

Differential effects of spermine and its analogues on the structures of polynucleotides complexed with ethidium bromide

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The interactions of spermine and polyamine analogues with synthetic polynucleotides of various base sequences complexed with ethidium bromide (EB) were investigated using measurements of fluorescence intensity and steady-state fluorescence polarization. Spermine and polyamine analogues displaced some but not all of the EB bound to poly(dA-dT)·poly(dA-dT) or poly(dG-dC)·poly(dG-dC), suggesting that polyamines may stabilize these polynucleotides in a conformation with reduced affinity for EB. Modifications of the aliphatic backbone of spermine have pronounced effects on its ability to displace

EB from poly(dA-dT)·poly(dA-dT) but not from poly(dG-dC)·poly(dG-dC). Spermine and some but not all of the polyamine analogues caused fluorescence depolarization when they interacted with the complex of EB and poly(dA-dT)·poly(dA-dT). Neither spermine nor any of the analogues, however, induced fluorescence depolarization in the complex of EB with poly(dG-dC)·poly(dG-dC) or poly(dA)·poly(dT). This suggests that spermine and some spermine analogues induce structural changes specific to alternating A-T sequences.

INTRODUCTION

The polyamines putrescine, spermidine and spermine are biological cations involved in regulating cell growth and differentiation [1–3]. *In vitro* studies have shown that the binding of spermine induces specific structural changes in DNA [4–7]. Molecular modelling and physicochemical studies suggest that these changes involve a bend in the axis of the DNA helix at specific sequences [8–13].

Ethidium bromide (EB) is a cationic dye that interacts cooperatively with specific DNA sequences by intercalating its phenanthridium ring between adjacent base pairs [14–17]. This interaction is characterized by an increase in the fluorescence quantum yield of EB [18]. At low ionic strength, a weaker non-intercalating binding is observed in addition to this strong binding [19,20]. The second binding is mainly electrostatic and appears to make only a minor contribution to the overall fluorescence enhancement [20,21]. The binding of EB also restricts its rotational degrees of freedom, increasing its steady-state fluorescence polarization [18]. Polarization is a function of the rotational motion of EB bound in strong and weak modes of the internal motions of DNA and of resonance energy transfer between bound dye molecules at high EB/P ratios [22,23].

Many compounds that bind to DNA, including non-intercalating agents such as spermine, can displace EB from EB–DNA complexes [24,25]. Cain et al. [24] have shown a correlation between the affinity of a given binding agent for DNA and the agent's efficiency at displacing EB. The higher efficiency of spermine in displacing EB from poly(dG-dC)·poly(dG-dC) than from poly(dA-dT)·poly(dA-dT) has therefore been interpreted as higher affinity of spermine for the former polynucleotide [25].

The interpretation of such results may not be straightforward, however, for two reasons. First, the affinity of EB for DNA and the co-operativity in its binding depend not only on DNA base sequence but also on environmental conditions such as buffer composition and ionic strength [26–28]. Second, the mechanism of EB displacement by non-intercalating agents may be non-competitive and instead may involve changes in DNA conformation.

Here we present the results of fluorimetric studies analysing the interactions of spermine and several polyamine analogues with synthetic polynucleotide–EB complexes. Steady-state fluorescence polarization, which is commonly used to determine the molecular mobility of the complex in the region of the bound dye, was used to probe conformational changes in EB–DNA complexes upon binding of spermine. Our results provide evidence for sequence specificity in the binding of spermine and changes in the structure of EB complexed with specific DNA sequences. Modifications in the aliphatic backbone of spermine also have an effect on these phenomena.

MATERIALS AND METHODS

Materials

Poly(dA-dT)·poly(dA-dT), poly(dA)·poly(dT) and poly(dG-dC)·poly(dG-dC) (Pharmacia, P-L Biochemicals, Milwaukee, WI, U.S.A.), sodium cacodylate and EB (Sigma Chemical Co., St. Louis, MO, U.S.A.) and spermine (Calbiochem, La Jolla, CA, U.S.A.) were purchased from commercial suppliers. Polyamine analogues were generous gifts from Dr. P. Bey (Marion Merrell Dow Research Institute, Cincinnati, OH, U.S.A.), Professor R. J. Bergeron (University of Florida,

Abbreviations used: EB, ethidium bromide; EB/P, ethidium bromide/DNA phosphate; abbreviations used for polyamine analogues are defined in Table 1.

