low salt concentrations, low temperatures, and/or high magnetic fields, non-Lorentzian line shapes have been observed for ^{23}Na in solutions of B-DNA (Nordenskiöld et al., 1984; Van Dijk et al., 1987). Under each of the conditions examined in the present study, the ^{23}Na spectrum can be fitted by a single Lorentzian line shape, whose width at half-height, $\Delta\nu^{Na}$, is therefore directly proportional to R, the transverse relaxation rate of ^{23}Na: $\pi\Delta\nu^{Na}$ (Hz) = R (s^{-1}). According to the two-state model for the association of sodium with DNA, R is given by the following equation, which accounts for the competition between $Co(NH_3)_6^{3+}$ and Na^+ for association with DNA

$$R = (r^0 - nr_{Co})(R_B - R_F)[P]/[Na] + R_F \quad (3)$$

where n and r^0 are as defined in the introduction, R_B is the

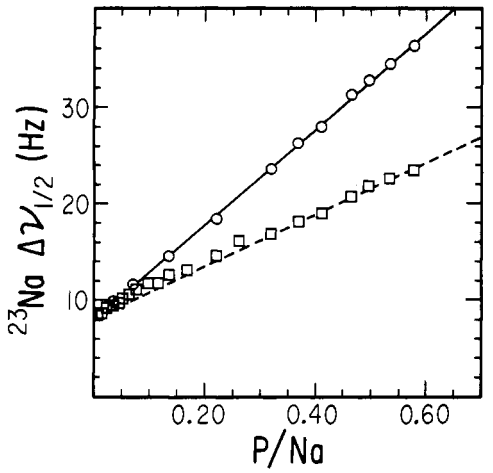

FIGURE 3: Variation in ^{23}Na line width with [P]/[Na] for a titration of NaDNA ([P] = 14.6 mM) with NaCl in the absence (○) and presence (□) of added $Co(NH_3)_6Cl_3$ at a ratio [Co]/[P] = 0.10. The upper line is a least-squares fitting of all points obtained in the absence of added $Co(NH_3)_6Cl_3$. The lower line is the least-squares fitting of the six data points corresponding to the lowest salt concentrations examined in the presence of $Co(NH_3)_6Cl_3$. The uncertainty in the ^{23}Na line widths is ±0.4 Hz.

(average) relaxation rate of ^{23}Na bound to DNA, R_F is the relaxation rate of free ^{23}Na, and [Na] is the *total* concentration of Na^+ in solution. Equation 3 is predicated on the condition that the rate of exchange of sodium nuclei between the two states is rapid in comparison to the relaxation rate in either state. For NaCl in DNA solutions the condition of rapid exchange has been verified previously by an analysis of the temperature dependence of the ^{23}Na relaxation rate (Bleam et al., 1983).

Plots of the ^{23}Na line width versus [P]/[Na] for titrations of NaDNA with NaCl in the presence and absence of cobalt hexaammine are shown in Figure 3. In the absence of this trivalent competitor, the ^{23}Na titration curve agrees with those determined previously (Anderson et al., 1978; Bleam et al., 1980, 1983). According to eq 3 (with $r_{Co} = 0$), the linearity of the upper titration curve in Figure 3 indicates the constancy of the slope $r^0[R_B - R_F]$. In the presence of $Co(NH_3)_6^{3+}$ ([Co]/[P] = 0.10), the slope of the titration curve is reduced, because the trivalent ions effectively displace sodium ions from the vicinity of DNA. The "initial" portion of the lower titration curve in Figure 3, corresponding to the addition of relatively small amounts of NaCl, is linear. It follows from eq 3 that $(r^0 - nr_{Co})(R_B - R_F)$ is constant, at least until $[Na^+]$ exceeds ~0.04 M. Apparently the initial additions of sodium do not displace any significant amount of cobalt hexaammine from the vicinity of DNA. Over this portion of the titration curve the only source of variation in R is the increase in the pool of free sodium. For $[Na^+]/[P] \gtrsim 3.0$ there appears to be an upward deviation in the titration curve, but this small effect would be difficult to analyze, because under these conditions most of the ^{23}Na line width is due to nuclei that are free in solution. If during the early stage of the titration ($[Na^+]/[P] < 3.0$) all of the cobalt hexaammine remains associated with DNA, then $r_{Co} = 0.10$, and the ratio of the slope of the initial portion of this titration curve to the slope of the upper curve in Figure 3 is predicted to be $1 - 0.10(n/r^0)$. The ratio of the slopes determined by linear least-squares fitting of the data in Figure 3 is 0.55 ± 0.05. Hence, $r^0/n = 0.23 \pm 0.01$. On the basis of the CC hypothesis (Manning, 1978) this ratio is predicted to be 0.76/3 = 0.25.

