Condensation of Chromatin: Role of Multivalent Cations†

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ABSTRACT: We have used electric dichroism to investigate the influence of multivalent cations upon the compaction of chicken erythrocyte chromatin from the unfolded, 10-nm fiber to the 30-nm solenoid and subsequent aggregation. The pattern of condensation, which consists of compaction plus aggregation, is found to be strikingly similar for a variety of cations of differing charge, including the physiologically important polyamines spermine and spermidine. With a few exceptions such as Cu^{2+} and Gd^{3+}, an optimally compacted fiber with reproducible hydrodynamic properties is produced prior to the onset of aggregation. We report the concentrations of di-, tri-, and tetravalent cations required for optimal condensation; in addition, for tri- and tetravalent cations, we were able to estimate the extent of charge neutralization produced by their binding to the optimally compacted fiber. The results show that the multivalent ion concentration required for optimal compaction decreases as cationic charge increases. In addition, the effect of a mixture of dilute mono- and multivalent cations on chromatin condensation is synergistic, rather than competitive as has been found for the multivalent cation induced condensation of DNA or the B → Z conformational transition. A simple calculation indicates that the entropy of ion uptake in chromatin condensation is surprisingly constant for a range of ionic conditions; this factor may be a dominant one in determining the folding equilibrium.

Packaging of DNA in eukaryotic cells is achieved by its folding in a hierarchical series of progressively more compact structures, the smallest of which is the nucleosome (Kornberg, 1974; Simpson, 1978), followed by the 30-nm diameter fiber (Finch & Klug, 1976) observed in condensed metaphase chromosomes (Ris & Kubai, 1970) as well as in dispersed interphase chromatin. The techniques used to study the 30-nm fiber include electron microscopy (Thoma et al., 1979; Olins & Olins, 1979 Rattner & Hamkalo, 1978), the scattering of X-rays (Sperling & Klug, 1977; Brust & Harbers, 1981; Langmore & Schutt, 1982), neutrons (Suau et al., 1979), and light (Campbell et al., 1978; Marion et al., 1981), electric and flow dichroism (McGhee et al., 1980, 1983; Lee et al., 1981; Lee & Crothers, 1982; Yabuki et al., 1982; Tjerneld et al., 1982), and photochemically detected linear dichroism (Mitra et al., 1984). A composite picture has now emerged from these studies [reviewed by Butler (1983)] of a somewhat irregular solenoidal helix of pitch 11 nm, diameter 30 nm, and approximately six nucleosomes per helical turn.

In vitro studies have indicated that maintenance of the 30 nm thick solenoidal structure is contingent on the presence of a monovalent cation concentration of at least 50 mM (Thoma et al., 1979) or, alternatively, a divalent cation concentration above 0.2 mM. In the absence of these ionic conditions, the 30-nm fiber unfolds progressively to a 10 nm thick fiber containing approximately one nucleosome per 11 nm of fiber length. The transition between these two forms of chromatin as a function of sodium ion concentration has been studied in a series of experiments by Thomas and associates (Bates et al., 1981; Butler & Thomas, 1980), using sedimentation techniques. However, no general study has been reported of multivalent cation induced chromatin condensation. There are persuasive biochemical reasons to seek such a characterization as a model for the chromatin unfolding which occurs in vivo in such fundamental processes as transcription (Foe et al., 1976; Blackburn & Chiou, 1981; Pruitt & Grainger, 1981; Spring & Franke, 1981; Andersson et al., 1982).

We have employed the technique of electric dichroism to examine systematically the influence of ionic variables on chromatin condensation. The combination of geometric information from dichroism amplitudes and hydrodynamic information from rotational relaxation rates provides an unusually detailed picture of the compaction and aggregation processes. In particular, measurement of the rotational relaxation time has an advantage over determination of sedimentation velocity in distinguishing compaction from aggregation, because both of these increase s values, whereas they have opposite effects on the rotational relaxation rate.

The ions we have chosen to investigate include Mg^{2+}, spermidine(3+), and spermine(4+), each of which may play a role in chromatin condensation in vivo; in addition, we have studied the influence of ionic radius by comparing Ca^{2+} and Ba^{2+} with Mg^{2+}. Among other divalent cations, Cu^{2+} shows a contrasting behavior, which is of interest in view of its putative role in the organization of metaphase chromosomes (Lewis & Laemmli, 1982). The stable d-block cation $Co(NH_3)_6^{3+}$ and the lanthanide Gd^{3+} were used to provide comparison with spermine(3+). Where possible, we compare our results on chromatin condensation with results on the B–Z transition of DNA (Behe & Felsenfeld, 1981) and the oligocation-induced condensation of DNA (Wilson & Bloomfield, 1979; Widom & Baldwin, 1980).

Materials and Methods

Chromatin from chicken erythrocytes was prepared and purified as described by McGhee et al. (1983), except for the following modifications: micrococcal nuclease digestion was carried out on 100 OD/mL of nuclei, with 25 units/mL enzyme (Boehringer) at 37 °C for 5 min. After the micrococcal nuclease digestion of the nuclear suspension was quenched with ethylenediaminetetraacetic acid (EDTA)[1] to 10 mM, the

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[1] Abbreviations: EDTA, ethylenediaminetetraacetic acid; Tris, tris(hydroxymethyl)aminomethane; SDS, sodium dodecyl sulfate.

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suspension was immediately diluted with 1–2 volumes of 1 mM Tris–0.1 mM EDTA buffer prior to overnight dialysis against the same buffer to lyse the nuclei. High yields of chromatin were obtained by this procedure. The sucrose gradient purified fractions of chromatin were dialyzed against several changes of dichroism buffer (0.3 mM NaCl, 0.2 mM Tris, 0.003 mM EDTA, pH 7.5), with multivalent cations added prior to measuring relaxation and dichroism data. For these experiments, we used chromatin of a fairly wide size distribution, 60 ± 20 nucleosomes long, in order to obtain sufficiently large quantities of material.

Chromatin samples prepared as above showed little loss of histone H5 as assayed by SDS gel electrophoresis, and the purified samples were stable in dichroism buffer at 4 °C for up to a week, as judged by reproducible dichroism signals.

Low-field electric dichroism experiments for relaxation measurements were carried out essentially as described by Marini et al. (1982). A rectangular exciting pulse of 2 kV, 150-μs duration, was applied across a dichroism cell with a 5-mm electrode gap. Data acquisition and processing were done with a Le Croy 2256 waveform digitizer interfaced to a Digital Equipment Corp. PDP-11/40 computer. The instrument operated in the signal-averaging mode; the computer collected and averaged each data set up to a predetermined number of sweeps, which ranged from 100 to 1000, depending on the signal amplitude, which determines the need for signal averaging. Sweeps were repeated at intervals of 150–400 ms, with reversal of the field polarity every eight pulses.