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Table 1 Polyamine analogues used in the present study

The IUPAC names and chemical formulae of polyamine analogues are listed along with their abbreviated names. The abbreviations are used throughout the text of the article.

Abbreviation used here	Systematic (IUPAC) name	Chemical formula
Spermine (3-4-3)	1,12-Diamino-4,9-diazadodecane	$\text{NH}_2\text{-(CH}_2\text{)}_3\text{-NH-(CH}_2\text{)}_4\text{-NH-(CH}_2\text{)}_5\text{-NH}_2$
4-4-4	1,14-Diamino-5,10-diazatetradecane	$\text{NH}_2\text{-(CH}_2\text{)}_4\text{-NH-(CH}_2\text{)}_4\text{-NH-(CH}_2\text{)}_4\text{-NH}_2$
3-3-3 (Norspermine)	1,11-Diamino-4,8-diazaundecane	$\text{NH}_2\text{-(CH}_2\text{)}_3\text{-NH-(CH}_2\text{)}_3\text{-NH-(CH}_2\text{)}_3\text{-NH}_2$
3-2-3	1,10-Diamino-4,7-diazadecane	$\text{NH}_2\text{-(CH}_2\text{)}_3\text{-NH-(CH}_2\text{)}_2\text{-NH-(CH}_2\text{)}_3\text{-NH}_2$
BESm	1,12-Bisethylamino-4,9-diazadodecane	$\text{C}_2\text{H}_5\text{-NH-(CH}_2\text{)}_3\text{-NH-(CH}_2\text{)}_4\text{-NH-(CH}_2\text{)}_3\text{-NH-C}_2\text{H}_5$
BE-4-4-4	1,14-Bisethylamino-5,10-diazatetradecane	$\text{C}_2\text{H}_5\text{-NH-(CH}_2\text{)}_4\text{-NH-(CH}_2\text{)}_4\text{-NH-(CH}_2\text{)}_4\text{-NH-C}_2\text{H}_5$
BE-3-3-3	1,11-Bisethylamino-4,8-diazaundecane	$\text{C}_2\text{H}_5\text{-NH-(CH}_2\text{)}_3\text{-NH-(CH}_2\text{)}_3\text{-NH-(CH}_2\text{)}_3\text{-NH-C}_2\text{H}_5$
BE-3-2-3	1,10-Bisethylamino-4,7-diazadecane	$\text{C}_2\text{H}_5\text{-NH-(CH}_2\text{)}_3\text{-NH-(CH}_2\text{)}_2\text{-NH-(CH}_2\text{)}_3\text{-NH-C}_2\text{H}_5$
4-4-4-4	1,19-Diamino-5,10,15-triazanonadecane	$\text{NH}_2\text{-(CH}_2\text{)}_4\text{-NH-(CH}_2\text{)}_4\text{-NH-(CH}_2\text{)}_4\text{-NH-(CH}_2\text{)}_4\text{-NH}_2$
Be-4-4-4-4	1,19-Bisethylamino-5,10,15-triazanonadecane	$\text{C}_2\text{H}_5\text{-NH-(CH}_2\text{)}_4\text{-NH-(CH}_2\text{)}_4\text{-NH-(CH}_2\text{)}_4\text{-NH-(CH}_2\text{)}_4\text{-NH-C}_2\text{H}_5$

Gainesville, FL, U.S.A.) and Professor K. Samejima and Dr. A. Shirahata (Josai University, Sakado, Saitama, Japan). The names and structures of polyamine analogues used in this study are shown in Table 1, and the abbreviations shown in the Table are used throughout this manuscript. All other reagents were reagent grade, and distilled deionized water was used as the solvent. All reagents other than polynucleotides were used without further purification. Experiments were carried out at room temperature in a low ionic strength buffer (5 mM NaCl, 1 mM sodium cacodylate, pH 7) to maximize the interaction of spermine and analogues with DNA.

