Ionic and Structural Specificity Effects of Natural and Synthetic Polyamines on the Aggregation and Resolubilization of Single-, Double-, and Triple-Stranded DNA†

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ABSTRACT: DNA condensation, precipitation, and aggregation are related phenomena involving DNA−DNA interactions in the presence of multivalent cations, and studied for their potential implications in DNA packaging in the cell. Recent studies have shown that the condensation/aggregation is a prerequisite for the cellular uptake of DNA for gene therapy applications. To elucidate the ionic and structural factors involved in DNA aggregation, we studied the precipitation and resolubilization of high molecular weight and sonicated calf thymus DNA, two therapeutic oligonucleotides, and poly(dA)·2Poly(dT) triplex DNA in the presence of the tetravalent polyamine spermine using a centrifugation assay, T_m measurements, and CD spectroscopy. The ability of spermine to provoke DNA precipitation was in the following order: triplex DNA > duplex DNA > single-stranded DNA. In contrast, their resolubilization at high polyamine concentrations followed a reverse order. The effective concentration of spermine to precipitate DNA increased with Na^+ in the medium. T_m data indicated the DNA stabilizing effect of spermine even in the resolubilized state. CD spectroscopy revealed a series of sequential conformational alterations of duplex and triplex DNA, with the duplex form regaining the B-DNA conformation at high concentrations (∼200 mM) of spermine. The triplex DNA, however, remained in a Ψ-DNA conformation in the resolubilized state. Chemical structural specificity effects were exerted by spermidine and spermine analogues in precipitating and resolubilizing sonicated calf thymus DNA, with N^4-methyl substitution of spermidine and a heptamethylene separation of the imino groups of spermine having the maximal difference in the precipitating ability of the analogues compared to spermidine and spermine, respectively. Therapeutically important bis(ethyl) substitution reduced the precipitating ability of the analogues compared to spermine. The effect of the cationicity of polyamines was evident with the pentamines being much more efficacious than the tetramines and triamines. These results provide new insights into the mechanism of DNA precipitation by polyamines, and suggest the importance of polyamine structure in developing gene delivery vehicles for therapeutic applications.

Several investigators have shown that multivalent cations, including polyamines, inorganic salts and complexes, and dehydrating solvents, can provoke the collapse of DNA to compact structural forms (*1*−*9*). Morphologically distinct DNA condensates, such as toroids and spheroids, are formed in the presence of polyamines and $Co(NH_3)_6^{3+}$ at very low concentrations (∼1 μg/mL) of relatively high molecular weight (>400 bp) DNA. This phenomenon has been studied as a model system for the packaging of DNA in viruses and to calculate the energetic forces involved in the collapse of the wormlike DNA chains to highly compacted forms (*10*). Wilson and Bloomfield (*8*) modified the counterion condensation theory, developed by Manning (*11*) and Record et al. (*12*), to determine the extent of DNA charge neutralization by multivalent cations and found that the collapse of DNA occurred at ∼89% phosphate charge neutralization by polyamines in aqueous solutions, although a slightly lower charge neutralization was sufficient for $Co(NH3)_6^{3+}$ (*8*). It is generally believed that the condensation of DNA is not associated with global conformational perturbations; however, the involvement of microstructural alterations cannot be ruled out at present. Interestingly, DNA sequences that are highly labile to undergo the right-handed B-DNA to left-handed Z-DNA transition facilitate the condensation of plasmid DNAs harboring these sequences (*13*).

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DNA precipitation and aggregation are related phenomena observed at high concentrations of DNA and triggered by the same agents that provoke condensation. In many cases, conformational alterations are found, especially the transition of B-DNA to the Ψ-DNA structure which is believed to be a tightly packaged, twisted, liquid-crystalline form of DNA (*14*−*19*). The CD spectrum of Ψ-DNA arises from the interaction of circularly polarized light with the highly ordered tertiary structure which depends on the proximity of the adjacent superhelical turns (*14*). The forces involved in the precipitation of DNA include charge neutralization as well as solvent characteristics, described by the Flory−Huggins parameter χ, which is the difference in free energy between solvent−solvent, polymer segment−segment, and solvent−segment interactions (*4*, *10*). DNA precipitation and aggregation by polyamines, polyaminolipids, and cationic lipids have acquired considerable importance in recent years as a model system to understand the mechanism of cellular uptake of oligonucleotides and recombinant gene constructs in gene therapy applications (*20*−*24*).

Pelta et al. (*3*) reported an interesting phenomenon involving precipitated DNA at high polyamine concentrations. They found that the addition of spermidine or spermine to calf thymus DNA fragments (∼146 bp) in the concentration range from 1 μg/mL to 1 mg/mL led to the precipitation of the DNA, followed by resolubilization of the precipitate at higher polyamine concentrations (>30 mM for spermidine and >70 mM for spermine in 10 mM Na^+). The DNA aggregates thus formed are anisotropic, containing cholesteric liquid-crystalline phases that flow spontaneously. We wished to examine the relevance of this phenomenon with therapeutically important oligonucleotides since electron microscopic studies suggest that condensation/aggregation is a prerequisite for transfection of oligonucleotides and plasmid vectors in living cells (*25*−*28*). Our previous studies (*29*) demonstrated that triplex DNA can undergo a Ψ-DNA transition; however, it is not known whether Ψ-DNA formation is associated with DNA precipitation. Thus, we examined the ability of spermine to precipitate and resolubilize single-stranded and triplex DNA, in addition to sonicated and unsonicated (native) calf thymus DNA. The single-stranded DNAs used in this study were two therapeutic oligonucleotides: (i) a 21-mer antisense oligonucleotide (sso21) targeted to c-*myc* mRNA, including its AUG translation initiation codon site (*30*); and (ii) a 37-mer oligonucleotide (sso37) directed to the promoter region of c-*myc* oncogene (*31*). As a model system for triplex DNA, we used poly(dA)·2poly(dT). Our results showed that the tendency of DNA to precipitate in the presence of spermine is related to the helical organization, with the following order: triplex DNA > duplex DNA > single-stranded DNA.

Structural specificity effects of polyamines on DNA stabilization, condensation, and conformational transitions have been reported by several investigators (*32*−*36*); however, no detailed studies on the role of polyamine structure in DNA precipitation/resolubilization phenomena have been reported so far. In the present study, we therefore examined the effects of three spermidine analogues and three bis(ethyl)-tetramine and two bis(ethyl)pentamine analogues of spermine on the precipitation and resolubilization of sonicated calf thymus DNA (please see Figure 1 for chemical structures of the compounds used in this study). Our results show that the efficacy of the polyamines to precipitate DNA increased with their cationicity. However, the polyamine structure, especially substitution at the imino group of spermidine and increased number of methylene groups between the imino groups of spermine, also exerted a remarkable effect on the ability of polyamines to precipitate and resolubilize DNA.

