all the superhelical turns are restrained in nucleosomes (Sinden et al., 1980).

The striking decondensation which chromatin undergoes in terminally differentiated cells cannot at present be explained within the framework of models relating structure with function; what might be the genetic program requiring such a large rearrangement respresents a difficult question. A close analysis of this effect should, however, take into account the possible interference of an extensive digestion process.

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Interactions of the Dimethyldiazaperopyrenium Dication with Nucleic Acids. 1. Binding to Nucleic Acid Components and to Single-Stranded Polynucleotides and Photocleavage of Single-Stranded Oligonucleotides†

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ABSTRACT: The binding of dimethyldiazaperopyrenium dication (**1**) with nucleosides, nucleotides, and single-stranded polynucleotides has been studied by photophysical methods. It has been shown that **1** may be a potential selective fluorescent probe for A- and/or T-rich polynucleotides. **1** efficiently cleaves oligonucleotides at guanine sites, under illumination with visible light, and therefore may be used as a sequence-specific artificial photonuclease.

Cationic planar molecules such as the acridine dyes (Neidle & Waring, 1983; Steiner & Kubota, 1983; Berman & Young, 1981; Zimmerman, 1986; Dougherty & Pilbrow, 1984) and neutral aromatic polycyclic molecules such as pyrene (Lianos & Georghiou, 1979; Zinger & Geacintov, 1988) are known to interact and form complexes with DNA and its constituents. In the present work, we have studied the interactions of the dimethyldiazaperopyrenium[1] dication (**1**) with nucleosides, nucleotides, and single-stranded polynucleotides. It has been

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[1] The diazaperopyrene is first described in the literature in German Patent 276.357 (June 1913). Aromatische Kohlenwasserstoffe (MC 18), Dr. E. Clar, Berlin Springer-Verlag, 1941.

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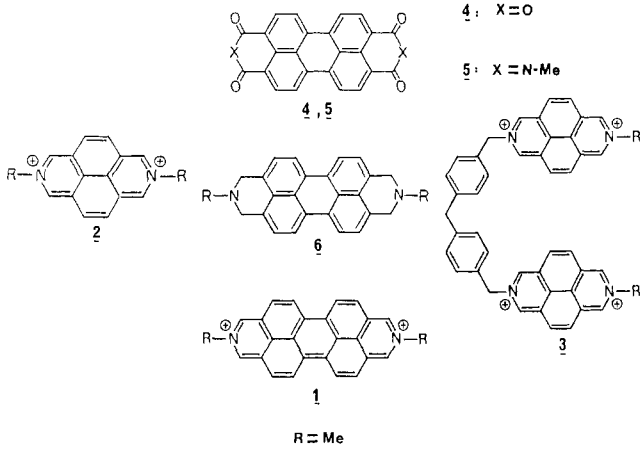
reported previously (Blacker et al., 1986) that the related dimethyldiazapyrenium (**2**) and the bis(diazapyrenium) derivative (**3**) bind to nucleic acids and effect their photocleavage under irradiation with visible light. The present paper will help to elucidate the origin of the attractive forces involved in the interactions of **1** with DNA constituents, as model systems for the study of DNA itself, presented in the following paper (Slama-Schwok et al., 1989). The finding of new molecules, such as **1**, which may complex efficiently small molecular units, and single-stranded polynucleotides is of interest for both its biochemical and biological applications: the potential use of **1** as a selective marker of single-stranded polynucleotides (ss) and the specific ss photochemical cleavage, showing that the dimethyldiazaperopyrene dication is an artificial single-stranded photonuclease.

Experimental Procedures

Synthesis of Dimethyldiazaperopyrenium Dichloride (**1**-Cl_2). The synthesis of dimethyldiazaperopyrenium dichloride involved three steps (Clar, 1941):



(*1*) *Synthesis of the Imide* **5** *from 3,4,9,10-Perylenetetracarboxylic Dianhydride* **4** (*Commercial Product of Aldrich*). Methylamine (10 mL, 40% aqueous solution) was added to a suspension of **4** (5 g, 12.8 mmol) in 100 mL of water. This mixture was stirred and heated to 90 °C for 1 h. After evaporation of the solvent, the crude imide product was dried by heating it in nitrobenzene, and two-thirds of the solvent was removed by distillation. The red solid was filtered, washed, and dried. The yield was 93% (5 g, 12 mmol): ^{1}H NMR (CF_3COOD) 8.87 ppm (m, 8 H), 3.79 ppm (s, 6 H). Anal. Calcd for $C_{26}H_{14}O_4N_2 \cdot 0.25H_2O$ (418): C, 73.94; H, 3.46; N, 6.62. Found C, 73.85; H, 3.12; N, 6.51.

(*2*) *Reduction of the Imide* **5**. $AlCl_3$ (1.6 g, 12 mmol) was dissolved in 30 mL of dry THF; $LiAlH_4$ (1.3 g, 33.6 mmol) was added. To this stirred and refluxed mixture was added the imide (2 g, 5 mmol) in several parts. During 2.5 h of reflux, the color changed from deep blue to yellow green. Excess $LiAlH_4$ was decomposed by addition of THF and water. After filtration, the solid was extracted several times with boiling $CHCl_3$. Collected filtrates (THF and $CHCl_3$) were evaporated in vacuo, and the crude diamine product **6** was crystallized from $CHCl_3$: yield 0.68 g (1.9 mmol) 40%; ^{1}H NMR ($CDCl_3$) 8.10, 8.06 ppm (2d, J = 8.5 Hz, 8 H), 3.92 ppm (s, 8 H, CH_2N), 2.61 ppm (s, CH_3, 6 H). Anal. Calcd for $C_{26}H_{22}N_2 \cdot 0.75H_2O$ (362): C, 83.06; H, 6.30; N, 7.45. Found: C, 82.99; H, 5.99; N, 7.34.