Preparation of polynucleotide solutions

Synthetic polynucleotides were dissolved in 1 mM EDTA, 1 mM Tris/HCl (pH 7), dialysed against high- and low-salt buffers with or without EDTA [12], and stored in 1 mM Tris/HCl (pH 7) at -20°C . The concentrations of nucleic acids were determined spectrophotometrically in terms of their nucleotide residues using average molar absorption coefficients at 260 nm of 6000 for poly(dA)·poly(dT), 6600 for poly(dA-dT)·poly(dA-dT) and 8400 for poly(dG-dC)·poly(dG-dC) [29].

Fluorimetric studies

Fluorescence (excitation 546 nm, emission 595 nm) was monitored in a Fluorolog 2 photon-counting spectrofluorimeter (Spex Industries Inc., Edison, NJ, U.S.A.) coupled to an IBM XT-compatible personal computer for collection and storage of data. All measurements were corrected for dilution caused by ligand addition.

Fluorescence titration of EB was carried out with poly(dA-dT)·poly(dA-dT), poly(dG-dC)·poly(dG-dC) and poly(dA)·poly(dT). Plots of fluorescence enhancement versus polynucleotide concentration were used to determine the amount of polynucleotide needed to achieve 95% of the maximum fluorescence enhancement caused by $2\ \mu\text{M}$ EB (results not shown). The concentrations used were $9.8\ \mu\text{M}$ for poly(dG-dC)·poly(dG-dC), $10.6\ \mu\text{M}$ for poly(dA-dT)·poly(dA-dT) and $22.2\ \mu\text{M}$ for poly(dA)·poly(dT). The concentrated polynucleotide solution was added sequentially to 3 ml of buffer containing $2\ \mu\text{M}$ EB in a fluorescence cuvette. The release of EB from the EB–nucleic acid complexes was studied by adding small volumes of concentrated solutions of spermine, spermine analogues or MgCl_2 as described elsewhere [25].

Fluorescence-polarization studies

The steady-state fluorescence polarization was measured using a Spex Fluorolog 2 spectrofluorimeter fitted with polarizing and analysing filters. The steady-state polarization (p) was calculated from the equation

$$p = (I_{vv} - GI_{vh}) / (I_{vv} + GI_{vh})$$

where I is the observed emission intensity with the first and second subscript referring to the vertical (v) or horizontal (h) orientation of the polarizer and analyser respectively and G is an instrumental factor given by $G = I_{hv}/I_{hh}$ [18].

The fractions of free and bound dye, determined from the fluorescence intensities as described previously [18], were used to calculate the polarization of bound EB. These calculations were performed using the equation for the polarization (p) of a multicomponent solution

$$(1/p) - (1/3) = \frac{1}{\sum \Phi_n / [(1/p_n) - (1/3)]}$$

where p_n is the polarization and Φ_n the fraction of the light emitted by the n^{th} species [30].

RESULTS

Fluorescence-intensity measurement

The addition of spermine to complexes of EB with poly(dG-dC)·poly(dG-dC) or poly(dA-dT)·poly(dA-dT) caused a decrease in the fluorescence intensity (Figures 1 and 2) and shifted the maximum of fluorescence emission to a higher wavelength (results not shown). For both EB–polynucleotide complexes, the fluorescence intensity reached a plateau at a level significantly above the fluorescence intensity of EB alone. The plateau reached with EB–poly(dG-dC)·poly(dG-dC), however, was significantly lower than that reached with EB–poly(dA-dT)·poly(dA-dT). Mg^{2+} released EB from those polynucleotides to an extent comparable with that of spermine (i.e. EB was released up to a point, and then plateaued), but at a concentration 1000 times as high as that required for spermine (results not shown).

Modifications of the spermine backbone changed the efficiency of the polyamine for displacing EB from poly(dA-dT)·poly(dA-dT) (Figure 1a and 1b). All of the analogues tested, except for 3-2-3 and its bis-ethylated derivative BE-3-2-3, were more efficient than spermine in displacing EB from poly(dA-dT)·poly(dA-dT).