An interactive computer program was used to analyze the dichroism data by determining the best fit of the decay portion of the signal to a sum of two exponentials:

$$y = A_1 e^{-t/\tau_1} + A_2 e^{-t/\tau_2} \quad (1)$$

where y is the normalized signal amplitude, $A_1 + A_2 = 1$, and τ_1 and τ_2 are the relaxation times. We defined the average relaxation time $\bar{\tau}$ as

$$\bar{\tau} = A_1\tau_1 + A_2\tau_2 \quad (2)$$

High-field experiments for the measurement of dichroism amplitudes were carried out as described by Lee and Crothers (1982). All experiments except those in which the field dependence was measured were done at 16 kV/cm. The field dependence of the dichroism amplitude was determined at intervals up to 27.5 kV/cm. The reduced dichroism ρ is defined as the difference of absorbance for light polarized parallel to the electric field, minus the absorbance for perpendicularly polarized light, divided by the absorbance of unpolarized light.

Care was taken to maintain the integrity of the samples during high-field measurements. A given sample was pulsed at most twice. Periodic checks against optical artifacts were made by measuring dichroism with the polarizer set at 0° and 90° to the electric field and ensuring that these dichroism signals were consistent with each other. Repeated high-voltage pulsing of the sample leads to the transient absorbance change which we reported earlier (Yabuki et al., 1982); although the correction procedure which we outlined leads to dichroism values consistent with those reported here, we now prefer to eliminate the artifact altogether by minimizing the high-voltage exposure of the sample.

Salts were added to chromatin samples of 250–1000 μL by addition from dilute stocks, with rapid but gentle mixing. Measured dichroism and relaxation properties were independent of time after mixing for periods of at least several hours on ice.

Experiments, unless otherwise stated, were carried out with chromatin of $A_{260} = 0.2 = 15$ μM DNA base pairs = 30 μM DNA phosphate; the measurement temperature was 22 °C unless otherwise specified.

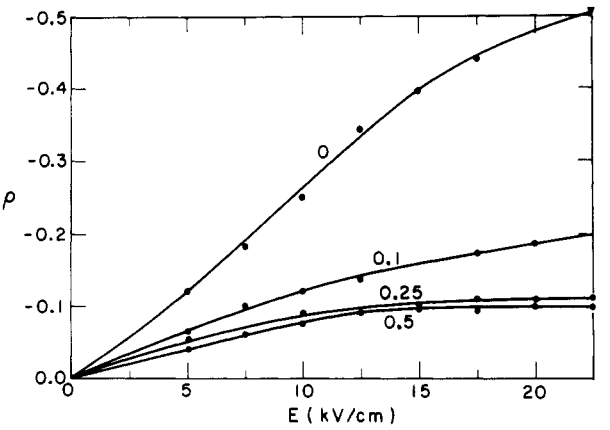

FIGURE 1: Electric field (E) dependence of the reduced dichroism ρ of erythrocyte chromatin ($A_{260} = 0.2$) in dichroism buffer (0.3 mM NaCl, 0.2 mM Tris, and 0.003 mM EDTA, pH 7.5) with added Mg^{2+} concentration of 0, 0.1, 0.25, and 0.5 mM, as indicated. $T = 22$ °C.

RESULTS

We report experiments of three main kinds: (1) We studied the electric field dependence of the dichroism amplitude of chromatin as a function of Mg^{2+} concentration. (2) We examined the influence of ionic conditions on the rotational relaxation rate of erythrocyte chromatin samples, using pulsed electric fields of 4 kV/cm. (3) Having established through the field dependence studies that 16 kV/cm provides sufficient field to saturate the orientation of chromatin stabilized by 250 μM Mg^{2+}, we used that value of the field strength to characterize the dependence of dichroism amplitude on ionic conditions. An electric field of 16 kV/cm does not yield the limiting or full-orientation dichroism of samples at lower Mg^{2+} concentrations, but because of the complex contributions of orientation and field-induced distortion to the high-field dichroism of unfolded chromatin, we chose not to attempt to extrapolate those data to infinite fields.

All three types of experiments lead to a picture of increasing chromatin compaction into the 30-nm fiber until an optimal divalent ion concentration of 250 μM is reached (at 0.5 mM monovalent ion), at which point aggregation becomes the dominant consequent of further increases in ionic concentration. The pattern, including the values of the rotational rate and dichroism amplitude at optimal ion concentration, is similar for tri- and tetravalent ions, except that substantially smaller ion concentrations are needed for optimal compaction. Ions of the same charge generally behave similarly, except for measurable differences in the tendency to cause aggregation.

Mg^{2+}-Induced Condensation. (a) Dichroism Amplitudes. The dichroism amplitude of chromatin condensed by Mg^{2+} reaches a plateau of −0.1 by about 15 kV/cm applied field, remaining unchanged at higher values of the field, but only if the $MgCl_2$ concentration is 250 μM or greater, as shown in Figure 1. At lower Mg^{2+} concentrations the dichroism continues to rise as the field increases; similar results are found for chromatin samples from other sources (Sen et al., 1986). Saturation of the dichroism at high field is the behavior expected for particles that orient by an induced dipole moment mechanism (O'Konski et al., 1959) and is the result actually found for chromatin fibers stabilized by chemical cross-linking to minimize electric field induced distortion (Lee et al., 1982). We conclude that full field-induced orientation of chromatin fibers is achieved for fields ≥15 kV/cm when the Mg^{2+} level

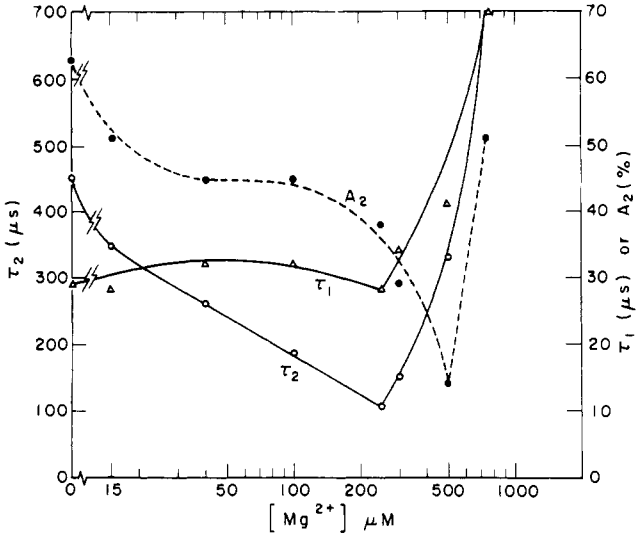

FIGURE 2: Dependence of the relaxation times and corresponding amplitudes (see eq 1) for erythrocyte chromatin on the concentration of added Mg^{2+}; dichroism buffer, T = 22 °C.

equals or exceeds that required (~250 μM) for full compaction (as will be described) and stabilization against field-induced distortion. This is in agreement with the recent study of chromatin folding using anisotropy decay of bound ethidium (Ashikawa et al., 1985), which indicates that about 300 μM Mg^{2+} is required for completion of folding.