(*3*) *Aromatization of the Amine* **6** *to the Dimethyldiazaperopyrenium Dication* **1**. The amine **6** (0.75 g, 2 mmol) was dissolved in 30 mL of warm acetic acid, stirred, and refluxed. NBS (1.39 g, 8 mmol) was added, and the mixture was heated under reflux for 30 min. A yellow-brown solid precipitated instantly after the addition of NBS. After cooling, the solid was filtered and dried in vacuo (yield, 1.7 g). The product, dissolved in water, was purified by passing it through an Amberlite CG-50(H), acid-form column (previously washed with water, 10% HCl solution, and then water). The solvent was evaporated in vacuo and the crude product (0.78 g) dried. It was then dissolved in a small volume of water, precipitated by addition of acetone, filtered, and dried in vacuo to give 0.66 g of **1**-Cl_2 (1.54 mmol), 77%: ^{1}H NMR (D_2O) 9.09 ppm (s, 4 H Ar), 7.06 ppm (m, 8 H Ar), 4.57 ppm (s, 6 H, CH_3). Anal. Calcd for $C_{26}H_{18}N_2Cl_2 \cdot H_2O$ (447.3): C, 69.80; H, 4.51; N, 6.26. Found: C, 69.45; H, 4.42; N, 6.32.

Chemicals. The chemicals used were commercial products (Merck): NaCl, sodium cacodylate (dimethylarsenic acid), HCl, KI, and 2,6-naphthalenedicarboxylate were analytical reagents. The synthetic polynucleotides, as well as the nucleosides and nucleoside mono- and triphosphates, were P-L Biochemicals products.

Two different oligonucleotides were used for the photocleavage experiments. Their sequences are

5' TGAGTGAGTAAAAAAAATGAGTGCCAA 3'

5' TCCTGATAAAGGAGGAGATGAAGAAAAAATGA 3'

Doubly distilled water was used for all the solutions. The cells used were either semimicro- or microquartz cells from Hellma (optical path of 1 cm for two faces and 0.4 or 0.2 cm for the perpendicular optical faces). In the case of high ionic concentration measurements, acryl cells (Sarstedt) were used to reduce the adsorption on the cell walls. The glass vessels used were preadsorbed with **1**. All stock solutions were kept in the dark at −20 °C.

Apparatus. The absorption spectra were recorded either on Kontron spectrophotometers, Model 860 or 820, or on a Perkin-Elmer 554 spectrophotometer. A Spex Fluorolog spectrofluorometer was used for the static emission experiments. The spectra were corrected for the wavelength dependence of the transmission and detection systems. The area of the fluorescence curves was then calculated to obtain relative fluorescence yields. The lifetimes were measured on an Edinburgh Instruments 199 single photon counting fluorometer, using a hyperbaric hydrogen-filled flash lamp, which gave an instrumental response profile width at half-maximum height of 1.1 ns. Computer programs were used to fit mono-, bi-, and triexponential decay kinetics to the decay data. The quality of the fit was judged by reduced χ^2 and Durbin–Watson tests. Each apparatus was thermostated; the temperature was 20 ± 1 °C unless otherwise stated.

Results and Discussion

Photophysical Properties of Dimethyldiazaperopyrenium Dication (**1**). (*a*) *Absorption Spectra*. The absorption spectrum of dimethyldiazaperopyrenium dication, shown in Figure 1A, is dependent on the dye concentration and on the ionic concentration. Two isosbestic points were observed at 447 and 510 nm. All spectroscopic results could fit the dimerization scheme

$$2M \rightleftharpoons^{K} M_2$$

The dimerization constant K was calculated to be (2.0 ± 0.2) × 10^5 M^{-1} from absorption measurements in the concentration range (1–100) × 10^{-6} M, at 0.25 M NaCl in the presence of 25 mM cacodylate (pH 7.5). The two curves shown in Figure

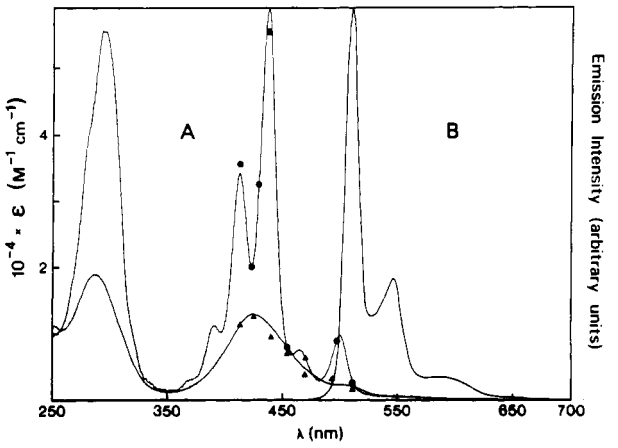


FIGURE 1: (A) Absorption spectra of the monomeric and dimeric forms of dimethyldiazaperopyrenium dication (**1**). Circles and triangles represent respectively the calculated extinction coefficients of the monomer and the dimer, in monomer and dimer units, from the absorption spectra recorded in the concentration range (1–100) × 10^{-6} M at 0.25 M NaCl and 25 mM cacodylate buffer, pH 7.5, taking $K = 2 \times 10^5$ M^{-1}. These circles and triangles are superimposed on the spectra measured respectively at 4×10^{-6} M dye, without buffer, using a light path of 1 cm, and at 3×10^{-5} M dye at 0.38 M NaCl and 38 mM cacodylate, using a light path of 0.2 cm. (B) Emission spectrum of **1**: [D] = 4×10^{-6} M, 0.25 M NaCl and 25 mM cacodylate, pH 7.5. The excitation wavelength was 414 nm; the excitation and emission slits were 0.6 nm.

Table I: Absorption Data of the Monomeric and Dimeric **1**

monomer[a]		dimer[b]		
λ (nm)	$\epsilon_m \pm 5\%$ (M^{-1} cm^{-1})	λ (nm)	$\epsilon_d \pm 5\%$ (M^{-1} cm^{-1})	transition
293	5.60×10^4	286	2.10×10^4	3 ← 0
390	1.11×10^4			2 ← 0
412	3.45×10^4	424	1.43×10^4	2 ← 0
	3.70×10^4[c]		1.25×10^4[c]	
438	5.94×10^4			2 ← 0
	5.7×10^4[c]			
465 (sh)	7.39×10^3			1 ← 0
501	1.00×10^4	505	2.57×10^3	1 ← 0

[a] ϵ_m were obtained from a Beer–Lambert plot without salt. [b] The extinction coefficients of the dimer were measured at 3.25×10^{-4} M, 0.375 M NaCl, and 37.5 mM cacodylate buffer. [c] Calculated extinction coefficients from absorption spectra at [D] = 1×10^{-6}–1×10^{-4} M at 0.25 M NaCl and 25 mM cacodylate, assuming a dimerization constant $K = 2 \times 10^5$ M^{-1}.