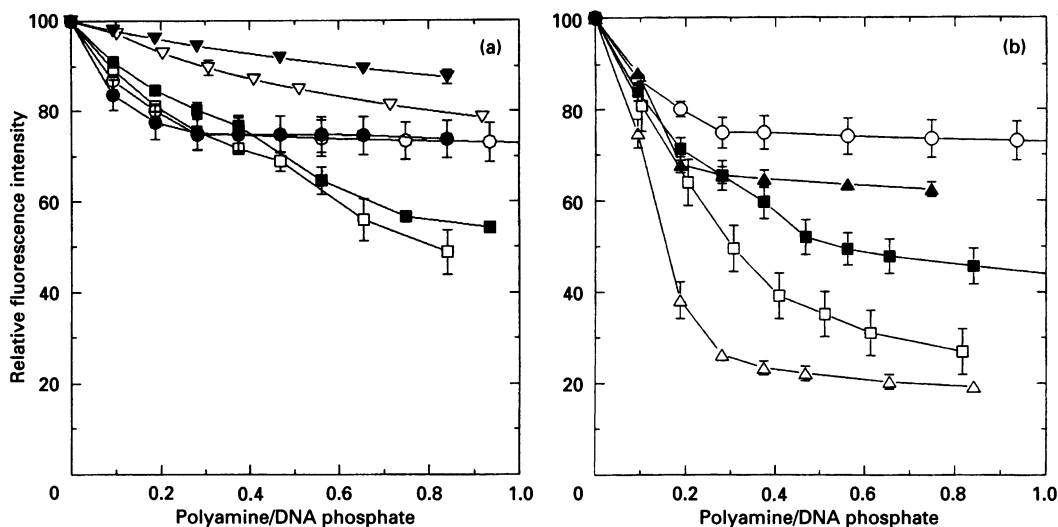


Figure 1 Changes in the fluorescence intensity of EB-poly(dA-dT)·poly(dA-dT) complexes in the presence of increasing concentrations of spermine or analogues

(a) Effect of spermine (○), BESm (●), 3-2-3 (▽), BE-3-2-3 (▼), 3-3-3 (□) or BE-3-3-3 (■). (b) Effect of spermine (○), 4-4-4 (□), BE-4-4-4 (■), 4-4-4-4 (△) or BE-4-4-4-4 (▲). Each point is an average of three observations with less than 5% variability. Error bars, where not visible, are smaller than the symbol size.

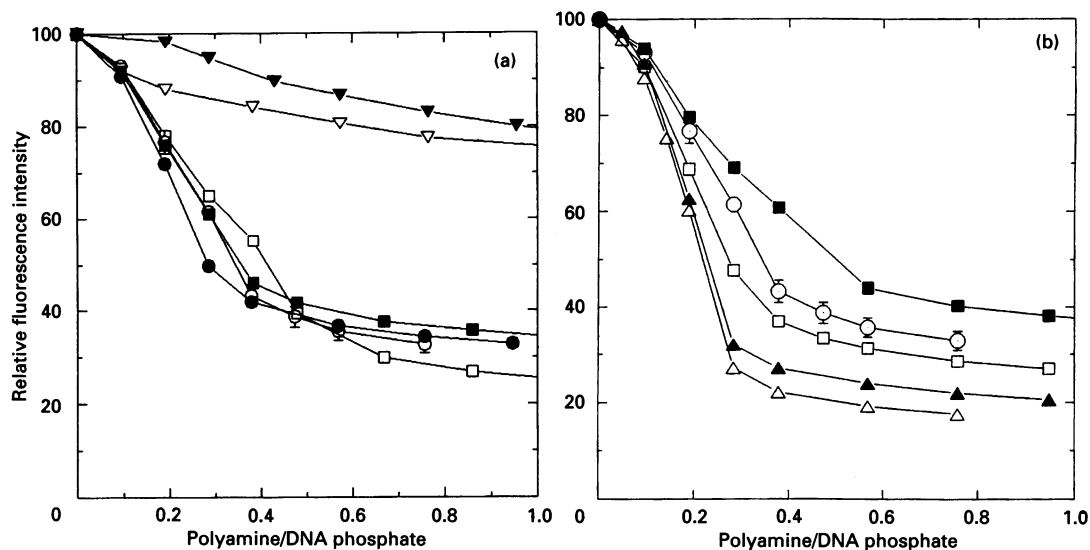


Figure 2 Changes in the fluorescence intensity of EB-poly(dG-dC)·poly(dG-dC) complex in the presence of increasing concentrations of spermine or analogues