The limiting chromatin dichroism values reported by McGhee et al. (1980, 1983) differ from ours because of the conditions of measurement and methods of analysis. They did not report field dependence curves for chromatin samples at Mg^{2+} concentrations above 100 μM Mg^{2+} and, therefore, presumably did not observe the dichroism saturation seen at high fields for higher divalent ion concentration, as shown in Figure 1. Hence, their limiting dichroism values represent the result of extrapolation to infinite field, whereas we take the view that the field dependence at suboptimal Mg^{2+} concentration includes contributions from field-induced distortion, which should not be included in the optical properties of the unperturbed fiber. Therefore, our limiting dichroism values reflect the high-field plateau seen for [Mg^{2+}] $\geq$ 250 μM.

(*b*) *Relaxation Behavior.* Figure 2 shows typical results on the resolution of the orientational relaxation spectrum of chromatin fibers into a sum of two exponentials, with relaxation times τ_1 and τ_2 and percent amplitude A_2 of the slower relaxation, given as functions of the Mg^{2+} concentration. At 250 μM Mg^{2+}, τ_2 reaches a minimum, corresponding to the highest degree of compaction that can be achieved. Under these conditions, the relaxation spectrum consists of two times which differ by about a factor 3, with approximately equal amplitudes and average $\bar{\tau}$ = 55 μs. These properties are consistent with expectation for the rotational relaxation of the 30-nm fiber on the basis of earlier measurements performed on stabilized and highly fractionated chromatin samples (Lee et al., 1981), considering the size (60 nucleosomes) and polydispersity (±20 nucleosomes) of the present samples. The sharp rise of τ_2 at higher Mg^{2+} concentrations doubtless results from aggregation processes, but it is difficult to ascribe a definite meaning to the individual relaxation components under other conditions.

In order to simplify the analysis, the remainder of the results are presented as the average relaxation rate $1/\bar{\tau}$, which exhibits the dependence on ion concentration shown in Figure 3. This choice has the virtue of focusing attention on the major trends: below 250 μM Mg^{2+}, increasing ion concentration causes the

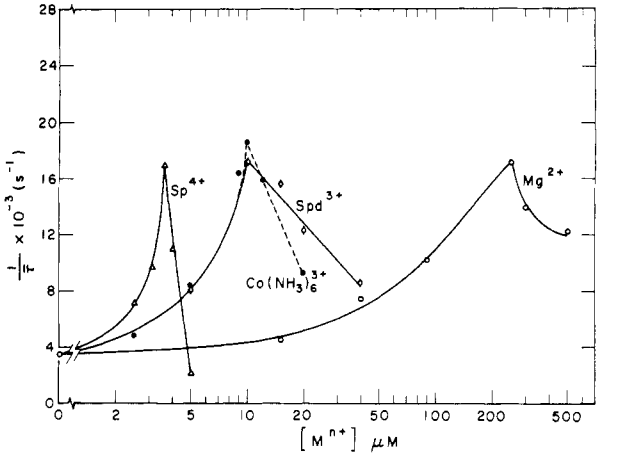

FIGURE 3: Dependence of average relaxation rate $1/\bar{\tau}$, where $\bar{\tau} = A_1\tau_1 + A_2\tau_2$, on added multivalent ion concentration, showing comparative results for Mg^{2+}, spermidine (Spd^{3+}), $Co(NH_3)_6^{3+}$, and spermine (Sp^{4+}).

average relaxation rate to increase, corresponding to increasing compaction, whereas above 250 μM Mg^{2+}, increasing ion concentration leads to a decreased relaxation rate. Given the accompanying monotonic increase in sedimentation rate at all ion concentrations (Butler & Thomas, 1980; Yabuki et al., 1982) the underlying process above 250 μM Mg^{2+} must be predominantly aggregation. Aggregation is also clearly indicated by the phenomenon which dictated the upper limit on the ion concentration for the curves in Figures 3 and 4, namely, the appearance of turbidity and very long relaxation times, characteristic of macroscopic aggregation.

We define the state of chromatin at the point of maximum rotational relaxation rate to be that of "optimal compaction", corresponding to the greatest extent of folding into the 30-nm fiber that can be observed before aggregation becomes the dominant consequence of further ion addition. We denote the concentration of a cation needed to bring about optimal compaction of chromatin as C_m, defined as the value which produces the maximum relaxation rate $1/\bar{\tau}$.

Condensation by Other Divalent Cations. Ca^{2+} and Ba^{2+} are indistinguishable from Mg^{2+} in the dependence of dichroism on field strength shown in Figure 1 (data not shown). The only observable difference in the relaxation rate profile is the finding (data not shown) that Ba^{2+} causess the smallest increase in relaxation time of chromatin beyond the point of optimal compaction and is thus the least effective of the three in inducing aggregation. This may result from the substantially larger ionic radius of Ba^{2+} compared to those of Mg^{2+} and Ca^{2+}, yielding a lower charge density on the surface of the Ba^{2+} ion.

Other divalent cations among the first period transition metals were examined for their ability to condense chromatin. With the exception of Cu^{2+}, these ions (Co^{2+}, Zn^{2+}, Mn^{2+}) behaved in a very similar fashion to Mg^{2+}, with only small differences in the concentration of cation at which the chromatin began to aggregate.

Cu^{2+}, however, showed variant behavior, as shown in Figure 4. At low concentrations, the relaxation rate of Cu^{2+}-condensed chromatin is significantly faster than observed for equivalent Mg^{2+}-condensed samples. However, above 50 μM concentration, Cu^{2+}-containing samples began to show the symptoms of aggregation, with macroscopic association terminating the experiment beyond 100 μM Cu^{2+}. As shown in Figure 5, Cu^{2+} is also more effective than Mg^{2+} in reducing the dichroism amplitude toward the values characteristic of the folded fiber. With naked DNA, Cu(II) is known to have

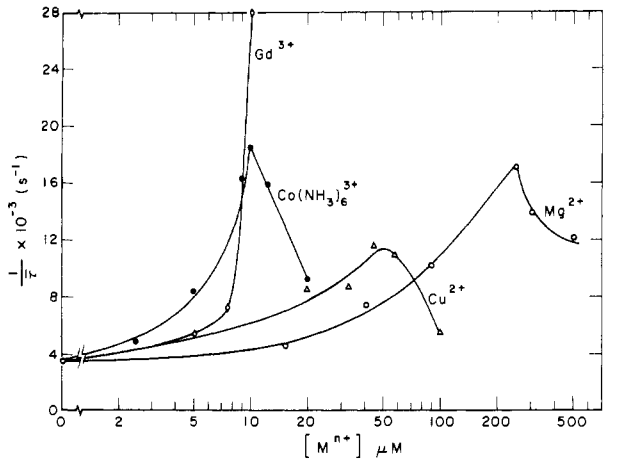

FIGURE 4: Results as in Figure 3, showing comparative behavior of Cu^{2+} and Gd^{3+}.