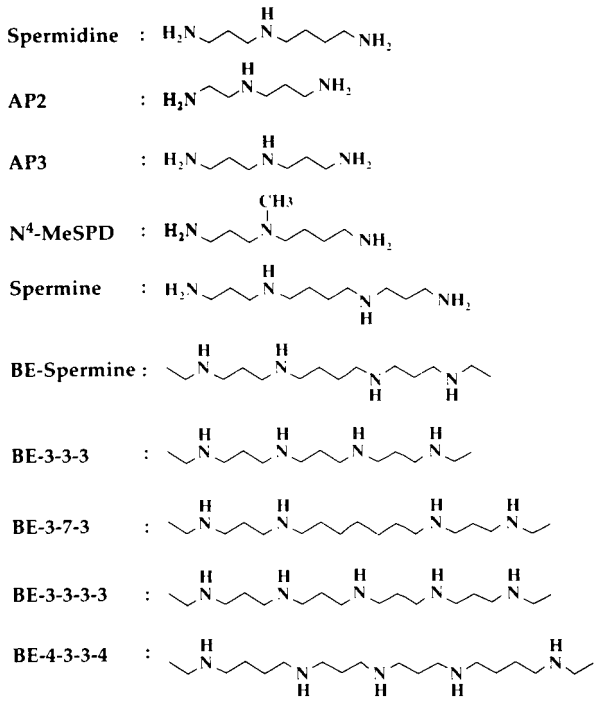

FIGURE 1: Chemical structures of polyamine analogues used in this study.

MATERIALS AND METHODS

Polyamines. Spermidine·3HCl and spermine·4HCl were purchased from Sigma Chemical Co. (St. Louis, MO). N^4-Methylspermidine·3HCl was synthesized and characterized as described previously (*29*). *N*-(2-aminoethyl)-1,3-propanediamine·3HCl and 3,3′-iminobispropylamine·3HCl were synthesized and purified as described by Thomas and Bloomfield (*35*). For convenience, these compounds are considered as aminopropyl derivatives of diamines and abbreviated as AP2 and AP3, respectively. Bis(ethyl) derivatives of spermine and its analogues, 3,7,12,16-tetrazaoctadecane·4HCl (BE-3-4-3 or BE-SPM), 3,7,11,15-tetrazaheptadecane·4HCl (BE-3-3-3), 3,7,15,19-tetrazahenicosane·4HCl (BE-3-7-3), 3,7,11,15,19-pentazahenicosane·5HCl (BE-3-3-3-3), and 3,8,12,16,21-pentazatricosane·5HCl (BE-4-3-3-4), were synthesized as described previously (*37*, *38*). The structures and purity of all polyamines were confirmed by elemental analysis, NMR, HPLC, and mass spectrometry. Concentrated solutions of polyamines were prepared in 10 mM cacodylate buffer (10 mM sodium cacodylate, pH 7.4, 0.5 mM EDTA), and appropriate volumes were used for precipitation experiments.

Ultrasonic Fragmentation of Calf Thymus DNA. Calf thymus DNA was purchased from Worthington Biochemicals (Freehold, NJ) and dissolved in 10 mM cacodylate buffer at a concentration of 2 mg/mL. The A_{260}/A_{280} ratio was determined to be 1.88, indicating that the DNA was free of protein contamination. Short DNA fragments were prepared by ultrasonication, following the procedure of Fukudome et

al. (*39*) using a Branson Sonicator (Branson Ultrasonics Corp., Danbury, CT). Agarose gel electrophoresis showed that the sonicated DNA was clustered around an average molecular size of ~145 bp. The sonicated sample was dialyzed extensively against 10 mM cacodylate buffer. The concentration of calf thymus DNA was determined by measuring the absorbance at 260 nm, and using the molar extinction coefficient (ϵ) of 6900 M^{-1} cm^{-1}.

Oligo- and Polynucleotides. The triplex-forming oligonucleotide that is capable of forming triplex DNA at the promoter sites of c-*myc* oncogene, 5′-GTGGTGGGGTGGTTGGGGTGGGTGGGGTGGGGT-3′ (sso37), and an antisense oligonucleotide that can hybridize with the *c-myc* mRNA, 5′-GAAGTTCACGTTGAGGGGCAT-3′ (sso21), were purchased from Oligos, Etc., Inc. (Wilsonville, OR), and were HPLC-purified. Both sso37 and sso21 were dissolved in 10 mM cacodylate buffer and dialyzed extensively against the same buffer before use. The molar extinction coefficients of sso37 and sso21 were 3.6×10^5 and 2.1×10^5 M^{-1} cm^{-1}, respectively.

Poly(dA) (MW 9.8×10^4) and poly(dT) (MW 8.9×10^4) were purchased from Pharmacia, Inc. (Piscataway, NJ). The polynucleotides were dissolved in 10 mM cacodylate buffer and dialyzed extensively against the same buffer. The concentration of polynucleotides was determined by measuring the absorbance of solutions in 10 mM cacodylate buffer, and molar concentration was calculated using the extinction coefficients of 8900 $M^{-1}cm^{-1}$ at 257 nm for poly(dA) and 9000 M^{-1} cm^{-1} at 265 nm for poly(dT). To prepare triplex DNA, poly(dA) and poly(dT) were mixed in 1:2 molar ratio in 10 mM cacodylate buffer and 10 μM spermine, incubated in a boiling water bath for 5 min, cooled to 22 °C, and allowed to equilibrate for 16 h at this temperature. Triplex DNA formation was confirmed by the characteristic double melting pattern and a strong negative peak at 210 nm in the CD spectrum (*40, 41*).

Centrifugation Assay for DNA Precipitation/Resolubilization Curves. Aliquots of the DNA were mixed with appropriate concentrations of NaCl and polyamines, and made up to 400 μL with the buffer. After vortexing for 15 s, the samples were incubated for 20 min at room temperature (22 °C) and centrifuged for 10 min at 11300*g* using a microfuge. The supernatant was separated and analyzed for the presence of DNA by determining the absorbance at 260 nm. All the absorbance measurements were performed with a microprocessor-controlled Beckmann DU 640 spectrophotometer at 22 °C, using a 350 μL quartz microcuvette (path length = 1 cm) at a wavelength of 260 nm. In most of our experiments, the concentration of DNA used was 0.75 $A_{260\ nm}$ unit. (There was no difference in the precipitation/resolubilization curves at a DNA concentration of 1.5 A_{260} units.) The amount of DNA in solution was calculated using the equation: percent DNA in solution = $(A_s/A_o) \times 100$; where A_s and A_o are the absorbance of the supernatant and control (in the absence of polyamines) at 260 nm, respectively.