(a) Effect of spermine (○), BESm (●), 3-2-3 (▽), BE-3-2-3 (▼), 3-3-3 (□) or BE-3-3-3 (■). (b) Effect of spermine (○), 4-4-4 (△), BE-4-4-4 (▲), 4-4-4-4 (□) or BE-4-4-4-4 (■). Each point is an average of two observations with less than 5% variability. Error bars, where not visible, are smaller than the symbol size.

The order of efficiency at displacing EB was as follows: BE-3-2-3 < 3-2-3 < spermine = BESm < BE-4-4-4-4 < BE-3-3-3 = 3-3-3 < BE-4-4-4 < 4-4-4 < 4-4-4-4.

Modification of polyamine structure had a smaller effect on the efficiency of displacing EB from poly(dG-dC)·poly(dG-dC). All analogues had a displacement efficiency comparable with spermine, except for 3-2-3 and BE-3-2-3, which had very low efficiencies compared with spermine (Figure 2a and 2b).

Steady-state fluorescence polarization

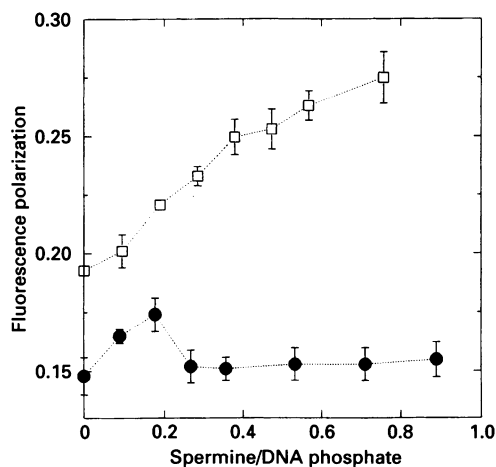
The fluorescence polarization of EB bound to DNA was substantially greater than that for free EB, and the fluorescence polarization of EB bound to poly(dG-dC)·poly(dG-dC) was higher than that of EB bound to poly(dA-dT)·poly(dA-dT) (Table 2).

The addition of spermine to the EB-poly(dG-dC)·poly(dG-

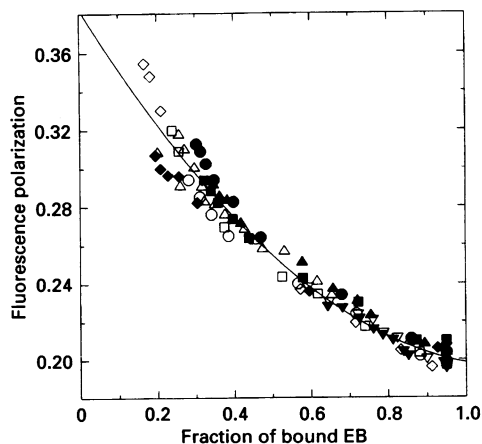
Table 2 Fluorescence polarization of free and bound EB

Steady-state fluorescence polarization values of free EB and EB complexed with polynucleotides were measured at room temperature in a solution containing 5 mM NaCl and 1 mM sodium cacodylate (pH 7) using a Spex Fluorolog 2 spectrofluorimeter fitted with polarizing and analysing filters. The EB/P ratio was 0.19 for poly(dA-dT)·poly(dA-dT) and 0.20 for poly(dG-dC)·poly(dG-dC).