The effect on $\Delta\nu^{Na}$ of adding $Co(NH_3)_6^{3+}$ to a solution containing NaDNA and NaCl is shown in Figure 4. Since

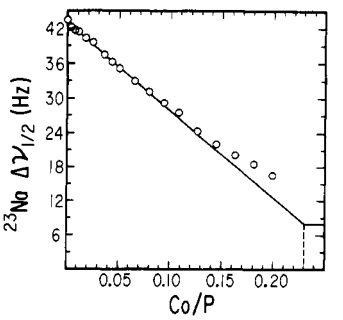

FIGURE 4: Dependence of the ^{23}Na line width on [Co]/[P] during a titration of NaDNA with $Co(NH_3)_6Cl_3$ ($[Na^+]$ = 0.03 M, [P] = 0.021 M). The solid line is the least-squares fitting of the first 12 points of the titration (for which [Co]/[P] < 0.1). The solid horizontal line corresponds to the line width of free $^{23}Na^+$.

the initial portion of this titration is linear, it can be analyzed readily in terms of the two-state model. From eq 1, it follows that r^0/n is the abscissa of the point at which the extension of the linear initial portion of the curve intersects the horizontal line corresponding to the line width of free sodium (Bleam et al., 1983). Application of this analysis to a linear least-squares fitting of the data in Figure 4 gives $r^0/n = 0.23 \pm 0.01$, within uncertainty the same as the value determined above from the slopes of the titration curves in Figure 3. This value is only slightly higher than that reported previously (Braunlin et al., 1986) as a result of analyzing a titration of the type shown in Figure 4. During the initial stage of a titration of this type, as the number of associated trivalent ligands steadily increases, they could tend to occupy particular regions on or near the DNA surface (without necessarily forming long-lived complexes). If the associated cobalt complexes preferentially displace associated sodium ions from regions where the ^{23}Na relaxation process is particularly effective, then R_B would vary in a way that could be impossible to discern by a two-state analysis of the linear initial portion of the titration curve. [The effects of such variations in R_B on estimates of r^0/n have been discussed in detail previously (Bleam et al., 1983).] However, during the initial stage of a titration of the type shown in Figure 3 (lower curve), the binding density of $Co(NH_3)_6^{3+}$ is *constant*, and hence these trivalent ions cannot progressively displace sodium ions from any regions near DNA where their relaxation rates could differ signficantly from the average. [The preceding analyses of the titration curves in Figures 3 and 4 require that all of the $Co(NH_3)_6^{3+}$ in solution is associated with DNA if $[Co]/[P] \lesssim 0.1$ and $[Na]/[P] \lesssim 3.0$. Under these conditions quantitative binding is indicated by other evidence, given in the following sections.]

The consistency between the values of r^0/n estimated from the analyses of the two different types of titrations (in Figure 3 and 4) supports the model assumptions invoked in analyzing both sets of data. In particular, the two-state model appears valid in the sense that R_B exhibits no variations, at least over the initial portions of the titration curves. Moreover, n, the stoichiometric coefficient reflecting the local ion-exchange equilibrium, does not vary with the relative amounts of the competing cations in the vicinity of DNA. Analyzing the effect of $Co(NH_3)_6^{3+}$ on ^{23}Na line widths does not permit a unique evaluation of n, because r^0 has not been determined independently. If $n = 3.0$, the valence of the competing ion, then the corresponding value of r^0 is 0.69 ± 0.03. [These numbers are upper bounds on the respective values calculated with the PB equation (Bleam, 1980).] More precise, quantitative information about the association of $Co(NH_3)_6^{3+}$ with DNA in the presence of NaCl can be obtained from ^{59}Co chemical shifts, which are reported in the following sections.