T_m Measurements. The melting experiments were performed using a Beckman DU 640 spectrophotometer. The T_m block consists of six cells, each with a volume of ca. 0.35 mL, of which the first was filled with buffer and used as the blank. Absorbance (*A*) versus temperature (*T*) profiles were obtained by increasing the temperature at a rate of 0.5 °C/min from 30 to 100 °C, with accumulation of A_{260nm} at every 30 s. T_m values were taken as the temperatures corresponding to half-dissociation of the complexes, and the reproducibility was within ±0.5 °C. The first derivative, d*A*/d*T*, of the melting curve was computer-generated and was also used for determining T_m. In the case of duplex and triplex DNA studies conducted here, the T_m values obtained from both methods did not differ by more than 1 °C. All experiments were carried out in 10 mM cacodylate buffer, with appropriate concentrations of NaCl and polyamines.

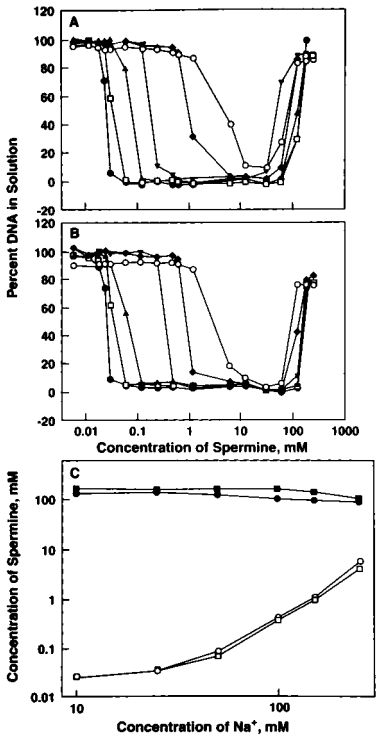

FIGURE 2: Precipitation/resolubilization of sonicated (A) and native (B) calf thymus DNA by spermine in the presence of 10 (●), 25 (□), 50 (▲), 100 (▼), 150 (◆), and 250 (○) mM Na^+. A logarithmic scale is used for spermine concentrations. Experiments were performed in 10 mM cacodylate buffer (pH 7.4). Higher concentrations of Na^+ were achieved by adding small volumes of 4 M NaCl solution. Panel C shows the concentrations of spermine required for 50% DNA precipitation (open symbols) and resolubilization (filled symbols) of sonicated (○, ●) and native (□, ■) calf thymus DNA. Logarithmic scale is used for both *X* and *Y* axes of panel C.

Circular Dichroism (CD) Experiments. The CD spectra were recorded using an Aviv 62DS circular dichroism spectrometer (Aviv Associates, Lakewood, NJ). All the measurements were made in 10 mM cacodylate buffer using 1 cm path length quartz cuvettes. The spectra were corrected by subtracting the signal from the buffer. Control experiments showed that none of the polyamines had measurable CD spectral characteristics.

RESULTS

Precipitation/Resolubilization, T_m, and CD Spectral Studies of Calf Thymus DNA in the Presence of Spermine. Figure 2 shows the effect of spermine on the precipitation and resolubilization of high molecular weight (A) and sonicated (B) calf thymus DNA. At low polyamine concentrations (<0.02 mM), there was no precipitation of DNA in 10 mM cacodylate buffer, as judged by the centrifugation assay. At higher concentrations of spermine, however, a decrease in

the absorbance at 260 nm of the supernatant was found, corresponding to the separation/precipitation of DNA from the solution phase. The DNA remained in the precipitated state from 0.05 to ~100 mM spermine; however, resolubilization of the precipitate occurred at higher concentrations, with almost 100% DNA in solution in the presence of 150 mM spermine. Ultrasonic fragmentation had no significant effect on the precipitation and resolubilization pattern of DNA under these ionic conditions. However, Na^+ had a significant effect on the concentration range at which spermine exerted the precipitating effect. The sharp precipitation and resolubilization curves found in these figures indicate a rapid phase separation process. To quantify the efficacy of spermine in these processes, we calculated its concentration necessary to precipitate [$EC_{50(ppt)}$] and resolubilize [$EC_{50(resol)}$] 50% of DNA.

The effect of Na^+ on the efficacy of spermine to precipitate and resolubilize calf thymus DNA is shown in Figure 2C in which the midpoint spermine concentrations are plotted against the concentration of Na^+ on a logarithmic scale. There was a 200-fold increase in $EC_{50(ppt)}$ as [Na^+] increased from 10 to 250 mM. The increase in [Na^+] decreased the effective precipitation range (Figure 2A,B); however, the precipitation was almost complete (>97%) for the whole range of Na^+ concentrations. The precipitation curves for high molecular weight and sonicated calf thymus DNA were almost indistinguishable.

The effective concentration of spermine for resolubilizing calf thymus DNA [$EC_{50(resol)}$] was less dependent on Na^+ (Figure 2C). Resolubilization was almost complete with 187 mM spermine between 10 and 100 mM Na^+ for high molecular weight and sonicated calf thymus DNA. There was a decrease in $EC_{50(resol)}$ at higher [Na^+].

We also examined the ability of spermine to precipitate calf thymus DNA in cacodylate buffer containing the approximate cellular levels of some common cations, i.e., 120 mM KCl, 10 mM NaCl, 2 mM $MgCl_2$, and 0.1 mM $CaCl_2$. The precipitation of DNA in this buffer was comparable to that in the presence of 150 mM Na^+ with >90% of DNA in the precipitated phase. However, spermine was very efficient to precipitate DNA under these physiologically relevant conditions, with an $EC_{50(ppt)}$ value of 0.475 mM compared to that of 1.05 mM in the presence of 150 mM Na^+. $EC_{50(resol)}$ values were comparable in both cases, 96.1 mM spermine at 150 mM Na^+ and 115 mM at physiologically compatible buffer conditions. Thus, the precipitation range was increased by the presence of KCl and divalent cations in the buffer, suggesting that the multivalent ions acted in concert to precipitate DNA from solution.

To examine the structure of resolubilized DNA, we next determined the T_m values of high molecular weight and sonicated DNA in the presence of different Na^+ (10, 25, 100, 150, and 250 mM) and spermine (0.015−150 mM) concentrations (Table 1). The T_m increased with Na^+, with a dT_m/d log [Na^+] value of 17 ± 0.4 for both high molecular weight and sonicated DNA. Our data showed that spermine stabilized duplex DNA at concentrations that retained the DNA in solution (<0.5 mM and >100 mM). However, the efficacy of spermine to increase the T_m decreased with Na^+. For example, ΔT_m in the presence of 150 mM spermine was 28.1 °C in 10 mM Na^+ in the buffer, whereas that in the presence of 250 mM Na^+ was only 3 °C. We examined the possibility that a shorter oligonucleotide (a duplex DNA of 37 base pair length) might show higher ΔT_m in the presence of 150 mM Na^+ and spermine. However, the ΔT_m values (3−5 °C) were comparable to that measured with sonicated calf thymus DNA (results not shown).