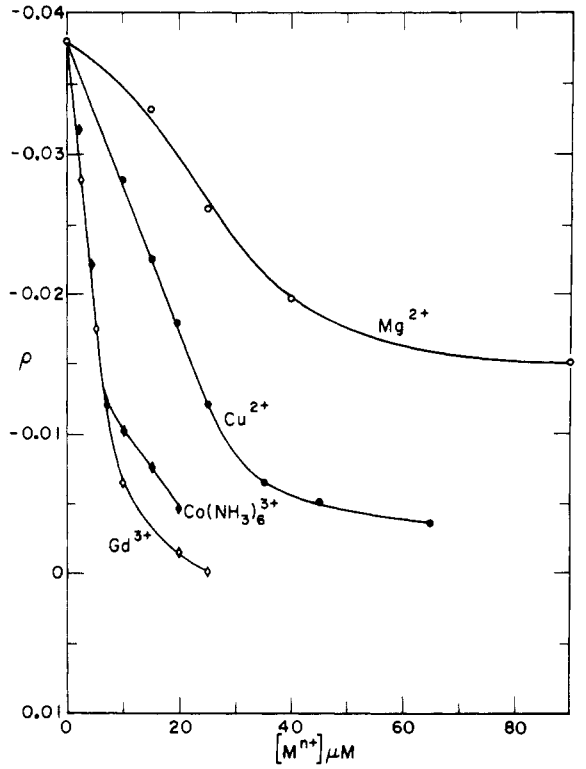

FIGURE 5: Dichroism amplitudes at 16 kV/cm of chromatin condensed by the addition of Mg^{2+}, Cu^{2+}, $Co(NH_3)_6^{3+}$, and Gd^{3+}. All measurements were made at 266 nm. The dichroism of chromatin condensed by Mg^{2+} reaches a plateau of −0.1 at approximately 250 μM Mg^{2+} (results not shown).

a high affinity for the bases (Wei et al., 1978) and also for soft nucleophilic ligands present in proteins such as sulfhydryls. The sole sulfhydryl in chicken erythrocyte nucleosomes (Olins et al., 1977) is thought to lie buried in the interior of the core, being generally inaccessible to the solvent. Thus, the condensation and aggregation brought about by Cu^{2+} is probably due to a combination of partial charge neutralization of DNA phosphates, along with the effects of binding to DNA bases and perhaps to non-histone proteins present in the purified chromatin.

Condensation by Trivalent and Tetravalent Ions. We examined the condensing behavior of three different cationic species with charge 3+: hexaamminecobalt(III), the hydrated lanthanide Gd^{3+}, and the polyamine spermidine(3+) (Spd^{3+}). The stably bound ammonia ligands in the hexaammine-

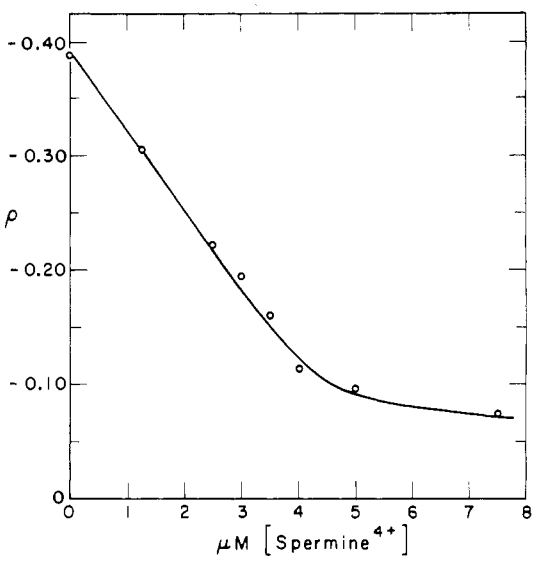

FIGURE 6: Dichroism amplitudes of chromatin measured at 266 nm, at a field strength of 16 kV/cm, against increasing concentrations of spermine.

cobalt(III) complex give it a substantially larger ionic size than Gd^{3+} (2.98 Å compared to 1.02 Å).

The dichroism amplitude profiles at fixed electric field (Figure 5) were remarkably similar for all three trivalent ions, being essentially identical for Spd^{3+} and hexaamminecobalt(3+), both of which gave dependences which were superimposable for a substantial ionic range on the curve for Gd^{3+}. The curves for $Co(NH_3)_6^{3+}$ and Spd^{3+} show a slight tendency to level off, beginning at approximately 5 μM, whereas the Gd^{3+} profile decreases to zero more rapidly.

The dependences of relaxation rate on ion concentration (Figures 3 and 4) have one common feature for the three ions: all cause optimal compaction of chromatin (A_{260} = 0.2) at 10 μM, approximately one trivalent ion per three DNA phosphates. Beyond that concentration, aggregation due to hexaamminecobalt(3+) begins, with a dramatic increase in $\bar{\tau}$, as cation concentration increases (Figure 3), whereas with Spd^{3+} aggregation proceeds more gradually.

Significant differences are seen with Gd^{3+}, for which the compaction–aggregation process appears to be cooperative (Figure 4). Between 9.0 and 10.5 μM Gd^{3+}, chromatin progresses from a state apparently less compacted than by comparable concentrations of Spd^{3+} or $Co(NH_3)_6^{3+}$ to a completely aggregated state with a millisecond relaxation time. Over a narrow concentration range near 10.0 μM, Gd^{3+} brings about a state of high apparent compaction, with $\bar{\tau}$ = 33 μs, the shortest we ever observed, compared to 55 μs for Mg^{2+}- or Spd^{3+}-compaction chromatin. Even small increases in $[Gd^{3+}]$ beyond this point bring about large-scale aggregation.

Spermine(4+) (Sp^{4+}) produces optimal compaction at only 3.6 μM (Figure 2); the dichroism amplitude profile (Figure 6) resembles that of Mg^{2+} or Spd^{3+} in that, although shifted to lower ion concentration, there is a similar leveling off of the signal dependence on ion concentration, and the final dichroism near the ion concentration required for optimal compaction is approximately −0.1, as with Mg^{2+}.