1A represent the absorption spectra of the monomer (in monomer units) and of the dimer (in dimer units). The absorption maxima of both monomer and dimer and their decadic extinction coefficients are presented in Table I. As can be seen from the different spectra presented in Figure 1A, the dimerization process is dependent on salt concentration; the shielding of the charges at high ionic concentration favors dimerization. The dimerization constant calculated for **1** is of the same order of magnitude as that reported for acridine orange, $K = 8.7 \times 10^4$ M^{-1} at 0.25 M NaCl (Kapuscinski & Darzynkiewicz, 1987).

(*b*) *Emission Spectra, Lifetime, Quenching by Halides.* The emission spectrum of **1** is shown in Figure 1B. It exhibits three bands at 508, 546, and 590 nm. The same spectrum is obtained independently of the excitation wavelength. The excitation spectrum shows the same bands as the absorption spectrum, i.e., 294, 392, 413, 439, 467, and 500 nm. The emission spectrum appears as the mirror image of the absorption. The energies of the three vibronic bands of the first transition can be calculated and correspond to 500, 466, and 422 nm. The fluorescence quantum yield of monomeric **1** has been measured as $\phi = 0.53 \pm 0.03$ by using 9-aminoacridine,

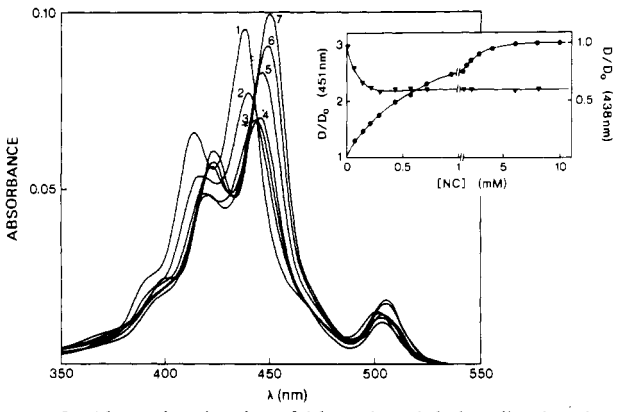


FIGURE 2: Absorption titration of **1** by 2,6-naphthalenedicarboxylate: [**1**] = 2.8×10^{-6} M, [NC] = 0, 7.3×10^{-5}, 1.5×10^{-4}, 2.2×10^{-4}, 4.4×10^{-4}, 9.4×10^{-4}, and 1.8×10^{-3} M, 0.25 M NaCl, 25 mM buffer, pH 7.5. These NC concentrations are symbolized respectively by the numbers 1–7 on the figure. (Insert) D and D_0 are the absorbances of bound and free **1**, observed at 451 nm (circles) and 438 (triangles), and D values are corrected for dilution. The light path was 1 cm.

quinine sulfate, and rhodamine 101 as references ($\phi = 0.96$, 0.545, and 1.00, respectively) (IUPAC, 1986; Kubota & Motoda, 1980). The quantum yield of the dimer is zero within experimental error. The decay of the monomer **1** is monoexponential: $\tau = 26.0 \pm 0.1$ ns in air-saturated solutions and $\tau = 27.35 \pm 0.10$ ns in N_2-saturated solutions. The bimolecular quenching constant of the lowest singlet state of **1** by oxygen is therefore estimated to be $k_q(O_2) = 7 \times 10^9$ M^{-1} s^{-1}, assuming an oxygen concentration of 0.28 mM in air-saturated solution.

The addition of NaI to the dye at constant ionic strength (0.1 M with $NaClO_4$) causes a quenching of the dye fluorescence. The Stern–Volmer plots are linear in the range 5–50 mM halide, leading to $K_{SV}(I^-) = 500$ M^{-1}. Using the measured lifetime of **1** in solution, the corresponding quenching constant was calculated to be $k_q(I^-) = 2.0 \times 10^{10}$ M^{-1} s^{-1}. This value is in agreement with a diffusion-controlled reaction for molecules of opposite charges.

Interaction of **1** *with Naphthalenedicarboxylate.* (*a*) *Binding Constants.* The binding of 2,6-naphthalenedicarboxylate (NC) to **1** was studied because NC is a negatively charged aromatic molecule that could engage in stacking interactions with positively charged **1**. The binding of NC by the dye was studied by absorption, and the titration curve is presented in Figure 2. Complex formation occurs in two steps as shown by the hypochromism of the absorption at low NC concentrations. At higher NC concentrations, the absorption spectrum is red-shifted from 414, 438, and 502 to 424, 451, and 506 nm.

The emission measurements also show two complexation steps: a small quenching of the fluorescence is observed for NC concentrations in the range (10–100) × 10^{-6} M and an increase of the fluorescence occurs in the millimolar range. The emission spectrum is red-shifted with respect to the free dye: it peaks at 515 nm, as compared to 508.5 nm. These spectroscopic properties of the dye complexed to NC present some similarities with those observed when it is bound to polynucleotides, as will be seen below. The proposed complexation scheme is

$$\mathbf{1} + \mathrm{NC} \rightleftarrows (\mathbf{1}\text{–}\mathrm{NC}) \quad (1)$$

$$(\mathbf{1}\text{–}\mathrm{NC}) + \mathrm{NC} \rightleftarrows (\mathbf{1}, (\mathrm{NC})_2) \quad (2)$$

The corresponding binding constants $K(1)$ and $K(2)$ have been calculated as $K(1) = 1.1 \times 10^4$ M^{-1} and $K(2) = 1.5 \times 10^3$ M^{-1}. Complexation of benzenepolycarboxylates and NC to the