Table 1: Effect of Spermine on the T_m of Sonicated Calf Thymus DNA before Precipitation and after Resolubilization

[Na^+] (mM)	[spermine] (mM)	T_m, (°C)[a]
10	0.0	64.8
	0.015	82.9
	0.02	84.5
	100	92.9
	150	92.9
25	0.0	76.8
	0.02	81.8
	100	90.4
	150	91.0
100	0.0	83.2
	0.2	83.2
	100	92.3
	150	92.3
150	0.0	86.4
	0.5	87.9
	100	91.4
	150	94.3
250	0.0	89.3
	0.5	90.2
	100	90.8
	150	92.3

[a] T_m values are the average of two independent determinations. The T_m values were reproducible within 0.5 °C. The T_m values for unsonicated DNA were not significantly different from that of sonicated DNA shown here.

We next recorded the CD spectra to identify conformational changes induced by spermine on calf thymus DNA under the conditions of precipitation and resolubilization. The CD spectrum of calf thymus DNA has a weak positive band at 222 nm, a strong negative band at 245 nm, and a strong positive band at 277 nm (Figure 3). There was no significant change in the CD spectrum of calf thymus DNA at spermine concentrations up to 0.015 mM. However, intense bands peaking at 218 and 282 nm were seen in the CD spectrum of calf thymus DNA in the presence of 0.03 mM spermine, suggesting the formation of a Ψ-DNA structure. The 282 nm band extended up to 350 nm, indicating the formation of an aggregated structure (*42*). The intensity of both positive bands increased with polyamine concentration, and maximal ellipticity was seen at 0.05 mM. This change in spectral intensity of calf thymus DNA might indicate conformational changes in the aggregated form. The result of our CD spectral studies of calf thymus DNA in the presence of high concentrations (>25 mM) of spermine is shown in panel B. With 25 mM spermine, the spectral characteristics were similar to those of the aggregated structure before precipitation, but with reduced intensity. The band intensities increased with polyamine concentration, which was maximum at 100 mM spermine. At this concentration, the molar ellipticity at 282 nm was ~6-fold higher than that observed for the condensed/aggregated structure before precipitation (0.1 mM spermine). However, progressive addition of spermine resulted in a decrease in the intensity of the positive band characteristic of Ψ-DNA. The CD spectra at >125 mM

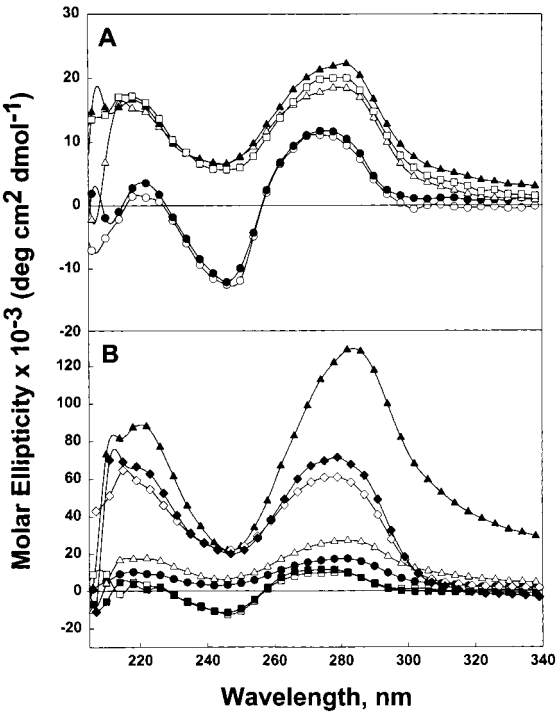

FIGURE 3: CD spectra of sonicated calf thymus DNA in the presence of spermine in a buffer containing 10 mM sodium cacodylate, 0.5 mM EDTA, pH 7.4. Concentrations of spermine are as follows. Panel A: 0 (○), 0.015 (●), 0.03 (△), 0.05 (▲), and 0.10 (□) mM; panel B: 25 (●), 75 (△), 90 (◇), 100 (▲), 110 (◆), 125 (□) and 200 (n) mM. Since the solutions were not centrifuged, panel B shows the CD spectra of either aggregated DNA (●, △) or resolubilized DNA (◇, ▲, ◆, □, ■).

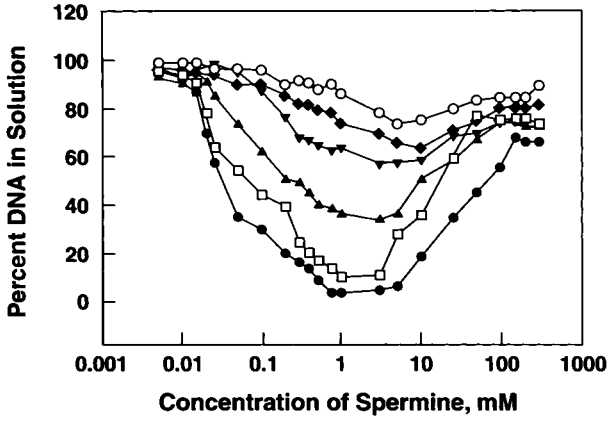

FIGURE 4: Precipitation/resolubilization curves of sso37 with spermine in the presence of 10 (●), 25 (□), 50 (▲), 100 (▼), 150 (◆), and 250 (○) mM Na^+. A logarithmic scale is used for spermine concentrations. Experiments were performed in 10 mM cacodylate buffer, pH 7.4.

were very similar to that of calf thymus DNA in the absence of polyamines, suggesting conformational similarity between the solution structures of DNA before precipitation and after resolubilization.

Effect of Spermine on sso37 and sso21. We next examined the ability of spermine to provoke the precipitation/resolubilization of two single-stranded and therapeutically important oligonucleotides (sso37 and sso21). As in the case of double-helical calf thymus DNA, sso37 and sso21 were also precipitated and resolubilized by spermine (Figures 4 and 5). However, the precipitation and resolubilization occurred in incremental steps over a wide range of spermine concentration, suggesting that the precipitation behavior might be different from that of duplex DNA. At 10 mM Na^+, sso37 precipitation was 95% complete with 1 mM spermine. The efficacy of spermine on sso37 precipitation decreased with increase in Na^+ in buffer, resulting in 42% precipitation at 3 mM spermine in the presence of 100 mM Na^+. At 150 and 250 mM Na^+, the amount of sso37 precipitated was only ~20−30%. The precipitation and resolubilization curves are almost symmetrical in appearance, with a shift of precipitation maxima to the right.