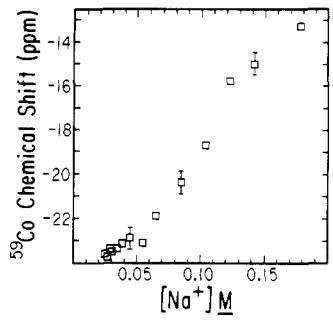

FIGURE 5: Dependence of the ^{59}Co chemical shift on [Na^+] for a titration of NaDNA with NaCl in the presence of added $Co(NH_3)_6Cl_3$ ([Co]/[P] = 0.10, [P] = 14.6 mM). These data correspond to the ^{23}Na data shown in the lower curve of Figure 3. The uncertainty in δ is ±0.5 ppm.

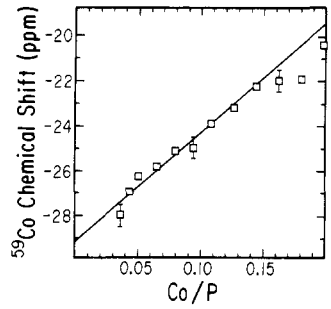

FIGURE 6: Dependence of the ^{59}Co chemical shift on [Co]/[P] during a titration of NaDNA with $Co(NH_3)_6Cl_3$. The variation in the ^{23}Na line width for this titration is shown in Figure 4. The solid line is the best linear fitting of the data for [Co]/[P] < 0.15. The uncertainty in δ (error bars) is ±0.5 ppm.

(D) Use of ^{59}Co NMR Chemical Shifts To Characterize the Association of $Co(NH_3)_6^{3+}$ with DNA. The variation in the chemical shift of ^{59}Co with [Na^+] is shown in Figure 5, which corresponds to the lower titration curve in Figure 3. The observed chemical shift is approximately constant until [Na^+] exceeds 0.04 M. These data, therefore, are consistent with the assumption (employed in the preceding analysis of the ^{23}Na line widths in Figure 3) that $Co(NH_3)_6^{3+}$ remains quantitatively bound during the initial stage of a titration with NaCl. This assumption is supported further by the analysis of cobalt hexaammine chemical shifts provided in the following section and by the results of some equilibrium dialysis experiments (Braunlin and Strick, unpublished results). The latter work indicates that the binding of $Co(NH_3)_6^{3+}$ to B-DNA is comparable in strength to that of the trivalent polyamine spermidine, for which the binding constant is greater than 5×10^3 M^{-1} at salt concentrations lower than 0.04 M. Recently, more extensive equilibrium dialysis measurements have been carried out in order to determine binding constants for the association of cobalt hexaammine with B-DNA over a wide range of salt concentrations (G. E. Plum and V. A. Bloomfield, unpublished results). These results indicate that more than 95% of the $Co(NH_3)_6^{3+}$ is bound for [Co]/[P] ≤ 0.1 under the conditions of Figure 5.

In Figure 6, a titration of NaDNA with $Co(NH_3)_6^{3+}$ is represented as a plot of the observed ^{59}Co chemical shift versus [Co]/[P]. Under the conditions of this titration ([P] = 0.02 M, [Na^+] = 0.03 M), quantitative binding of $Co(NH_3)_6^{3+}$ is expected for [Co]/[P] < 0.1, in view of the equilibrium dialysis measurements cited above and the preceding discussion of the results shown in Figures 3–5. If all of the cobalt complex were associated with DNA, then, according to eq 2, the observed chemical shift would be constant, $\delta = \delta_B$, and the titration curve in Figure 6 would be horizontal for [Co]/[P] < 0.1.

Table I: Binding of $Co(NH_3)_6^{3+}$ to DNA at [Na^+] = 0.133 M

[Co]/[P]	δ^a	δ_B	δ_F	r_{Co}	$r_{Co}/[Co]_f$
0.028	−22.6	−27.8	−0.96	0.022	240
0.055	−20.8	−26.9	−0.94	0.042	190
0.111	−17.2	−25.5	−0.84	0.074	110
0.166	−15.0	−24.2	−0.82	0.100	90
0.277	−11.5	−22.5	−0.71	0.136	60

[a] Relative to the chemical shift of 0.1 M $Co(NH_3)_6^{3+}$ in 99.8% D_2O at 21.4 °C. The uncertainty in δ is estimated to be ±0.5. The resulting uncertainties in r_{Co} are always less than 0.005. Uncertainties in $r_{Co}/[Co]_f$ are shown in Figure 7.

Instead, it appears that δ_B itself varies with the cobalt hexaammine binding density. [If the data shown in Figure 6 are fitted to eq 2 with constant δ_B, the extent of $Co(NH_3)_6^{3+}$ association predicted by this fitting is far less than that detected by equilibrium dialysis experiments under comparable conditions (G. E. Plum and V. A. Bloomfield, unpublished results).]