Sample	Fluorescence polarization (number of experiments)
Free EB	0.048 ± 0.015 (15)
+ poly(dA-dT)·poly(dA-dT)	0.143 ± 0.008 (10)
+ poly(dG-dC)·poly(dG-dC)	0.191 ± 0.006 (10)

**Figure 3** Changes in the fluorescence polarization of EB–polynucleotide complexes in the presence of increasing concentrations of spermine

Effect of spermine on the fluorescence polarization of EB complexed with poly(dA-dT)·poly(dA-dT) (●) or with poly(dG-dC)·poly(dG-dC) (□). Each point is an average of three observations with less than 5% variability. Error bars, where not visible, are smaller than the symbol size.

**Figure 4** Fluorescence polarization of EB bound to poly(dG-dC)·poly(dG-dC) in the presence of spermine or analogues

Correlation between the polarization and the fraction of EB bound to poly(dG-dC)·poly(dG-dC) in the presence of spermine (○), BESm (●), 3-2-3 (▽), BE-3-2-3 (▼), 3-3-3 (□), BE-3-3-3 (■), 4-4-4 (△), BE-4-4-4 (▲), 4-4-4-4 (◇), BE-4-4-4-4 (◆) or Mg²⁺ (△).

dC) complex caused a monotonic increase in fluorescence polarization (Figure 3). Similar increases were observed on addition of the polyamine analogues or Mg²⁺ (results not shown). With spermine, the analogues and Mg²⁺, increases in fluorescence polarization of EB–poly(dG-dC)·poly(dG-dC) were directly related to decreases in the fraction of bound EB (Figure 4).

When spermine was added to the EB–poly(dA-dT)·poly(dA-dT) complex, however, polarization initially increased but then decreased (Figure 3). This depolarization occurred when the fraction of bound EB was less than 75% (Figure 5a). A similar depolarization was observed when the bis-ethylated derivatives of spermine (BE-3-4-3) or norspermine (BE-3-3-3) were added to the EB–poly(dA-dT)·poly(dA-dT) complex (Figure 5a). Addition of BE-4-4-4-4 to the poly(dA-dT)·poly(dA-dT)–EB complex induced a depolarization slightly different from that observed with spermine (Figure 5b). No depolarization was seen with 3-2-3, BE-3-2-3, 3-3-3, 4-4-4, BE-4-4-4, 4-4-4-4 or Mg²⁺ (Figure 5a and 5b).

When spermine or Mg²⁺ was added to EB–poly(dA)·poly(dT), no depolarization was observed (Figure 6).

DISCUSSION

The binding of spermine and its analogues to DNA causes a partial release of bound EB from DNA. The fluorescence intensity of the EB–polynucleotide complexes in the presence of spermine or the analogues plateaued above the fluorescence intensity of free EB. This indicates that a fraction of EB remains bound to DNA even at a spermine/nucleotide ratio equal to 1. The fraction of bound EB remaining is dependent on the base sequence of the polynucleotide, is smaller in poly(dG-dC)·poly(dG-dC) than in poly(dA-dT)·poly(dA-dT), and is independent of spermine or analogue concentration. Although spermine has higher affinity for G-C than for A-T sequences, shown in gel-filtration [31] and equilibrium-binding studies [32], it may be erroneous to correlate directly the extent of EB released with the affinity of spermine for a specific base sequence. The affinity of spermine analogues for calf thymus DNA, as determined from u.v. melting studies, does not always correlate with the extent of bound EB released from DNA [33].

At least two explanations for the incomplete release of EB are possible. The first is that polyamines may reduce the number of binding sites on DNA for EB, as has been found in EB binding to tRNA in the presence of spermine [35]. The second possibility is that polyamines may reduce the affinity of EB for DNA. Mg²⁺ decreases the apparent equilibrium binding constant of EB for DNA mainly through a conformational change in DNA, which increases the rate constant for dissociation but does not alter the number of binding sites [18]. The polycationic nature of spermine might allow a similar mechanism, in which spermine stabilizes a DNA conformation with decreased affinity for EB.