Effect of Chromatin Concentration. The relation between the charge of a given cation and the concentration C_m of that ion that causes optimal chromatin compaction is not immediately apparent on inspection. Formulation of a thermodynamic relationship requires that we separate the added ion concentration into contributions from the bound and free ions, the latter (C_0) defined as the multivalent ion concentration

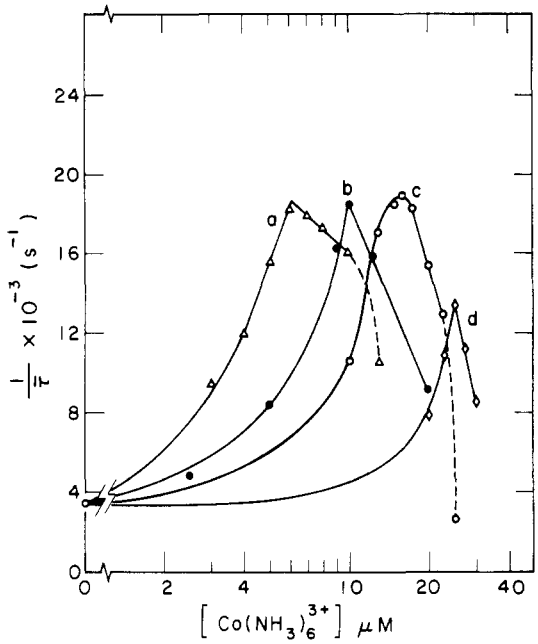

FIGURE 7: Average relaxation rate for varying chromatin concentrations, in response to added hexaamminecobalt(3+). Plotted as $1/\bar{\tau} \times 10^{-3}$ vs. the concentration of added $Co(NH_3)_6^{3+}$; DNA phosphate concentration (in μM): (a) 15; (b) 30; (c) 60; (d) 130.

C_m in the limit as the chromatin concentration, and hence the bound ion concentration, approaches zero. In a plot of C_m vs. chromatin concentration, the intercept yields C_0, and the slope gives the number of multivalent ions bound per chromatin phosphate.

In the case of Mg^{2+}, the concentration C_m remained constant within experimental error at 200–250 μM when the chromatin concentration was varied. This implies that the free concentration C_0 is approximately 200–250 μM but leaves us unable to calculate the concentration of bound ions, except to state that most of the added Mg^{2+} remains unbound. Hence, at least at chromatin concentrations up to 130 μM DNA phosphate, the critical factor in determining the extent of folding is the added Mg^{2+} concentration, not the ratio of Mg^{2+} to DNA phosphate.

For tri- and tetravalent cations, a substantial fraction of the added ion becomes bound, and measurement of C_m as a function of chromatin concentration becomes possible. Figure 7 shows the profiles of average relaxation rate $1/\bar{\tau}$ against hexaamminecobalt^{3+} concentration at chromatin concentrations of 15, 30, 60, and 130 μM DNA phosphate respectively. For the three lower concentrations, very similar optimally compacted conformations (with $\bar{\tau} \approx 58$ μs) result. At the highest concentration, on the other hand, aggregation processes begin to dominate before full compaction has taken place. These and similar results for C_m are plotted vs. chromatin concentration in Figure 8. C_0 for spermine(4+) and hexaamminecobalt(3+) are found to be 1 and 7 μM, respectively. A similar experiment with hexaamminecobalt(3+) at a higher Na^+ concentration of 3.5 μM was also performed and is plotted in Figure 8; note that the amount of hexaamminecobalt(3+) required for optimal compaction decreases as the Na^+ concentration increases.

Two-Cation Systems. Nearly all of the experiments described so far were carried out at a constant monovalent ion concentration of 0.5 mM. The effect of sodium ion concentration on the value of C_m for Mg^{2+} and hexaamminecobalt(3+) was examined systematically by varying the concentration of Na^+ from 0.5 to 15 mM, using a single value

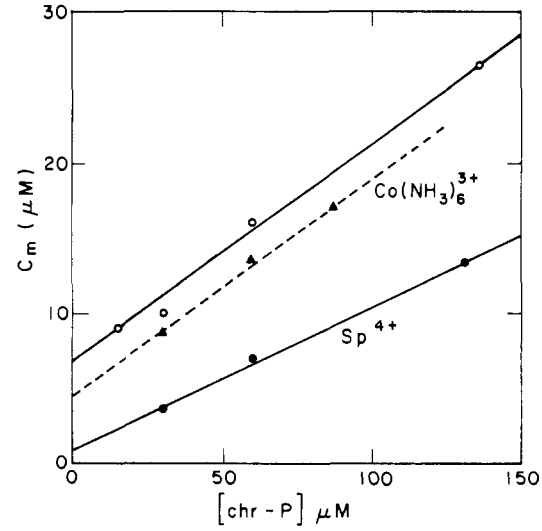

FIGURE 8: Optimally compacting concentration C_m of multivalent ions plotted vs. concentration of chromatin DNA phosphate. (○) $Co(NH_3)_6^{3+}$ at 0.05 mM Na^+ concentration; (△) $Co(NH_3)_6^{3+}$ at 3.5 mM Na^+ concentration; (●) spermine(4+) at 0.05 mM Na^+ concentration.

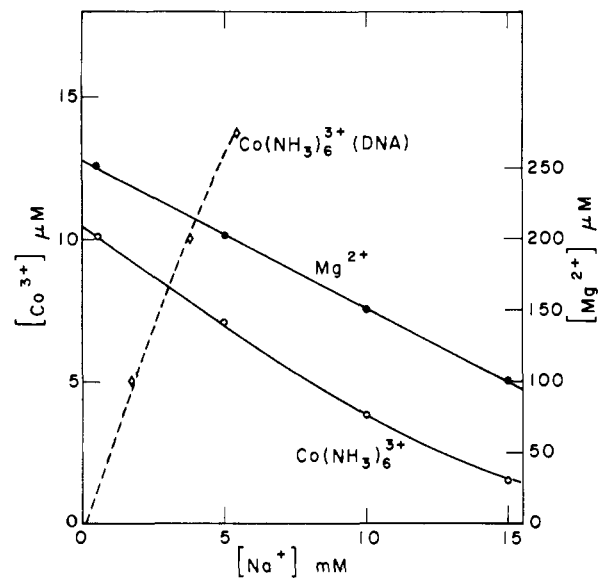

FIGURE 9: Phase diagram for the optimal compaction of chromatin at 30 μM DNA phosphate concentration, showing variation of the multivalent ion concentration C_m as a function of the monovalent ion concentration. The dashed line shows the corresponding trend for cation-induced condensation of DNA, adapted from Widom and Baldwin (1980).

of 30 μM DNA phosphate for the chromatin concentration.

The results, shown in Figure 9, clearly indicate that the effects of monovalent and higher valence cations on chromatin condensation are additive rather than competitive, at least in the regime of low monovalent ion concentration: a lower concentration of multivalent ion is required for condensation as the Na^+ concentration is increased. Because of the intrinsic limitations of electric field orientation, we were not able to measure above 15 mM in total ion concentration. However, the shapes of the two curves indicate that both lines might meet the x axis, corresponding to zero multivalent ion concentration, somewhere above 20 mM Na^+ concentration. This is consistent with the fact that above this concentration Na^+ is able to condense chromatin on its own; recently Ashikawa et al. (1985) reported that approximately 20 mM Na^+ is sufficient to complete the Na^+-induced folding of erythrocyte chromatin.