The most likely physical origin of the dependence of δ_B on the cobalt hexaammine binding density is heterogeneity in the magnetic environments sampled by $Co(NH_3)_6^{3+}$ adjacent to DNA. Such heterogeneity does not necessarily imply the existence of well-defined specific binding sites for $Co(NH_3)_6^{3+}$ on DNA, nor is it inconsistent with extensive translational diffusion of $Co(NH_3)_6^{3+}$ parallel to the helical axis. Radially localized $Co(NH_3)_6^{3+}$ may be highly mobile, but on average it may be in transient contact with certain features of the DNA molecule (such as charged phosphate groups) more often than with others (such as the edges of the base pairs accessible in the helical grooves). There is NMR evidence indicating that the tetravalent ligand spermine is highly mobile when associated with DNA (Wemmer et al., 1985). On the other hand, previously published analyses of the quadrupolar relaxation rates of Mg^{2+} (Rose et al., 1980) and of Ca^{2+} (Braunlin et al., 1987) suggest at least some degree of localized binding on the DNA surface. Interactions of associated cobalt hexaammine with the high local concentration of sodium ions near DNA also could affect the magnitude of δ_B but probably to a much smaller extent than interactions with phosphates or other groups on DNA. Even if there is no specific binding to particular sites, the proximity of cobalt hexaammine might bring about local changes in the B-DNA structure (prior to the phenomenon of collapse) that could in turn affect δ_B. Whatever the physical origin of the apparent dependence of δ_B on the binding density of $Co(NH_3)_6^{3+}$, this effect can be either described with sufficient accuracy or effectively minimized in the analyses of ^{59}Co chemical shifts that are presented in the following section.

(E) Use of ^{59}Co Chemical Shift Measurements To Quantify the Thermodynamics of the Association of $Co(NH_3)_6^{3+}$ with DNA. In solutions containing high salt (NaCl) concentrations, the partial extent of association of cobalt hexaammine with DNA can be determined by using eq 2 to analyze the concentration dependence of the ^{59}Co chemical shift. This analysis also requires information about the dependence of δ_B on r_{Co} and the dependence of δ_F on [Cl^-]. Figure 6 indicates that δ_B can be described empirically as a linear function of r_{Co}. Substituting this expression for δ_B into eq 2 and solving the resulting quadratic equation give r_{Co} as a function of δ and δ_F. This expression can be solved for r_{Co} by introducing the dependence of δ_F on [Cl^-] shown in Figure 1. (For excess [Cl^-], δ_F is independent of [$Co(NH_3)_6^{3+}$].) This approach was taken to analyze a titration of NaDNA with $Co(NH_3)_6Cl_3$ at high salt ([Na^+] = 0.133 M). The results of this analysis are summarized in Table I, and the variation in r_{Co} is represented

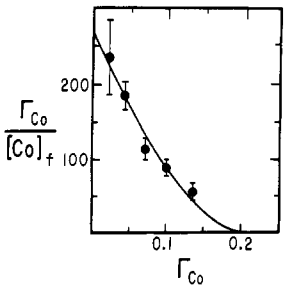

FIGURE 7: Scatchard plot for the binding of $Co(NH_3)_6^{3+}$ to DNA. During the titration [P] decreased slightly (from 17.8 to 17.2 mM); $[Na^+]$ was maintained at 0.133 M. The solid curve represents the best fit to the data of the equation of McGhee and von Hippel (eq 4). The binding parameters thus obtained are $K = 270 \pm 80$ M^{-1} and $l = 4.3 \pm 1$. The error bars shown for $r_{Co}/[Co]_f$ reflect uncertainties in δ. (The uncertainty in the abscissa, r_{Co}, is always less than 0.005.)

as a Scatchard plot in Figure 7.