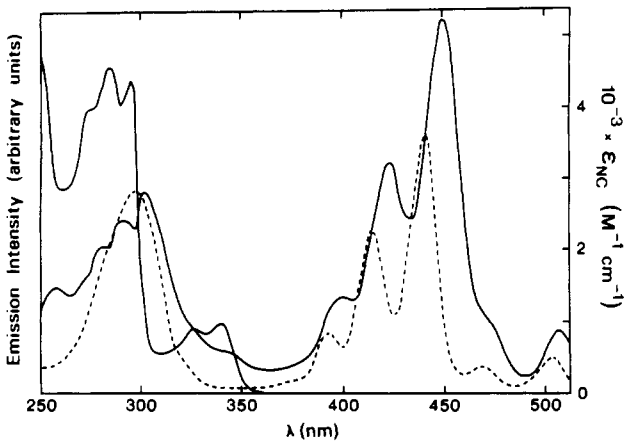


FIGURE 3: Energy transfer from NC to **1**: Excitation spectra (left scale), recorded at 515 nm in the presence of 2.6×10^{-4} M NC (solid line) and with 1.8×10^{-6} M **1** alone (dashed line) at 3 °C. The absorption spectrum of NC is superimposed on the excitation spectra (solid line, right scale). The optical paths were 0.2 cm for the excitation and 1 cm for the emission.

related dimethyldiazapyrenium (**2**) and its dimer derivative **3** has been reported (Blacker et al., 1987), and binding constants comparable to the above values have been obtained. The structure of these complexes has been suggested to be of the face-to-face type, accounting for the strong shielding observed in NMR for the protons of both partners.

(*b*) *Energy Transfer.* The complexation of NC to **1** has been revealed by red shifts of both absorption and emission spectra with respect to free **1**. These spectroscopic modifications may be interpreted by the creation of a hydrophobic environment close to the dye. The analysis of the results shows the formation of a complex, at high NC concentrations, in which one molecule of **1** is bound to two NC ligands. Here we show that an energy-transfer process occurs from NC (donor) to **1** (acceptor), in agreement with the proposed face-to-face complex geometry. Energy-transfer processes from the nucleic bases to several intercalating molecules have been reported (Weill & Calvin, 1963; Sari et al., 1986). Singlet–singlet energy transfer may occur if there is a close proximity between the donor and the acceptor, since the kinetic constant of this process depends on $1/R^6$ (Förster, 1947). In addition, their emission and absorption spectra should overlap. The existence of energy transfer can be revealed by measuring the emission spectrum of the complexed dye at different excitation wavelengths and also by recording the excitation spectrum, monitored at an emission wavelength where only **1** emits. In the present case, the emission spectrum of NC, whose quantum yield is $\phi_D = 0.14$, peaks at 355 and 371 nm and presents a shoulder at 390 nm. The corrected excitation spectrum, measured at the maximum emission wavelength of the complexed dye **1** (515 nm), in the presence of 2.7×10^{-4} M NC (1.8×10^{-6} M dye) at 0.25 M NaCl, presents an enhanced fluorescence at 280, 290, 328, and 340 nm, as compared to that observed upon excitation in the absorption spectrum of **1**. These maxima correspond to the peaks of the NC absorption as can be seen from Figure 3. The enhancement is dependent on temperature: the effect decreases at 20 °C as compared to −3 °C.

This dependence is probably due to larger binding constants at lower temperature. The analysis of the excitation spectrum of the NC·**1** complex shows that energy transfer occurs from NC to **1**. The apparent fluorescence yield of **1** as a function of the excitation wavelength increases in the absorption band of NC. A reference wavelength of 390 nm was chosen, since NC does not absorb at this wavelength. The wavelength dependence of the emission yield was characterized by the ratio $Q(\mathrm{UV})/Q(390)$.

$$Q(\mathrm{UV})/Q(390) = \frac{(I(\mathrm{UV})_b/I(\mathrm{UV})_f)(\mathrm{OD}(\mathrm{UV})_f/\mathrm{OD}(\mathrm{UV})_b)}{(I(390)_b/I(390)_f)(\mathrm{OD}(390)_f/\mathrm{OD}(390)_b)}$$

$I(\mathrm{UV})$ represents the emission intensity at a given wavelength in the UV spectral region, OD(UV) is the absorbance at the same wavelength, and subscripts f and b represent the free and bound property, respectively. $\mathrm{OD}(\mathrm{UV})_b$ is the difference absorbance, assuming no absorption change of NC upon complexation to **1**. A $Q(\mathrm{UV})/Q(390)$ ratio larger than 1 indicates energy transfer. The $Q(\mathrm{UV})/Q(390)$ plot shows two maxima (in the range 300–390 nm), 325 and 345 nm with $Q(325) = 2.5 \pm 0.3$ and $Q(345) = 2.2 \pm 0.2$. The critical distance R_0 has been calculated from the absorption spectrum of complexed **1** (Figure 2), which shows a large overlap with the emission spectrum of NC. This overlap is peaking at 390 and 415 nm, the overlap integral is $J = 1.1 \times 10^{-14}$ M^{-1} cm^3. The critical distance R_0 is calculated to be 2.6 nm by using an average orientation factor $\langle k \rangle^2 = 0.66$. This large distance allows total transfer between stacked molecules. Under conditions in which the electrostatic attractions are partly shielded, hydrophobic interactions between the aromatic rings of the donor and the acceptor are an important factor of binding. They keep the two species in proximity and allow an energy-transfer process to occur.

Binding of **1** *to Nucleosides.* (*a*) *Binding of Diazaperopyrenium* (**1**) *to Adenosine.* Complex formation between **1** and adenosine occurs in two steps, similar to the binding to NC. Hypochromism of the absorption is observed in the millimolar concentration range, $D_{max}/D_0 = 0.55$. At higher adenine concentrations, the absorption spectrum is red-shifted, peaking at 420, 445, and 505 nm. The emission spectrum of the second complex, at high adenosine concentrations, is red-shifted with respect to the free dye, peaking at 514.5 nm. The relative emission yield of this complex is 0.70 ± 0.05 compared to the yield of the free dye. A complexation scheme similar to that described above for NC is thus proposed, leading to $K(1) = 340 \pm 50$ M^{-1} and $K(2) = 64 \pm 10$ M^{-1}.

(*b*) *Binding of Diazaperopyrenium* (**1**) *to Thymidine.* Only one complexation step can be evidenced in the concentration range 0.5–50 mM thymidine. The absorption spectra present three isosbestic points at 377, 420, and 490 nm. The absorption maxima obtained at high thymidine concentrations are observed at 415, 442, and 503 nm. The emission spectrum is only slightly red-shifted ($\lambda_{max} = 510 \pm 1$ nm). An isoemissive point is present at 540 nm. The fluorescence intensity is about the same for the complex as for the free dye. Only an approximate value of the binding constant can be calculated, since the measurements are limited by the solubility of thymidine, $K(1) = 15$ M^{-1}.