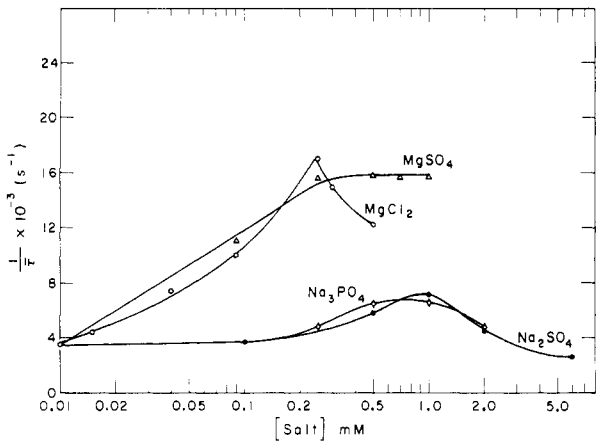

FIGURE 10: Chromatin relaxation rate $1/\bar{\tau}$, in response to added anions, plotted vs. added concentration of salt.

Interestingly, solutions containing millimolar concentrations of NaCl showed a very slow component in the field-free decay of the dichroism signal, whose amplitude appeared to increase with the number of pulses. This effect was not found with sodium phosphate or sodium sulfate. Samples containing NaCl were therefore pulsed much less to minimize this effect. Even so, samples containing 15 mM NaCl gave optimal values of $\bar{\tau}$ somewhat larger than those obtained under conditions of low sodium.

The observed behavior of two-cation systems for chromatin condensation stands in direct contrast with the behavior of such systems as oligocation-induced DNA condensation and the B to Z transition of poly(dG-dm^{5}C), induced by the cation hexaamminecobalt(3+). For DNA condensation, the observed behavior of the two-ion system is shown by the dotted line in Figure 9, adapted from the Na^+–hexaamminecobalt^{3+} phase diagram reported by Widom and Baldwin (1980). This kind of approximately linear dependence of the concentration of the higher valence ion on the lower valence ion is in accordance with the ion condensation formalism of Manning (1978). Clearly, that same formalism will not adequately describe the inverse concentration dependence which we find for chromatin condensation.

Effect of Multivalent Anions. It is evident that one of the factors involved in the condensation of chromatin is the neutralization of the negative charges carried by the DNA backbone. Chromatin, however, consists of DNA complexed with lysine- and arginine-rich histones. Although chromatin has a net negative charge, with an estimated 40–50% of the DNA phosphodiester charges neutralized by the histones (Elgin & Weintraub, 1975), it is conceivable that shielding of the positive histone charges could have an influence on the condensation of chromatin. The effects of Na_2SO_4, Na_3PO_4 (pH 7.5), and $MgSO_4$ upon chromatin condensation are shown in Figure 10. Na_2SO_4 and Na_3PO_4 (in which, at pH 7.5, the phosphate anion has an effective charge near 2–) show the unusual behavior of slightly compacting chromatin at ~1 mM concentration. In the same ionic range, NaCl shows no comparable effect (not shown). $MgSO_4$ compacts chromatin as well as $MgCl_2$ but does so over a much larger ionic range, from 0.1 to 1 mM (Figure 10), implying a lesser tendency to induce aggregation.

Temperature Dependence of Chromatin Aggregation. The temperature dependence of the condensation process was examined by using hexaamminecoablt(3+) with 30 μM chromatin phosphate between 7.0 and 25.5 °C. In this entire range of temperature, optimal compaction took place at the same concentration of hexaamminecoablt(3+), 9.95 ± 0.05 μM. Thus, there is effectively no influence of temperature on the observed equilibrium (there is also no time dependence of C_m), and we can say that $\Delta H \approx 0$ for the process.

Discussion

General Considerations. In summary of our results, we find that tri- and tetravalent ions are more effective in producing condensation of chromatin fibers than are divalent species. Condensation seems in all cases to consist of two processes, an intramolecular compaction and an intermolecular aggregation. Provided chromatin concentrations are below approximately 100–120 μM DNA phosphate, compaction is the dominant process at low concentrations of added cations. Continued addition of multivalent ions to a chromatin sample results in an increase of the rotational relaxation rate to a maximum value, corresponding to the highest degree of compaction that the fiber can achieve, which we call the state of optimal compaction. Further addition of multivalent ions yields a decrease in rotation rate, corresponding to aggregation. The concentration of a given ion required to produce the state of optimal compaction decreases as the charge on the ion increases.

The properties of optimally compacted chromatin seem not to be dependent on the nature or charge of the condensing ion. The average relaxation time $\bar{\tau}$ is in most cases close to 55 μs for chromatin of the average size (60 nucleosomes) studied in our experiments, a value that is in accord with earlier experiments on the rotational relaxation of fixed chromatin fibers (Lee et al., 1981). Furthermore, the relaxation spectrum, characterized by τ_1 and τ_2, is reasonable in view of the polydispersity (±20 nucleosomes) of the samples and the strong dependence of relaxation rate on fiber length (Lee et al., 1981). Finally, the reduced dichroism amplitude at 16 kV/cm in the optimally compacted state produced by tri- and tetravalent ions is close to the value −0.1 found for Mg^{2+}-compacted fibers.

Since chromatin condensation is brought about by the addition of cations to the negatively charged chromatin fiber, it is evident that the primary underlying phenomenon is one of charge neutralization or screening by counterion binding or association. In this sense the process is similar to the cation-induced condensation of DNA, for which Widom and Baldwin (1980) and Wilson and Bloomfield (1979) have reported that approximately 90% of the DNA charges must be neutralized in order to cause condensation. A related process is the B → Z transition of double-helical poly(dG-dm^{5}C), for which Behe and Felsenfeld estimated that ~85% of the DNA charges must be neutralized.