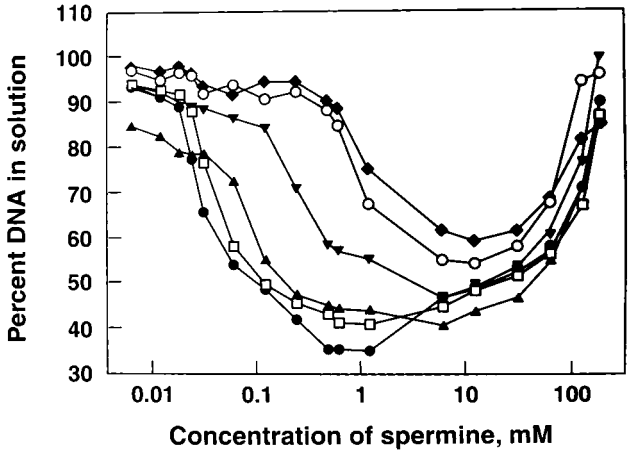

FIGURE 5: Precipitation/resolubilization curves of sso21 with spermine in the presence of Na^+ concentrations of 10 (●), 25 (□), 50 (▲), 100 (▼), 150 (○), and 250 (◆) mM. A logarithmic scale is used for spermine concentrations. Experiments were performed in 10 mM cacodylate buffer, pH 7.4.

Figure 5 shows that the ability of spermine to precipitate sso21 was not as efficient as that of sso37. At 10 mM Na^+, only 65% precipitation of sso21 occurred with a midpoint spermine concentration of 0.625 mM. The increase in [Na^+] had less effect on the amount of sso21 precipitation compared to that of sso37. Thus, 45% of sso21 was precipitated at 150 mM Na^+.

In the next series of experiments, we examined the effects of spermine on the CD spectra of sso37 and sso21. In 10 mM cacodylate buffer, the CD spectrum of sso37 had two positive bands at 210 and 260 nm and a negative band at 242 nm (Figure 6A). Initially, the peak intensity of the 260 nm band increased by ~2-fold in the presence of 0.015 mM spermine. On increasing the polyamine concentration further, the intensities of all three bands decreased, indicating the collapse of DNA. A red shift of about 5 nm was observed for the 260 nm positive peak at 10 mM spermine. In the resolubilized state (>100 mM spermine), the CD spectrum showed peak maxima at 210, 242, and 260 nm, with ellipticity values close to that found in the absence of spermine, suggesting that the conformational states of sso37 before precipitation and after resolubilization are similar, and that the structural changes were reversible.

The CD spectral changes of sso21 in the presence of spermine are depicted in Figure 6B. In 10 mM cacodylate buffer, its CD spectrum had two positive bands at 220 and 270 nm and a negative band at 245 nm. Increasing the polyamine concentration to 0.02 mM resulted in a small increase in ellipticity of the 270 nm band, which then decreased with further increase in polyamine concentration, without significant changes in the intensity of the 245 nm band. In the resolubilized state (>100 mM spermine), the

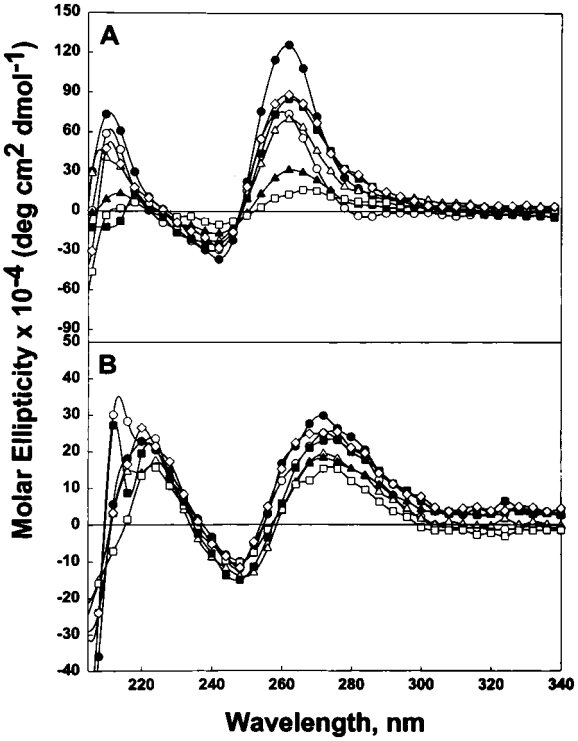

FIGURE 6: CD spectra of sso37 (A) and sso21 (B) in the presence of spermine in 10 mM cacodylate buffer. Concentrations of spermine are 0 (○), 0.015 (●), 0.05 (△), 0.1 (▲), 10 (□), 100 (■), and 200 (◇) mM for panels A and B.

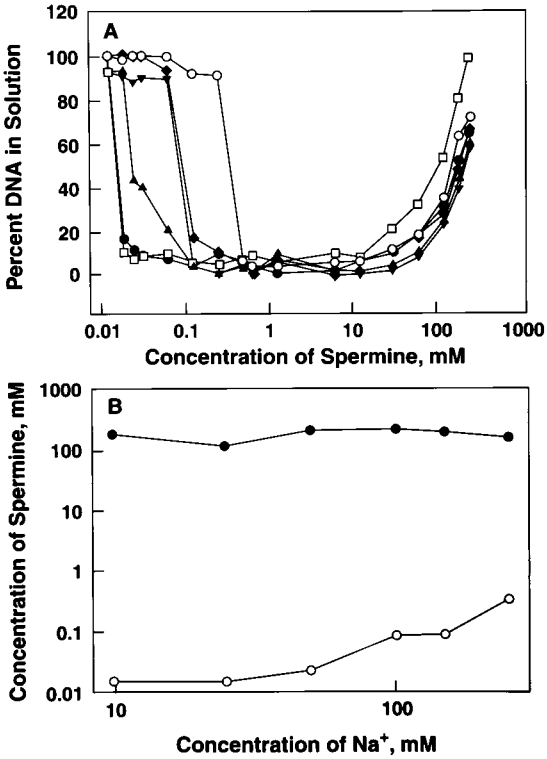

FIGURE 7: Precipitation/resolubilization curves of poly(dA)·2poly(dT) by spermine in the presence of Na^+ concentrations of 10 (●), 25 (□), 50 (▲), 100 (▼), 150 (◆), and 250 (○) mM (panel A). A logarithmic scale is used for spermine concentrations. In panel B, the midpoint polyamine concentrations for precipitation [$EC_{50(ppt)}$, open circles] and resolubilization [$EC_{50(resol)}$, filled circles] are plotted in a log−log scale.

intensity of the 270 nm band retained its original intensity, suggesting the reversible nature of these transitions. Although there are subtle differences in the CD spectra of sso21 and sso37, the trends for each oligonucleotide seem to be similar (initial increase in intensity, followed by a decrease with more spermine).