(*c*) *Complexation to 5-Bromodeoxyuridine.* This nucleoside was studied to investigate whether there is a static quenching of the fluorescence due to the heavy bromine atom. Complexation occurs for the concentration range 2–50 mM BrdU, and a quenching of the fluorescence is observed. The emission spectrum of the complex is red-shifted to 512 nm. The data have been treated according to the scheme proposed by Georghiou (Badea & Georghiou, 1976), leading to a binding constant in the ground state of 90 M^{-1} and to a quantum yield of the complex relative to that of the free dye of 0.30 ± 0.05. The heavy bromine atom causes a quenching of the relative quantum yield of the complexed dye from $I/I_0 = 1$ for thymidine to 0.3 for BrdU.

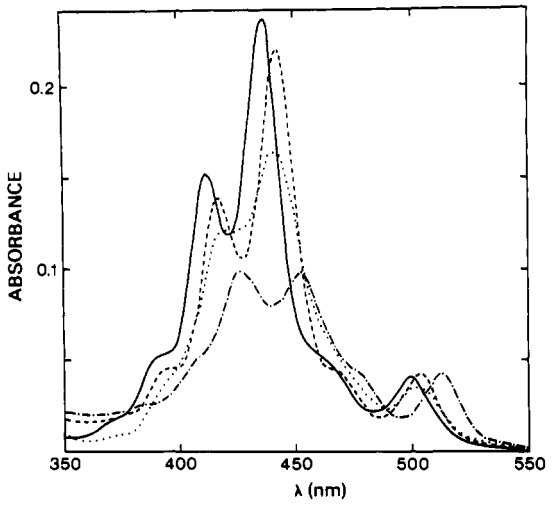


FIGURE 4: Comparison of the absorption spectrum of **1** complexed to AMP, poly(dT), poly(dA) and free **1**: [**1**] = 8.6×10^{-6} M [(free) solid line]; [AMP] = 10 mM (dashed line); [poly(dA)] = 0.15 mM (dot-dash line); [poly(dT)] = 0.8 mM (dotted line).

Table II: Comparison of the Association Constant for the Binding of Dyes to AMP in the Presence of 0.1 M NaCl

	dye					
	PF	AO	9AA	10-MeA	P	**1**
K(AMP) (M^{-1})	150[a,b]	120[b]	190[b]	42[b]	52[c]	4400

[a] Badea and Georghiou (1976). [b] Kubota et al. (1979). [c] Lianos and Georghiou (1979).

Binding of **1** *to the Nucleotides AMP and ATP.* The binding of **1** to AMP and ATP was studied by absorption measurements at 0.1 M NaCl and 10 mM cacodylate buffer, pH 7.5. As already observed for adenosine, the binding occurs in two steps, the first one in the concentration range (1–10) $\times 10^{-5}$ M and the second one in the millimolar range. In this first step, the overall shape of the spectrum is unchanged, but a hypochromism is observed. The maximal hypochromism is $D_{max}/D_0 = 0.63$ and 0.82 for ATP and AMP, respectively. Higher hypochromism is observed at 10 mM NaCl ($D_{max}/D_0 = 0.45$ for both AMP and ATP). The absorption spectra of the final forms are red-shifted, peaking at 418, 444, and 504 nm for ATP and AMP (shown in Figure 4). The absorption spectra of **1** complexed to either adenosine or AMP at high ionic concentration (0.1 M NaCl) are comparable. According to the same scheme described for NC, the binding constants are $K(1) = (4.4 \pm 0.6) \times 10^3$ and $K(2) = 60 \pm 15$ M^{-1} for AMP $K(1) = (1.3 + 0.3) \times 10^4$ and $K(2) = 250 + 40$ M^{-1} for ATP. The complexation of ATP at 10 mM NaCl leads to larger binding constants, especially for the first complex, due to higher electrostatic interactions: $K(1) = (2.3 \pm 0.3) \times 10^5$ and $K(2) = (1.4 \pm 0.3) \times 10^3$ M^{-1}. These values may be compared to the complexation of other dyes, such as proflavine (PF), acridine orange (AO), 9-aminoacridine (9AA), 10-methylacridinium (10MeA), and pyrene (P) (Table II). Thus, the first binding constant to AMP is much higher for **1** than for the acridine dyes; note that acridine dyes are monovalent cations, so that the electrostatic binding energy is smaller. Nevertheless, hydrophobic effects may also contribute to the higher figures obtained in the case of **1**. The second binding constant of **1** to adenosine is of the same order of magnitude as for the complexation of pyrene to AMP (Lianos & Georghiou, 1979). In both cases, it is the binding constant of a charged species with a neutral one, and the hydrophobic interactions should be of the same order of magnitude, since the purine rings are much smaller than either pyrene or **1**.