Our results reveal an important difference between chromatin condensation and these other two processes: For DNA condensation and the B → Z transition, monovalent and multivalent ions are competitive, meaning that higher concentrations of multivalent ions are required for the transition when the monovalent ion concentration is increased. In contrast, monovalent and multivalent ions have synergistic effects on the condensation of chromatin, at least at monovalent ion concentrations below 15 mM. According to the formalism of Manning (1978), only multivalent ions are able to produce the extent of charge neutralization needed for DNA condensation or the B → Z transition, and monovalent ions are therefore competitive because they displace multivalent ions, thus reducing the net charge neutralization. Clearly, the same rules do not apply to chromatin condensation; a possible underlying reason for the contrast is the fact that chromatin can be condensed by monovalent ions alone. The lack of competition between mono- and multivalent ions may imply that the regions of chromatin whose partial neutralization is essential for

Table I: Comparison of Chromatin Condensation with Other Transitions[a]

	C_0 (μM)[b]		
ion	chromatin condensation	DNA condensation	B ⇌ Z transition
Na^+	30 000		700 000
Mg^{2+}	200		600
Ca^{2+}/Ba^{2+}	200		600
$Co(NH_3)_6^{3+}$	7	7.5	5
Spd^{3+}	7	27	50
Gd^{3+}	7		
Sp^{4+}	1		2
	estimated 90–100% charge neutralization required	90% charge neutralization required	85% charge neutralization required
	Ion-Specific Effects		
	relatively unimportant	important	important

[a] Results on DNA condensation and the B ⇌ Z transition of poly(dG-dm^{5}C) are taken from Widom and Baldwin (1980), Wilson and Bloomfield (1979), and Behe and Felsenfeld (1981). [b] C_0 is the (free) ion concentration required for the indicated transition.

folding do not have a sufficiently large negative charge density to produce ion condensation of the type considered by Manning (1978).

Another significant general difference between chromatin condensation and the transitions previously studied is its lack of specific ion effects, with a few exceptions such as Gd^{3+}. For example, $Co(NH_3)_6^{3+}$ and Spd^{3+} differ appreciably in their influence on DNA condensation and the B → Z transition (Table I) but are essentially indistinguishable in their ability to cause chromatin compaction.

Entropy of Ion Binding in Chromatin Folding. The lack of temperature dependence of the multivalent ion concentration at optimal compaction (C_m) implies that ΔH for the folding process is essentially zero. Furthermore, since C_m is the ion concentration just sufficient to cause compaction, the free energy of folding must also be zero at an ion concentration near C_m; we approximate that concentration by C_m. Hence, the entropy of folding must also be nearly zero at C_m. Summed over all the physical processes involved in condensation, the favorable and unfavorable entropy changes must therefore roughly balance.

The various components into which we can divide the net entropy of the folding process include (a) the entropy of mono- and multivalent ion binding, which is expected to be unfavorable, (b) the entropy of folding the fiber, also likely to be unfavorable, (c) the entropy of water release from salt-bridge formation, expected to be favorable, and (d) a potentially favorable entropy contribution if there is a hydrophobic interaction between the H1 globular domains in the folded fiber. We might expect entropy contributions b and d to be roughly invariant over the range of cations and ionic conditions used in these experiments, but one expects the entropy terms that are dependent on electrostatic effects to vary with solution conditions.

Some simplifying assumptions allow us to estimate how the entropy of ion binding, ΔS_{ib}, varies with ionic conditions; the surprising feature of the results is the constancy of the estimated ΔS_{ib} evaluated at C_m for different condensing cations. This calculation is possible only if the free multivalent ion concentration C_0 and the fractional charge neutralization produced by the multivalent ion are known from the measurements, which is the case for condensation by spermine(4+) at 0.5 mM Na^+ concentration and by hexaamminecobalt^{3+} at 0.5 and 3.5 mM Na^+ concentration.

One expects the surface charge density of the chromatin fiber to increase upon folding from the 10-nm fiber to the 30-nm fiber, because the diameter increases only threefold while the length decreases about sixfold, assuming conversion from a cylindrical structure with one nucleosome per 11 nm to a solenoid with six nucleosomes per 11 nm. According to the theoretical treatment of Weisbach and Gueron (1981), association of counterions with the surface of cylindrical polyelectrolytes varies with the square of surface charge density. Consequently we expect net binding of cations to occur in the process of folding from the 10- to the 30-nm fiber:

$$\text{unfolded} + n_1 M^{Z^+_1} + n_2 M^{Z^+_2} \rightleftharpoons \text{folded} \qquad (3)$$

where monovalent and multivalent cations are represented by $M^{Z^+_1}$ and $M^{Z^+_2}$, respectively, and n_1 and n_2 are the number of moles of each taken up.

We now make the simplifying assumption that the molar ratio of the ions taken up in the folding process is the same as their molar ratio in the composition of ions bound to the folded state; this seems a reasonable first approximation if the increment in ion binding is small in the folding process.

From the slopes in Figure 8 we calculated that 0.09 mol of spermine(3+) and 0.14 mol of hexaamminecobalt(3+) bind per mole of DNA phosphate at the point of optimal compaction. Considering the charges on the respective cations, we find that 36% and 42% of the total negative charges of optimally folded chromatin are neutralized by spermine(4+) and hexaamminecobalt(3+), respectively; surprisingly, the measured charge neutralization by hexaamminecobalt(3+) is little affected by a sevenfold change in the monovalent ion concentration. An additional 50% of the charges are assumed to be neutralized by the positively charged histone residues (Elgin & Weintraub, 1975), leaving 8–14% of the charges to be neutralized, at least in part, by the monovalent ions. We assume in addition that the total fractional charge neutralization at the point of optimal compaction is independent of ionic conditions. Given the sum of the charge neutralizations by histones and hexaamminecobalt(3+) (92%), the total charge neutralization must lie between 92% and 100%; since the calculation is not very sensitive to the particular value chosen, we illustrate the results for the assumption of 100% neutralization.

The entropy of ion binding depends on the free concentrations C_1 and C_2 of monovalent and divalent ions, respectively, according to

$$\Delta S_{ib} = R n_1 \ln C_1 + R n_2 \ln C_2 \qquad (4)$$

We let $n_1 = \theta_1 \beta$ and $n_2 = \theta_2 \beta$, where θ is the number of mono- or multivalent ions bound per DNA phosphate, and β is an unknown factor that is proportional to the change in charge neutralization in the folding process. Therefore

$$\Delta S_{ib} = R\beta\theta_1 \ln C_1 + R\beta\theta_2 \ln C_2 \qquad (5)$$

from which the ion binding entropies are readily calculated. The results, collected in Table II, show that the contribution of ion binding to the entropy of folding is remarkably constant at about $-2.3\beta R$ for the different ionic conditions.

This analysis can be extended to condensation by monovalent ions alone by taking the entropy of Na^+ binding to be equal to $2.3\beta R$ and letting the critical concentration C_0 of Na^+ be an unknown. The resulting value of C_0 = 15 mM is somewhat lower than the expected range of 30–60 mM, although in reasonable agreement with the value of 10–20 mM

Table II: Parameters Characterizing Ion Binding to Optimally Compacted Fibers[a]

	ion		
	Sp^{4+} (0.5 mM M^+)	Co-$(NH_3)_6^{3+}$ (0.5 mM M^+)	Co-$(NH_3)_6^{3+}$ (3.5 mM M^+)
free multivalent ion concentration, C_0 (μM)	1	7	4.8
multivalent ions bound per DNA phosphate, θ_2	0.09	0.14	0.14
fraction of DNA charge neutralized by multivalent ion, ν_2	0.36	0.42	0.42
assumed fraction of DNA charge neutralized by monovalent ion	0.14	0.08	0.08
calculated entropy of ion binding	$-2.3\beta R$	$-2.3\beta R$	$-2.2\beta R$

[a] R is the gas constant, and β is an unknown factor that is proportional to the difference in ion binding between folded and unfolded forms of the fiber under conditions of optimal compaction.

recently reported by Ashikawa et al. (1985) for folding avian erythrocyte chromatin.