Effect of Spermine on Triplex DNA. We also examined the effects of spermine on the solution structural transitions of poly(dA)·2poly(dT) triplex. Figure 7A shows that the addition of spermine facilitated the precipitation of triplex DNA at >0.02 mM concentrations in the presence of 10 mM Na^+. Almost 100% precipitation occurred at all Na^+ concentrations; however, Na^+ affected the effective range of spermine concentration needed for precipitation. Resolubilization occurred at >100 mM spermine. The precipitation curves were sharp, whereas the resolubilization curves were incremental with increase in spermine concentration. Among the DNAs studied, triplex DNA required the lowest concentration of spermine for precipitation and the highest concentration for resolubilization. The midpoint curves showed an increase in $EC_{50(ppt)}$ with Na^+; however, $EC_{50(resol)}$ had only a marginal effect on increasing Na^+ (Figure 7B).

We next examined the effects of spermine on the CD spectrum of triplex DNA. In 10 mM cacodylate buffer, the CD spectrum of poly(dA) and poly(dT) mixed in a 1:2 molar ratio had a positive band at 220 nm, a negative band at 245 nm, and two weak positive bands at 258 and 280 nm (Figure 8). In the presence of 0.010 mM spermine, the characteristic 220 nm band reversed in sign, and an intense negative band was seen at 210 nm. The intensity of the 258 nm band increased while that of the 280 nm band decreased. These changes are characteristic of triplex DNA formation (*29*). With 0.025 mM spermine, the CD spectrum changed to

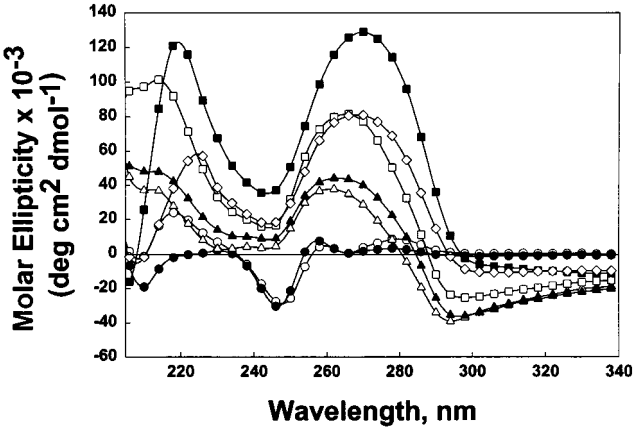

FIGURE 8: CD spectra of a poly(dA) and poly(dT) mixture (1:2 ratio) in the presence of spermine in 10 mM sodium cacodylate buffer. The spermine concentrations are 0 (○), 0.01 (●), 0.025 (△), 0.075 (▲), 0.15 (□), 100 (■), and 200 (◇) mM.

intense positive bands centered at 217 and 265 nm, a characteristic feature of Ψ-DNA. Maximum intensity of these bands was observed at 0.15 mM spermine in triplex DNA solution before precipitation. The band intensities decreased with further addition of spermine, and the CD signal almost disappeared at 1 mM (not shown in Figure 8), suggesting complete precipitation of triplex DNA. The Ψ-DNA CD spectrum showed maximum peak intensities at 100 mM spermine; however, the peak intensity was reduced by 2-fold at 200 mM, and the CD spectrum had the characteristics of a Ψ-DNA spectrum recorded at 0.15 mM spermine. This result suggests similarities in the conformation of spermine-

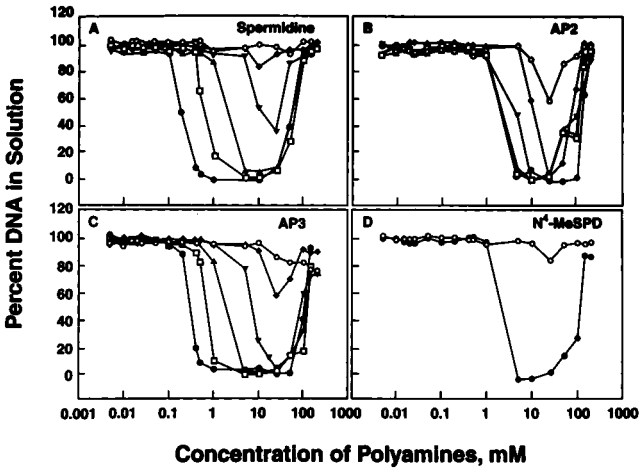

FIGURE 9: Precipitation/resolubilization curves of sonicated calf thymus DNA with spermidine (A), AP2 (B), and AP3 (C) in the presence of 10 (●), 25 (□), 50 (▲), 100 (▼), 150 (◆), and 250 (○) mM Na^+. N^4-MeSPD (D) was used only with 10 (●) and 150 (○) mM Na^+ concentrations. A logarithmic scale is used for polyamine concentrations.

bound triplex DNA before precipitation and after resolubilization. However, the broad negative band (~295 nm) for the aggregated structure before precipitation almost disappeared for the Ψ-DNA spectrum of the resolubilized DNA.

Effect of Chain Length Modification and Bis(ethyl) Substitution of Polyamines on the Precipitation/Resolubilization of Sonicated Calf Thymus DNA. To delineate the ionic and structural specificity effects of polyamines on DNA precipitation, we used three groups of compounds: (I) triamines: spermidine, AP2, AP3, and N^4-methylspermidine; (II) tetramines: BE-3-3-3, BE-3-4-3, and BE-3-7-3; and (III) pentamines: BE-3-3-3-3 and BE-4-3-3-4. The bis(ethyl)-polyamine analogues are being developed as chemotherapeutic agents for different forms of cancer (*43*).