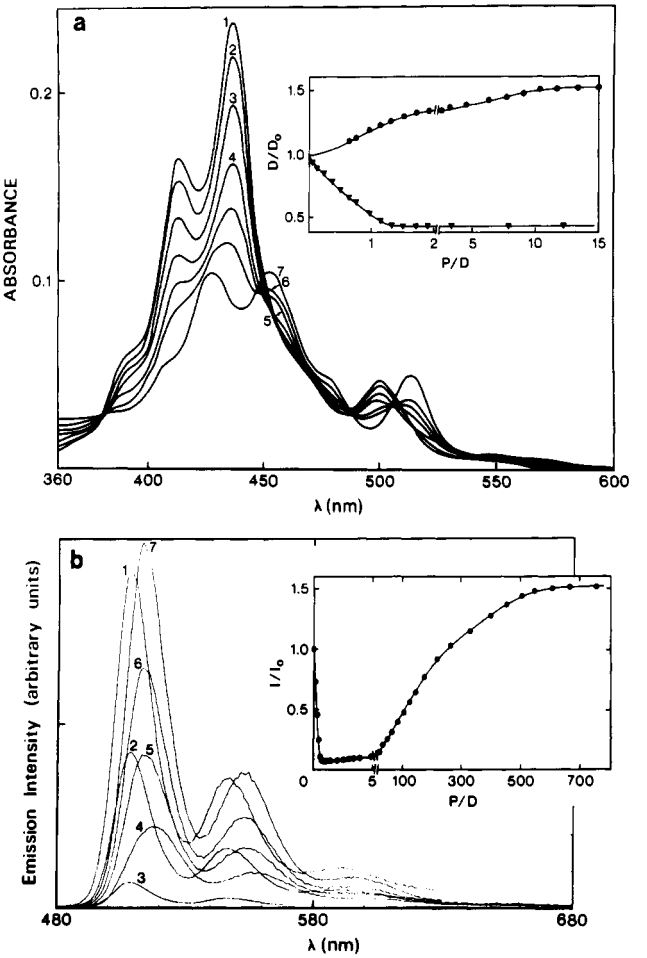


FIGURE 5: Titration of **1** by poly(dA), 0.25 M NaCl and 25 mM cacodylate, pH 7.5. (a) Absorption: [**1**] = 7.2×10^{-6} M, [poly(dA)] = 0, 2.2×10^{-7}, 1.6×10^{-6}, 3.4×10^{-6}, 5.1×10^{-6}, 6.4×10^{-6}, 5.1×10^{-4} M (numbers 1–7). (Insert) D/D_0 is calculated at 438 nm (triangles) and at 453 nm (circles), corrected for dilution. (b) Emission: Excitation wavelength of 449 nm; excitation and emission slits of 0.6 nm. [**1**] = 6.6×10^{-6} M, P/D (expressed in phosphate units) = 0, 0.3, 1.0, 23.6, 76.3, 165.4, 543 (numbers 1–7).

Binding of **1** *to Single-Stranded Polynucleotides.* (*a*) *Absorption Measurements.* The binding of **1** to single-stranded nucleotide homopolymers has been studied by absorption measurements. A typical titration curve is shown in Figure 5a, for the titration of **1** by poly(dA), at 0.25 M NaCl and 25 mM cacodylate buffer, pH 7.5, at 20 °C. As in the case of the mononucleotides, the titration presents two steps, occurring at different P/D: in the first step, the absorption decreases (hypochromism); in the second step, at higher P/D, a large red shift of the spectrum is observed, peaking at 427, 453, and 513 nm. The overall shape of the final spectrum is changed.

The titration curves of **1** by the polypurines studied, i.e., poly(dA), poly(rA), and poly(dG), present the same features as those described above. The spectroscopic properties of these complexes are compared in Table III. Comparison to the corresponding mononucleotides and nucleosides shows some general differences. The hypochromism in the first step as well as the red shifts in the second step observed is more pronounced in the case of the polynucleotides (Figure 4). The titration curve observed when **1** is complexed to polypyrimidine, poly(dT), is quite different. The hypochromicity is much lower: $D_{max}/D_0 = 0.42$ and 0.75 for poly(dA) and poly(dT), respectively. The red shifts are smaller: the final

Table III: Absorption and Steady-State Fluorescence Data for **1** Bound to Different Single-Stranded Polynucleotides

polynucleotide	D/D_0-(438)[a] hypochr	$D/D_0(\lambda_{max})$[a] hyperchr	λ_{max} abs	$\epsilon(\lambda_{max})$ (M^{-1} cm^{-1})	isosbestic point at P/D < 7	isosbestic point at P/D > 7	relative fluorescence quantum yield
poly(dA)	0.42	1.51	453	1.67×10^4	380, 453	505	1.54
poly(rA)	0.38	1.73	453	1.84×10^4	455, 510	414, 438, 505	
poly(dT)	0.75	1.10	445	2.38×10^4			1.12
poly(dG)	0.34	0.64	456	6.70×10^3			

[a] Extrapolated values for P/D = ∞.

Table IV: Fluorescence Data for **1** Bound to Different Single-Stranded Polynucleotides

polynucleotide	[1] × 10^{-6} M	P/D	τ_1 (ns)	B1 (%)	τ_2 (ns)	B2 (%)	τ_3 (ns)	B3 (%)	χ^2	DW
1 alone		0	25.86 ± 0.05						1.18	1.816
poly(dA)	6.00	125	29.5 ± 0.1	19.1	5.41 ± 0.15	22.3	0.60 ± 0.07	58.6	0.96	1.929
	3.00	250	29.7 ± 0.1	27.9	5.7 ± 0.2	18.4	0.64 ± 0.09	53.7	1.01	1.800
poly(dT)	10.00	80	28.5 ± 0.1	54.7	11.7 ± 0.7	8.5	0.54 ± 0.11	36.7	1.24	1.710

absorption spectrum presents maxima at 422, 445, and 505 nm. The solutions of **1** in the presence of low concentrations of polynucleotides, typically at P/D = 2–6, show an instability, evidenced at constant temperature 20 °C: there is a slow decrease of the whole absorption spectrum, in the visible as well as in the UV region, indicating a coprecipitation of the dye and the polymer. This precipitation is total or partial, on a time scale of several hours, depending on P/D. A similar phenomenon was previously reported for acridine orange and some other intercalators (Kapuscinski & Darzynkiewicz, 1984; Armstrong et al., 1970).

The addition of low concentrations of ss polymers causes a sharp increase of light scattering, compared to the scattering of dye-free polymer. These findings may be attributed to the formation of a three-dimensional network, due to inter- and intrapolymer electrostatic cross-linkings. This network has a much lower solubility than similar dye-free solutions.

(*b*) *Emission Measurements.* A typical fluorometric titration curve of **1** by poly(dA) is shown in Figure 5b. Two steps may also be evidenced. In the first one, for low P/D, there is a large quenching of the dye fluorescence, without any spectral shift. A second step occurs at higher P/D: red shifts of the spectra are observed, as well as an enhancement of the fluorescence. The emission yield of this complex is 1.54 ± 0.15 compared to the free dye. This yield, when **1** is bound to poly(dA), is higher than the value obtained when **1** is complexed to adenosine. The fluorescence yield in the presence of poly(dT) is 1.12 ± 0.11 compared to the free dye, which is similar to the value obtained with thymidine. The fluorescence of **1** bound to poly(dG) is completely quenched. The quenching of fluorescence by guanines, in the case of G-C-rich polynucleotides (Steiner & Kubota, 1983) and also in the case of mono- and dinucleotides (Lianos & Georghiou, 1979; Badea & Georghiou, 1976; Kubota et al., 1979; Georghiou, 1981), has been reported for a number of intercalating molecules. It has been related to the redox property of guanine, which is the best electron donor among the bases.