The remarkable feature of this simple calculation is its ability to predict the ionic conditions needed for optimal folding of chromatin simply on the basis of an estimation of the entropy of ion binding. According to the calculation, tetravalent ions are more effective than trivalent species in inducing folding because fewer tetravalent ions must be taken up to achieve the required increment in charge neutralization. Since fewer ions are required, uptake and folding must occur at a lower free tetravalent ion concentration in order to hold constant the ΔS_{ib} term. It is surprising that the sum $\Delta G' = -T\Delta S_{ib}$ of all other contributions to the folding free energy appears to be nearly constant over the range of conditions examined. (Recall that the total free energy of folding $\Delta G = \Delta G' + T\Delta S_{ib}$ is nearly zero at C_m.) One should, however, be wary of the possibility that compensating changes occur in $\Delta G'$ and ΔS_{ib}, the latter perhaps through changes in the parameter β or the degree of charge neutralization required for optimal folding, with the result that the behavior of the system appears simpler than it really is.

The strong effect of low concentrations of polyamines upon the higher order structure of chromatin may have significant consequences in vivo. In the normal intracellular ionic environment we would expect standard chromatin, with a full complement of core histones and H1 to be fully condensed. However, transcriptionally active segments, which may have lost H1 or undergone selective histone modification, could favor the unfolded state, but to a degree dependent on the concentration of multivalent ions.

As a general rule, we can state that any factor that increases the negative charge density on chromatin fibers should contribute to the tendency to unfold. This includes histone modifications such as acetylation which remove positive charges and also ADP-ribosylation of chromatin proteins (Wong et al., 1984). Newly synthesized RNA adds substantial negative charge, and on this basis it is not surprising that rapidly transcribing chromatins do not appear to form the characteristic 30-nm fiber (Labhart & Koller, 1982; Andersson et al., 1982).

The increased ability of Cu(II) to produce both compaction and aggregation of chromatin when compared with other divalent ions is of interest in view of the putative role of copper in the physiological condensation of chromatin. It has been proposed that the Cu^{2+} ion in vivo might offer more specific properties than Ca^{2+} or Mg^{2+}, thus allowing independent control of chromosome condensation, without substantially affecting other cellular processes (Butler, 1983).

Finally, a word about aggregated chromatin, the end product of most of our experiments: this is likely to be the closest approximation of chromatin as it is found in vivo, and the study of aggregated chromatin in vitro may provide the clearest picture of in vivo processes involving chromatin.

Registry No. Spd, 124-20-9; Sp, 71-44-3; Na, 7440-23-5; Mg, 7439-95-4; Ca, 7440-70-2; Ba, 7440-39-3; $Co(NH_3)_6^{3+}$, 14695-95-5; Gd, 7440-54-2; Cu, 7440-50-8.

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Influence of DNA-Binding Drugs on Chromatin Condensation†

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ABSTRACT: We have used transient electric dichroism to study the ability of DNA-binding drugs to affect the folding of chromatin from the 10- to the 30-nm fiber, either by themselves or in conjunction with multivalent cations. Variables considered include the cationic charge of the drug, the comparative influence of intercalation and groove binding as modes of interaction, and the effect of bis-intercalation compared to mono-intercalation. In parallel with our findings with other cations, we observe that a drug must have a charge of 3+ or greater in order to condense chromatin at concentrations substantially lower than the concentration of chromatin, measured in base pairs. Drugs of low charge, whether groove binders or mono- or bis-intercalators, are unable to condense chromatin on their own. Bis-intercalators of high charge, however, are extremely efficient condensers, being able to cross-link chromatin with greater efficiency than polyamines of corresponding charge. When Mg^{2+} is used in combination with bis-intercalators of high charge, the order of addition of the two determines whether compaction or cross-linking is favored. Finally, the antibiotics actinomycin D, daunomycin, and distamycin, despite varied modes of binding to DNA, all inhibit the compaction of chromatin beyond a critical point in a remarkably similar manner.

A number of small organic molecules are able to bind to DNA, among them dyes and various antibiotics. Many of these have pharmacological properties and can therefore be regarded as drugs. Over the years a great deal of research has been done toward understanding the interaction of these drugs with DNA and the dynamics of the drug–DNA complexes (Neidel & Waring, 1983).

A frequently encountered, and certainly the most-studied, mechanism of binding of small molecules to DNA is intercalation. In this mechanism, flat molecules of polycyclic or heterocyclic aromatic substances can be inserted between the base pairs. Drugs that interact with DNA in this fashion are, among others, ethidium bromide, the actinomycins, and the antitumor drugs of the anthracycline family such as daunomycin and adriamycin. Other drugs, notably oligopeptides such as distamycin and netropsin, attach themselves to the outside of the helix, in the small groove. This kind of binding can be very strong and is frequently several orders of magnitude stronger than intercalative binding.

Other widely accepted mechanisms of binding include the stacking of planar molecules outside of the DNA helix. In all of these different modes of binding, electrostatic interactions between the ligand molecule and DNA provide a variable, and in some cases important, contribution to the binding energy.

In higher cells DNA exists in the form of the nucleoprotein complex chromatin in which the DNA is complexed with histone and non-histone proteins. In order to understand the pharmacology of DNA-binding drugs in higher organisms, it is necessary to understand the interactions of these drugs with chromatin. In most somatic cells, chromatin is a dynamic substance undergoing various modifications of structure in response to the metabolic state of the cell. The most fundamental of these dynamic processes is perhaps the reversible folding and unfolding of the chromatin fiber between the 30-nm-thick condensed form to a form lacking higher order organization, one that is identified with high rates of gene transcription (Labhart & Koller, 1982; Andersson et al., 1982).

In the preceding paper (Sen & Crothers, 1986) we have used electric dichroism to study the condensation behavior of isolated and size-fractionated chromatin fibers in response to a number of physiologically important ions of different charge. Having established the basic physical factors that underlie chromatin condensation in vitro, we are now in a position to study the influence of extrinsic agents such as DNA-binding drugs on chromatin condensation. We have taken three approaches to this problem: to study the effect of intercalative drugs as compared to that of groove-binding drugs; to study the effect of mono-intercalative drugs compared to that of bis-intercalative drugs; to study the effect of cationic charge

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