Triamines. Figure 9 shows the effect of Group I compounds on the precipitation and resolubilization curves of sonicated calf thymus DNA. Almost 100% precipitation occurred at 0.4 mM spermidine in the presence of 10 mM Na^+. Resolubilization was complete at ~100 mM spermidine under the same ionic conditions. The precipitation curve shifted to the right with increased [Na^+], and a reduction in precipitation also occurred. At 250 mM Na^+, only ~5% DNA precipitated in the presence of 1−50 mM spermidine. AP3 followed a similar pattern; however, significant differences in the precipitation and resolubilization curves were observed with AP2. At high Na^+ concentrations (150−250 mM), spermidine and AP3 could not provoke the aggregation of DNA, whereas AP2 could induce precipitation. Although close concentration ranges were observed for spermidine and AP3 for precipitating DNA, the concentrations required for resolubilization were 2-fold higher for AP3 compared to spermidine. AP2 behaved in an entirely different manner: an increase in Na^+ concentration between 10 and 100 mM had only a negligible effect on the percentage of DNA precipitated. With 150 mM Na^+ in the medium, there was 40% DNA precipitation. When midpoint curves were compared (result not shown), spermidine and AP3 did not show appreciable differences in their ability to precipitate DNA. However, there were clear differences in their ability to resolubilize the precipitated DNA.

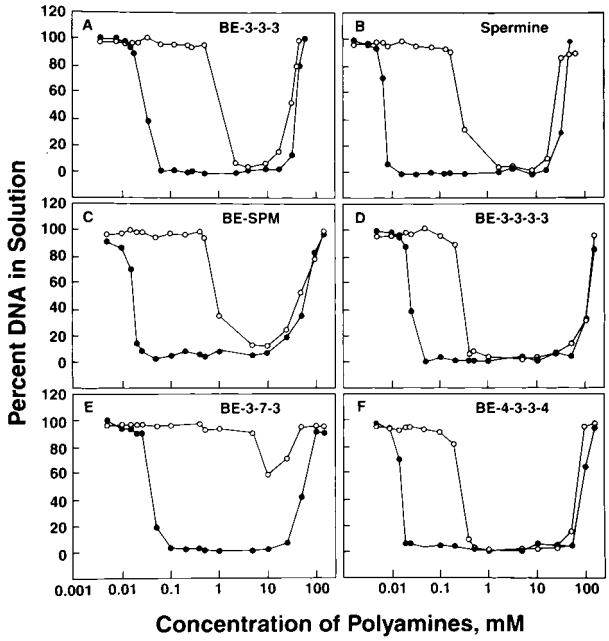

FIGURE 10: Precipitation/resolubilization curves of sonicated calf thymus DNA with BE-3-3-3 (A), spermine (B), BE-3-4-3 (BE-SPM) (C), BE-3-3-3-3 (D), BE-3-7-3 (E), and BE-4-3-3-4 (F) in the presence of 10 (●) and 150 (○) mM Na^+. A logarithmic scale is used for polyamine concentrations.

As in the case of spermine, we conducted the centrifugation assay in a physiologically compatible buffer (120 mM KCl, 10 mM NaCl, 2 mM $MgCl_2$, and 0.1 mM $CaCl_2$) with different concentrations of spermidine. The results were similar to that observed in the presence of 150 mM Na^+; i.e., DNA precipitation was negligible (~5%) in this buffer.

N^4-Methyl substitution of spermidine had a dramatic effect on the DNA precipitating efficacy of spermidine. Almost complete precipitation occurred over a narrow concentration range (1−5 mM) of N^4-methylspermidine at 10 mM Na^+ concentration, although $EC_{50(ppt)}$ was increased by 14-fold compared to that of spermidine (Figure 9D). At 150 mM Na^+, there was only ~18% precipitation, with a 5-fold excess of N^4-methylspermidine compared to that of spermidine. This result suggests the importance of the central imino group of spermidine in provoking structural transitions leading to the precipitation of DNA.

Bis(ethyl)tetramines. Figure 10 (panels A, C, and E) shows that the bis(ethyl)tetramines are excellent precipitating agents for sonicated calf thymus DNA at 10 mM Na^+. $EC_{50(ppt)}$ values were 0.05, 0.02, and 0.07 mM for BE-3-3-3, BE-3-4-3 (BE-SPM), and BE-3-7-3, respectively, indicating a structural specificity effect. $EC_{50(ppt)}$ values were 1.36 and 0.96 mM, respectively, for BE-3-3-3 and BE-3-4-3 at 150 mM Na^+, whereas the precipitation was less than 50% with BE-3-7-3. The resolubilization curves were less dependent on [Na^+], and values were 48, 64, and 73 mM for BE-3-3-3, BE-3-4-3, and BE-3-7-3 at 10 mM Na^+. For comparison, $EC_{50(ppt)}$ values were 0.085 and 0.44, respectively, for spermine at 10 and 150 mM Na^+. The $EC_{50(resol)}$ values were 37 and 55 mM at 150 mM Na^+ (panel B). These results show that alterations in the central methylene bridging region, as in BE-3-3-3 (panel C) and BE-3-7-3 (panel E), had no major effect on DNA precipitation at 10 mM Na^+; however, the hydrophobic effect of BE-3-7-3 became prominent at 150

Table 2: Effect of Spermine Analogues on the T_m of Sonicated Calf Thymus DNA before Precipitation and after Resolubilization

[Na^+] (mM)	[BE-3-7-3] (mM)	T_m (°C)	[BE-3-3-3-3] (mM)	T_m (°C)	[Be-4-3-3-4] (mM)	T_m (mM)
10	0.0	64.8	0.0	64.8	0.0	64.8
	0.02	82.9	0.02	81.5	0.01	84.4
	50	82.0	100	87.9	100	89.4
	100	85.7	150	87.5	150	88.8
150	0.0	86.5	0.0	86.5	0.0	86.5
	1.0	87.4	0.2	87.4	0.2	88.8
	50	84.9	100	87.4	100	87.4
	100	85.4	150	88.1	150	89.4

mM Na^+. In addition, bis(ethyl) substitution decreased the ability of a polyamine to precipitate DNA.

Bis(ethyl)Pentamines. Figure 10 also shows the efficacy of BE-3-3-3-3 (panel D) and BE-4-3-3-4 (panel F) to precipitate and resolubilize calf thymus DNA. Among the polyamines used in this study, these pentamines are the most efficient agents in precipitating calf thymus DNA, with almost complete precipitation in the range of 0.025−80 mM at 10 mM Na^+ buffer. The $EC_{50(ppt)}$ values calculated from these curves were 0.04 and 0.02 mM at 10 mM Na^+ and 0.44 and 0.43 mM at 150 mM Na^+, respectively, for BE-3-3-3-3 and BE-4-3-3-4. The corresponding $EC_{50(resol)}$ values were 171 and 85 mM in the presence of 150 mM Na^+.

Our T_m measurements (Table 2) showed that all of the bis(ethyl)polyamines were capable of stabilizing duplex DNA at 10 mM Na^+. However, their stabilizing effects were negligible at 150 mM Na^+. Therefore, the mechanism of bis(ethyl)polyamine involvement in duplex DNA stabilization may be different from the mechanism of their action in precipitating DNA.