The absorption and emission results may be interpreted as follows: the first step of complexation is attributed to the condensation and stacking of the dye along the polymer, which acts as a polyelectrolyte. This external binding is manifested by the hypochromism of the absorption and the quenching of the fluorescence. These spectroscopic properties are similar to those of the dimeric **1** in solution. The aggregation of the dye in the electrostatic field of the polymer is plausible, since the dimerization constant of the dye in polymer-free solution is high. This cooperative binding originates from electrostatic interactions between the positively charged dye and the negatively charged phosphate groups and from the dimerization tendency of **1** in solution. Such condensation and stacking phenomena have been invoked in the case of acridine orange, which has a somewhat lower dimerization constant (Kapuscinski, 1987). This first step may be characterized by three parameters, in the analysis proposed by McGhee and Von Hippel (K_1, ω_1, n_1). As can be seen from Figure 5b, this binding is strongly cooperative. The second step is attributed to the formation of an "internal" complex, where **1** is inserted between adjacent bases. This location of the dye may explain the difference in the red shifts obtained in the case of polypurines, which are known to efficiently stack, and the polypyrimidines, which have a much less ordered structure. This internal complex may be related to the partial intercalation complex, suggested in the case of proflavine bound to single-stranded polynucleotides (Dourlent & Hélène, 1971).

This second complex has a different location compared to the first aggregated species. It should be described by a set of parameters K_2, ω_2, and n_2. The quantitative treatment of these titration curves is problematic, since aggregation is very important and practically quantitative in the concentration range measurable experimentally. It is thus very difficult to evaluate the values of K_1 and ω_1 of the first step and, therefore, to obtain the figures for K_2 and ω_2.

Qualitatively, the product $K_1\omega_1$ seems much larger than $K_2\omega_2$, and therefore at low P/D, the aggregation is observed. At large P/D, an internal complex is obtained, which means that K_2 is larger than K_1. In this case the polymer is in a large excess compared to the dye concentration. The dye is thus "isolated" on the polymer, and the cooperativity terms are not dominant.

(*c*) *Lifetimes.* The comparison of the emission decay of **1**, free and complexed to poly(dA) and to poly(dT), is presented in Figure 6 and Table IV. Whereas the lifetime of the free dye is monoexponential, the decays in the presence of poly(dA) and poly(dT) are best fitted by a triexponential kinetic scheme. The long-lifetime component of 29.6 ± 0.1 ns is longer than for free **1**, which is 26.0 ± 0.1 ns. Its weight is larger with increasing P/D and is higher with poly(dT) than with poly(dA) at comparable P/D. The intermediate lifetime is 5.6 and 11.7 ns for poly(dA) and poly(dT), respectively. The weight of this component decreases when P/D increases. The shortest component is 0.6 ± 0.1 ns. Its weight is 40–55%.

The long-lifetime component is attributed to an internal complex. Its weight at P/D = 80–250 is still low, reflecting the dye distribution between aggregates (responsible for the shortest lifetime), externally bound dye in its monomeric form (to which we attribute the intermediate lifetime), and the

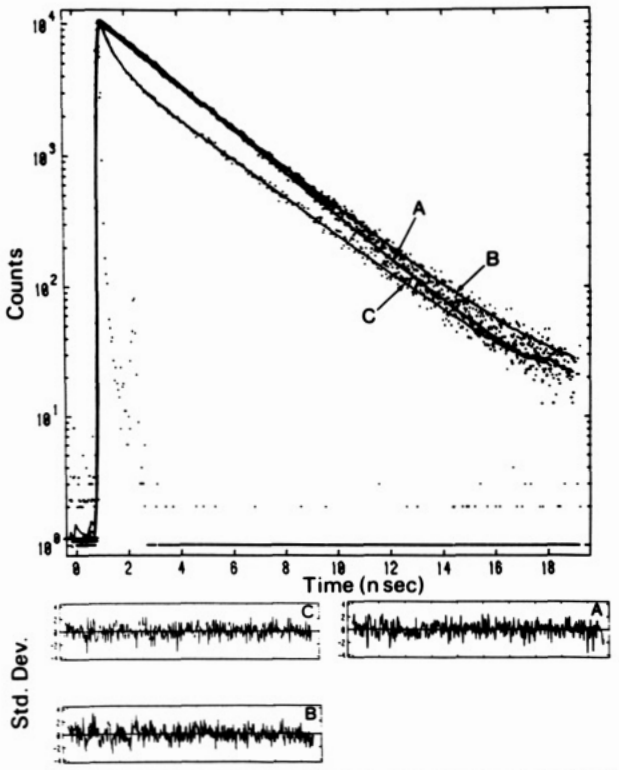


FIGURE 6: Decays of free and bound **1**: (A) free **1**; (B) bound to poly(dT), P/D = 80; (C) bound to poly(dA), P/D = 125. The lamp profile and the residuals are presented. The analysis is shown in Table IV.

internal complex. The attribution of the shortest component to dimeric and/or to higher aggregation forms is justified, since the dimeric free dye is only very weakly fluorescent.

Photocleavage of Single-Stranded Polynucleotides by **1**. Photocleavage of single-stranded oligodeoxynucleotides by **1** was observed after irradiation under visible light, at 0.1 M NaCl and 10 mM cacodylate, 0 °C. Both oligomers showed important cleavage, occurring mainly at the guanine sites. Only little cleavage is observed at other bases. The cleaved fragments migrate as those obtained during the Maxam–Gilbert procedure. Therefore, they carry a 3′-phosphate terminal group. The cleavage observed at guanines is strongly enhanced after treatment with 1 M piperidine at 90 °C for 20 min (Figure 7). This reaction reveals that, to a large extent, guanines were modified by irradiation in the presence of **1** without cleavage of the phosphodiester bonds. These alkali-labile sites most probably result from photooxidation of guanines.

The cleavage efficiency at a particular site reflects both the binding of **1** and the reactivity of the bases with the photoexcited ligand. Guanines appear to be by far the most reactive bases. The results presented in Figure 7 show that the reactivity of guanines is dependent on neighboring bases. The effect seems to extend over several bases, since one of the two AGA sequences is cleaved more efficiently than the other. The sequence TGA, which appears twice, is only weakly cleaved. Further studies on longer DNA fragments should provide more information on the role of base sequence on guanine photomodification.