Because minor changes in the lengths of the aliphatic chains of spermine greatly alter its efficiency at displacing EB, it appears that the carbon chain length of the polyamine moiety is critical to its interaction with poly(dA-dT)·poly(dA-dT). Bis-ethylation of the analogues either had no effect or decreased their efficiency at displacing EB. No obvious correlation can be drawn between the structure of a spermine analogue and its efficiency in releasing EB from poly(dA-dT)·poly(dA-dT). Structural changes had a smaller effect on the release of EB from poly(dG-dC)·poly(dG-dC). With both poly(dA-dT)·poly(dA-dT) and poly(dG-dC)·poly(dG-dC), 3-2-3 and BE-3-2-3 were very inefficient at displacing EB. These two analogues have very low pKs and very low affinities for DNA [33,36–38], which may explain their poor ability to release EB.

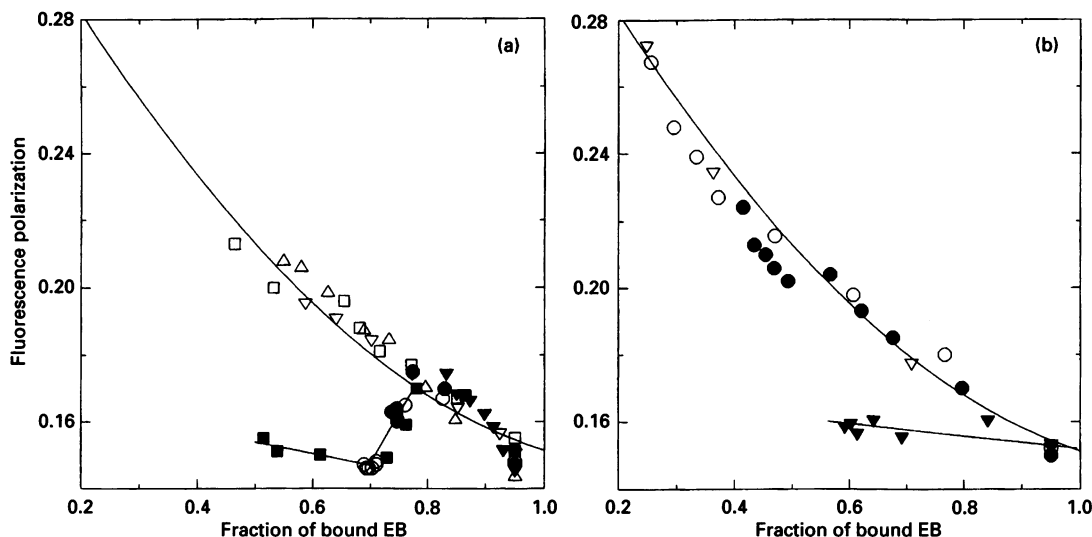


Figure 5 Fluorescence polarization of EB bound to poly(dA-dT)·poly(dA-dT) in the presence of spermine or analogues

Correlation between the polarization and the fraction of EB bound to poly(dA-dT)·poly(dA-dT) in the presence of (a) spermine (○), BESm (●), 3-2-3 (▽), BE-3-2-3 (▼), 3-3-3 (□), BE-3-3-3 (■), Mg²⁺ (△) and (b) 4-4-4 (○), BE-4-4-4 (●), 4-4-4-4 (▽) or BE-4-4-4-4 (▼).

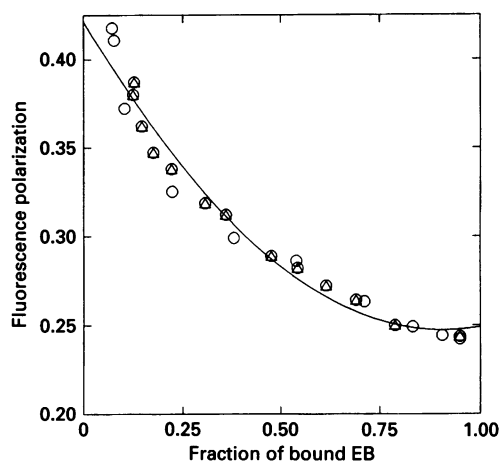


Figure 6 Fluorescence polarization of EB bound to poly(dA)·poly(dT) in the presence of spermine

Correlation between the polarization and the fraction of EB bound to poly(dA)·poly(dT) in the presence of spermine (○) or Mg²⁺ (△).