DISCUSSION

This report presents the results of our detailed studies on the precipitation and resolubilization of calf thymus DNA by spermidine, spermine, and several of their structural analogues. Cationicity of the polyamines is the overriding factor governing the ability of polyamines to precipitate DNA, with the pentamines being more efficacious than the tetramines, which in turn are better than the triamines. The $EC_{50(ppt)}$ was strongly dependent on the monovalent ion concentration in the medium, whereas $EC_{50(resol)}$ was less dependent on Na^+ concentration. Our results showed no significant difference in the precipitating behavior of sonicated and unsonicated calf thymus DNA. In this respect, the precipitation phenomenon differs from "DNA condensation", which is defined as the collapse of DNA to aggregates/precipitates of finite size and morphology (*10*). In addition, DNA condensation is mainly found with duplex DNA of >400 bp size, whereas precipitation is observed even with a 21 bp single-stranded DNA (sso21).

Several theories have been advanced to explain the precipitation and resolubilization of DNA in the presence of polyamines (*3, 4, 10*). Phase separation is believed to occur due to poor solvent condition of the medium toward DNA as the concentration of the multivalent cations (polyamines) increases in the medium. When phase separation occurs, DNA is considered to be in the nonpolar or less polar phase, and the solvent phase becomes polar. The fluidity of the ordered phase suggests that the most probable binding of spermine would be along the strands, rather than interstrand cross-linking as suggested by Schellman and Parthasarathy (*34*), and binding along strands would allow the strands to slip past one another. After precipitation, the increase in polyamine concentration would increase the osmotic stress of the medium. By virtue of the nonpolar nature of polyamines above a threshold concentration, the osmotic stress would drive the polyamines to gain entry into the nonpolar DNA phase. The increase in polyamine concentration around DNA would decrease the number of binding sites per polyamine and disrupt the liquid-crystalline order of DNA. Moreover, the binding of polyamines with DNA in solution phase is delocalized as observed by the NMR (*44*) and photoaffinity cleavage experiments (*45*). Thus, the rapid collapse of order by increased polyamine binding makes the DNA hydrophilic enough to dissolve in polar aqueous medium, resulting in resolubilization. As soon as the DNA dissolves in aqueous medium, the competitive counterion binding of Na^+ and polyamines could be reestablished to maintain the equilibrium. This might be the reason the resolubilization process is insensitive to the ionic strength of the medium.

Previous investigations of DNA condensation and aggregation were carried out using duplex DNA (*1*−*10*). We extended these studies by using short oligonucleotides (sso21 and sso37) which are important to develop gene therapy applications (*46*−*49*). The increase in ionic strength of the medium had less involvement on the fraction of sso21 precipitated; however, sso37 precipitation was suppressed by increased [Na^+]. The single-stranded DNAs have the highest degree of conformational freedom in space, and the binding of polyamine is less likely to alter this freedom. Plum and Bloomfield (*50*) examined the binding of polyamines with single-stranded poly[d(AT)] and found that the binding affinities of polyamines were much lower to single-stranded DNA compared to double-stranded DNA. This difference was attributed to the charge spacing on these different forms: double-stranded DNA, 1.7 Å, and single-stranded DNA, 4.3 Å. Despite the single-stranded nature of sso21 and sso37, there were significant differences in their precipitation behavior in the presence of spermine which can be attributed, in part, to sequence specificity in the interaction of DNA with polyamines. Preferential binding of polyamines to certain DNA sequences has been reported by Marquet et al. (*51*). These findings are important to understand the mechanism of oligonucleotide uptake by living cells because cationic lipids and polyaminolipids are often used to facilitate oligonucleotide uptake (*25*−*28, 52*−*56*).

Our studies further demonstrate that triplex DNA formation facilitates the precipitating tendency of DNA. Triplex DNA as a structural entity has assumed much significance in recent years as a potential strategy for gene inactivation (*41, 57*−*62*). Among the three different groups of DNAs studied herein, spermine requirement for precipitation is the lowest (0.015 mM) and that for resolubilization is the highest (300 mM) for triplex DNA, providing the largest concentration range for precipitated DNA. Previous studies have shown that triplex DNA can undergo a facile transition to the Ψ-DNA structure in the presence of polyamines (*29*). Our present data show that the Ψ-DNA structure is retained even after resolubilization. A possible explanation for the enhanced aggregation of triplex DNA might be the increased affinity

of spermine for this form because of the decreased charge spacing (1.1 Å) imposed by the association of the third strand through the major groove of duplex DNA.

Another important feature of the present study is the demonstration of polyamine structural specificity effects in the precipitation/resolubilization of DNA. Among spermidine and its analogues studied, spermidine was the most effective triamine in precipitating DNA at low Na^+ concentrations, with the efficacy in the order of spermidine ≫ AP3 ≫ AP2 > N^4-MeSPD. This trend reversed gradually with increase in Na^+ concentration, and AP2 became more effective than spermidine at high Na^+ concentrations. A similar situation was observed with spermidine and its analogues on the melting of DNA (*35*), and the observed differences were attributed to charge density considerations and ionic strength dependence of p*K* values (*32*). Methylation of spermidine (i.e., N^4-MeSPD) altered the precipitation behavior drastically. The methyl group may be sterically preventing the DNA−DNA interactions necessary for multimolecular collapse resulting in precipitation/aggregation.

Our T_m data confirmed the known stabilizing effects of polyamines on duplex DNA even in the resolubilized phase (*35*). Although ΔT_m values were very significant with polyamine addition at low Na^+ concentrations, these values became negligible at high (150 and 250 mM) [Na^+], suggesting a complex interplay of Coulombic repulsions and ion condensation (*35*). Alternately, these small ΔT_m values at high Na^+ concentrations (150 and 250 mM) may not reflect the relative amounts of spermine and Na^+ bound with DNA because the molecular interactions could easily be altered at the elevated temperatures used in the melting studies. In addition, the melting temperature of the model system (calf thymus DNA) has reached a plateau before any test ligand has been added, and hence the system is insensitive to added ligand.

In summary, the present work shows the structural specificity effects of natural polyamines and their synthetic analogues on the precipitation and resolubilization of single-, double-, and triple-stranded DNA. The efficacy of polyamines to induce precipitation followed the order: pentamines > tetramines > triamines. The helical nature of DNA was important in the precipitation/resolubilization phenomenon. The order of precipitation was triplex > duplex > single-stranded DNA, and the resolubilization followed the reverse order. Duplex melting studies indicated that the DNA is stabilized by polyamines not only in the precipitation phase but also in the resolubilized state. The ionic concentration of the medium had a profound influence on the precipitation phenomenon, whereas it had only negligible effect on the resolubilization phenomenon.

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