Many DNA-binding ligands induce base damages upon visible light irradiation, which lead to cleavage reactions after treatment under alkaline conditions (Nielsen et al., 1988, and references cited therein). Dimethyldiazaperopyrenium (**1**) is one of few ligands that can induce cleavage reactions at neutral pH.

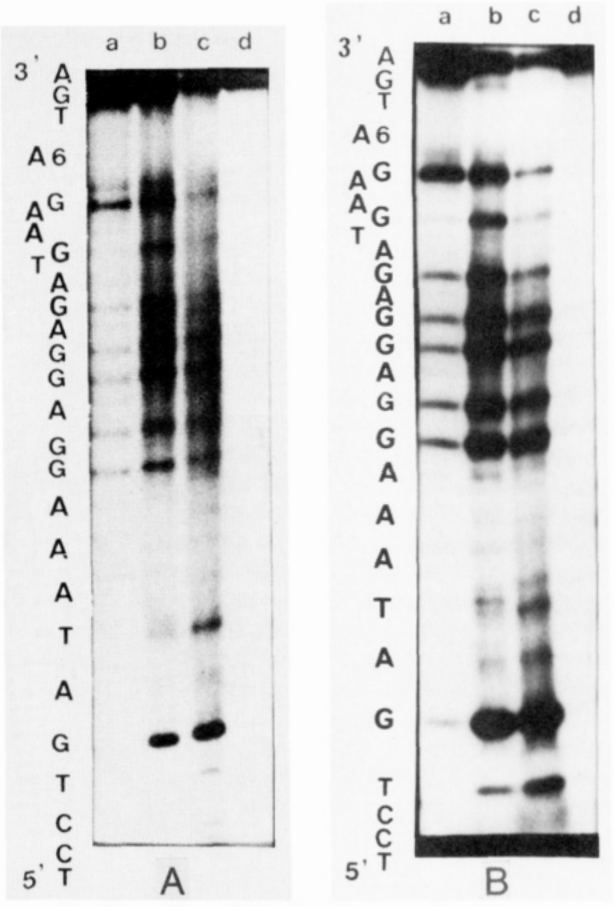


FIGURE 7: Comparison of the photocleavage of the single-stranded 32-oligomer by **1** in neutral and alkaline media: 15-min irradiation with a Hg 150-W lamp with a Schott cutoff filter GG400; air-saturated solutions, in 100 mM NaCl and 10 mM cacodylate, pH 7.0 at 0 °C. The concentration of the oligomer, labeled at its 5′ extremity, was 54.4 μM in phosphate units. The electrophoresis was performed by using 20% polyacrylamide gel. (A) (lanes a–c) Oligomer irradiated in the presence of [**1**] = 1.7, 8.6, and 17 μM; (lane d) irradiated oligomer alone. (B) (lanes a–c) The samples of (A) were treated in 1 M piperidine, at 90 °C, for 20 min to reveal alkali-labile sites; (lane d) irradiated oligomer alone treated with piperidine.

Conclusions

This work has shown that **1** may complex small molecular units with binding constants much larger than those of the cationic acridine dyes. This high affinity is probably related to the dicationic nature and to the hydrophobic interactions with the nucleic bases, as reported in the case of other polycyclic aromatic molecules such as pyrene, its derivatives, and coronene (Zinger & Geacintov, 1988; Spassky & Sigman, 1985; Bromley et al., 1986).

It has been suggested that **1** may be stacked between adjacent bases in ss polymers. It has been shown that **1** has a high fluorescence yield, when bound to either poly(dA) or poly(dT), but this emission is totally quenched in the case of poly(dG). *Therefore, the dimethyldiazaperopyrenium may be used as a selective fluorescence probe of A- and/or T-rich single-stranded polynucleotides.*

The dimethyldiazaperopyrenium dications are able to cleave selectively single-stranded oligomers, under irradiation with visible light. Most of the dyes previously reported to cleave under irradiation require alkaline treatment before cleavage occurs. Cleavage by **1** occurs at neutral pH and involves mainly guanine bases. Alkali-labile photomodification is also generated at the same sites. *The dimethyldiazaperopyrenium might be therefore used as a sequence-specific artificial nuclease.*

Registry No. **1**, 118891-85-3; **1**-Cl_2, 118891-86-4; **1** (dimer), 118920-10-8; **4**, 128-69-8; **5**, 5521-31-3; **6**, 188-97-6; NC, 1141-38-4; AMP, 61-19-8; ATP, 56-65-5; BrdU, 59-14-3; TGAGTGAGTAAAAAAAATGAGTGCCAA, 112603-07-3; TCCTGATAAAGGAGGAGATGAAGAAAAAATGA, 118977-32-5; I^-, 20461-54-5; poly(dA), 25191-20-2; poly(rA), 24937-83-5; poly(dG), 25656-92-2; poly(dT), 25086-81-1; adenosine, 58-61-7; thymidine, 50-89-5.

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Interactions of the Dimethyldiazaperopyrenium Dication with Nucleic Acids. 2. Binding to Double-Stranded Polynucleotides†

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ABSTRACT: The interactions of dimethyldiazaperopyrenium dication (**1**) with DNA have been studied by spectroscopic methods: absorption, static and dynamic fluorescence, and linear dichroism. **1** binds strongly to DNA at 250 mM NaCl, with a higher affinity for G-C pairs as compared to A-T pairs. The dye fluorescence is enhanced when it is bound to A-T pairs, whereas the emission is quenched in the vicinity of G-C pairs. Evidence for intercalation has been obtained via energy transfer and linear dichroism measurements.

Inside living cells, DNA represents an important target for different types of drugs. These drugs either bind in one of the grooves of the double helix or intercalate between base pairs. Binding of drugs on DNA may affect the biological activity in several ways: structural effects related to the binding, primarily noncovalent interactions of the drugs with duplex DNA; functional effects in which kinetics of DNA-dependent enzymes may be altered; DNA damages induced by the drug redox reactions due to either thermal or photochemical processes.

Since Lerman's intercalation model (Lerman, 1961), a large number of molecules have been shown to intercalate, for example, acridines (Neidle & Waring, 1983; Dougherty & Pilbrow, 1984), anthracyclines (Aubel-Sadron & Londos-

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