The association of multivalent ligands with a rodlike polyion frequently has been modeled in terms of an equation originally derived by McGhee and von Hippel (1974). To describe the association of $Co(NH_3)_6^{3+}$ with DNA, this equation can be written

$$\frac{r_{Co}}{[Co]_f} = K_{obsd}(1 - lr_{Co})\left(\frac{1 - lr_{Co}}{1 - (l-1)r_{Co}}\right)^{l-1} \quad (4)$$

where l is a parameter that reflects repulsive interactions among cobalt complexes that are "bound" to DNA, K_{obsd} is the overall observed binding constant on a per phosphate basis, and $[Co]_f$ is the concentration of free $Co(NH_3)_6^{3+}$. In Figure 7 the solid line represents the best fitting of the points to eq 4, for which the binding parameters are $l = 4.3 \pm 1$ and $K_{obsd} = 270 \pm 80$ M^{-1}. This value of l conforms to the empirical relationship $l = Z + 1$, which has been inferred by a variety of studies of the association of Z-valent ligands with DNA (Braunlin et al., 1982; McGhee & von Hippel, 1974; Record et al., 1976). The value of K_{obsd} for $Co(NH_3)_6^{3+}$ is the same, within experimental uncertainty, as K_{obsd} for the trivalent polyamine spermidine at the same salt concentration (Braunlin et al., 1982). This agreement provides another indication that the association of these structurally diverse oligocations with DNA is governed primarily by electrostatic interactions. It is important to recognize that using eq 4 to analyze a titration of the type represented in Figure 7 does not necessarily require that cobalt hexaammine ions associated with DNA be covalently bound at particular "sites" on a linear "lattice". In fact, for a model in which "bound" ligands are taken to be free sliding (Woodbury, 1982), the binding isotherms are exactly conformable to those generated with eq 4. Apparently this equation can provide an accurate description of the association of $Co(NH_3)_6^{3+}$ with DNA in terms of the binding constant, K_{obsd}, and a single additional parameter, l, that reflects any anticooperative effects, including purely electrostatic interactions.

A titration of the type represented in Figure 7 does not provide information about the dependence of K_{obsd} on the concentration of NaCl. The salt dependence of K_{obsd} can be determined by analyzing a titration in which both NaCl and $Co(NH_3)_6Cl_3$ are added in proportions that minimize variations in r_{Co}. Minimal changes in r_{Co} tend to minimize the effects of uncertainties about the values of δ_B and l on an analysis of the titration curve. (If both r_{Co} and l are roughly constant, then according to eq 4 K_{obsd} is directly proportional to $[Co]_f$.) For a titration of this type the ^{59}Co chemical shifts are given in Table II, together with the results of analyzing

Table II: Salt Dependence of $Co(NH_3)_6^{3+}$ Binding to DNA[a]

$[Na^+]$ (M)	[Co]/[P]	δ	δ_B	δ_F	r_{Co}[b]	log K_{obsd}[c]
0.060	0.0556	−26.0	−26.3	−2.64	0.055	3.76
0.060	0.0834	−24.6	−25.1	−2.62	0.082	3.75
0.071	0.0834	−23.7	−25.3	−2.29	0.078	3.22
0.081	0.0834	−23.1	−25.4	−1.99	0.075	3.04
0.092	0.0834	−22.3	−25.6	−1.76	0.072	2.85
0.091	0.111	−20.8	−24.6	−1.74	0.092	2.87
0.102	0.111	−20.4	−24.7	−1.49	0.091	2.81
0.112	0.111	−19.4	−24.9	−1.28	0.085	2.65
0.133	0.111	−18.0	−25.3	−0.88	0.078	2.46
0.132	0.139	−16.9	−24.5	−0.86	0.094	2.52
0.153	0.139	−15.2	−25.0	−0.23	0.084	2.32
0.173	0.139	−13.5	−25.5	−0.23	0.073	2.11
0.172	0.167	−12.9	−24.9	−0.21	0.085	2.18
0.221	0.167	−9.7	−25.9	+0.34	0.064	1.85
0.219	0.222	−9.1	−25.1	+0.36	0.083	1.90
0.310	0.278	−4.4	−26.2	+1.32	0.057	1.48

[a] 16.2–18.3 mM DNA phosphate. [b] The uncertainty in r_{Co} is always less than ±0.003. [c] The units of K_{obsd} are M^{-1}. The uncertainties in log K_{obsd} are shown for representative points in Figure 8.