The increase in steady-state fluorescence polarization of the EB-DNA complex seen on addition of spermine and the analogues may be explained by a reduction in resonance energy transfer. Such energy transfer between closely bound dye molecules, seen when EB/P is greater than 1/75 [39], is an important source of depolarization in these complexes. The release of EB from the polynucleotide is therefore accompanied by a reduction in the energy transfer and an increase in polarization. This explanation is supported by the inverse correlation between the fluorescence polarization and the amount of EB bound to poly(dG-dC)·poly(dG-dC) (Figure 5) or poly(dA)·poly(dT) (Figure 6).

With poly(dA-dT)·poly(dA-dT), in contrast, a consistent depolarization was observed when spermine, BE-3-4-3, BE-3-3-

3 or BE-4-4-4-4 had released about 25% of the bound EB. There was no direct relationship between the efficiency of displacement and depolarization. In fact, although 3-3-3 and BE-3-3-3 displaced EB with the same efficiency, only BE-3-3-3 induced depolarization. Because none of the analogues affected the fluorescence lifetime of the EB-DNA complex (results not shown), the reason for depolarization is not obvious. The polynucleotides used in this study are longer than their persistence length. Thus the fluorescence polarization is a result of the motion of the dye, either alone or with its adjacent base pairs, and not the result of the movement of the polynucleotide molecule. Therefore changes in polarization must represent a change in the dye environment [23,40,41]. An increase in the number of degrees of freedom of the dye, generated by local structural changes at its binding site, is the most probable explanation for the depolarization.

We conclude that fluorescence depolarization caused in complexes of EB and poly(dA-dT)·poly(dA-dT) by spermine and some of its analogues mirror changes in the structure of poly(dA-dT)·poly(dA-dT), but that these changes are not found in complexes of EB and poly(dG-dC)·poly(dG-dC) or poly(dA)·poly(dT). Computer simulations of spermine binding to DNA suggest that spermine binds to the major groove of both alternating purine-pyrimidine and homopolymeric sequences, but that it bends the major groove and widens the minor groove only in the alternating sequences [9,10]. This widening may increase the mobility of EB bound in this region, causing depolarization [15-17]. Other structural changes specific for poly(dA-dT)·poly(dA-dT) sequences such as looped and/or alternating B-helical structures may also be responsible for the observed changes in fluorescence polarization. The depolarization we observed only with poly(dA-dT)·poly(dA-dT) complexed with EB is, to our knowledge, the only experimental evidence of spermine-induced structural changes specific for alternating A-T sequences as compared with alternating G-C or homopolynucleotide poly(dA)·poly(dT).

The lack of similar distortions in G-C sequences is consistent with previous studies using u.v. spectroscopy, hydrogen-

deuterium exchange and electric birefringence [12,29]. Marquet and Houssier [29] interpreted the differential effect of spermine on electric dichroic signals as a consequence of spermine-induced bending of alternating A-T sequences and stiffening of alternating G-C sequences. The observed stiffening of poly(dG-dC)-poly(dG-dC), however, might have resulted from conversion into the left-handed Z form, which would be likely under the conditions of low ionic strength used in their studies [42-44]. B-Z transition, however, was neither expected nor observed under our conditions (results not shown). The difference in the effect of spermine on alternating A-T and G-C sequences may be related to the greater flexibility of the alternating A-T sequences [45,46], which is supported by the relatively lower fluorescence polarization for EB bound to AT than to G-C sequences (Table 2).

The ability of polyamines to induce B-Z transition and bending in specific DNA sequences might be important in nucleosome phasing or chromatin condensation. Moreover, tracts of alternating A-T sequences in the genomes of many species, often close to or at eukaryotic promoters [47], indicate that specific structural changes in these sequences may have a role in the regulation of transcription and spermine may take an important part in such regulations.

Our results confirm that the number and distribution of positive charges on a polyamine analogue have profound effects on its binding to DNA and on its ability to induce conformational changes in specific DNA sequences. The inability of some analogues to mimic spermine might explain their inability to support cell growth in the absence of naturally occurring polyamines [48,49]. Our results may therefore assist in the search for polyamine analogues that inhibit growth by replacing the structural but not the functional characteristics of natural polyamines.

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