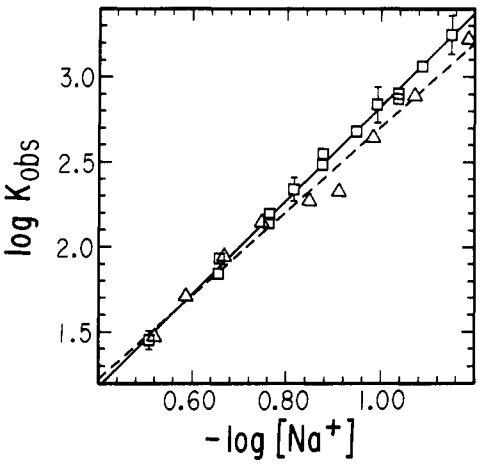

FIGURE 8: Plot of log K_{obsd} vs $-\log [Na^+]$ for the binding of $Co(NH_3)_6^{3+}$ to DNA. Values for K_{obsd} were obtained from ^{59}Co chemical shift measurements as described in the text. Squares represent the determinations shown in Table II for the salt dependence of δ. The solid line through these points represents the linear least-squares fitting, for which log $K_{obsd} = -2.7(\pm 0.1) \log [Na^+] + 0.1(\pm 0.2)$. The triangles are from the data of Figures 3 and 5 for a NaCl titration of NaDNA in the presence of a constant amount of added $Co(NH_3)_6Cl_3$. The best linear least-squares fitting of these data (dashed line) is log $K_{obsd} = -2.5(\pm 0.2) \log [Na^+] + 0.2(\pm 0.4)$.

the data with eq 2 and 4. (In applying eq 4, l was set equal to 4.3, the value obtained from fitting the Scatchard plot in Figure 7.) The salt dependence of the binding constants given in Table II is represented in Figure 8 by a plot of log K_{obsd} vs $-\log [Na^+]$. The best-fitted straight line for these data is given by log $K_{obsd} = -2.7(\pm 0.1) \log [Na^+] + 0.1(\pm 0.2)$. Also included in Figure 8 are values of log K_{obsd} obtained from an analysis of the titration of NaDNA with NaCl shown in Figure 5, for which [Co]/[P] is constant but r_{Co} steadily decreases. For these data the best-fitted straight line is given by log $K_{obsd} = -2.5(\pm 0.2) \log [Na^+] + 0.2(\pm 0.4)$. Clearly, the slopes and intercepts of the plots in Figure 8 agree, within uncertainty.

In Figure 8 the dependence of log K_{obsd} on $-\log [Na^+]$ and the small intercept (at 1 M Na^+) indicate that the association of $Co(NH_3)_6^{3+}$ with DNA is primarily electrostatic, driven entropically by the release of thermodynamically associated sodium ions. According to a theory of this ion exchange based on the CC hypothesis (Record et al., 1976, 1978), the number of thermodynamically associated sodium ions released by the association of one Z-valent ligand is given by $d \log K_{obsd}/d \log [Na^+] = -0.88Z$. Thus, for $Co(NH_3)_6^{3+}$ this effective stoichiometric coefficient is predicted to be 2.64, which falls be-

tween the best-fitted slopes of the lines in Figure 8. The magnitude and salt dependence of K_{obsd} for $Co(NH_3)_6^{3+}$ are close to those reported previously for the association with DNA of other trivalent ligands, including spermidine (Braunlin et al., 1982) and trilysine (Record et al., 1976). This comparison provides further evidence that the association with DNA of $Co(NH_3)_6^{3+}$, like that of other structurally diverse trivalent ligands, is governed primarily by electrostatic interactions.

Concluding Remarks

Under the conditions investigated in the present study, both the qualitative characteristics of the relaxation rate and chemical shift of ^{59}Co and the quantitative results of analyzing the concentration dependence of δ_{Co} indicate that the strong association between $Co(NH_3)_6^{3+}$ and helical DNA is primarily a consequence of electrostatic attraction. If some type of specific binding does control the intramolecular collapse of DNA, it must predominate only under a narrow range of conditions. Although our results give no indication of specific interactions between $Co(NH_3)_6^{3+}$ and helical B-DNA in solution, in the solid state there is crystallographic evidence for the formation of a well-defined complex between $Co(NH_3)_6^{3+}$ and atoms of the phosphate and guanine groups of DNA in the Z conformation (Gessner et al., 1985). The possible existence of such a complex in aqueous solution may be investigated by means of ^{59}Co NMR. Further studies are planned to investigate the role of $Co(NH_3)_6^{3+}$ in driving the B–Z conformational transition and other conformational changes of DNA.

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Registry No. $Co(NH_3)_6^{3+}$, 14695-95-5; Na, 7440-23-5; Co, 7440-48-4.

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