enzyme contour (Darst et al., 1989). This model shows a cleft that is presumed to be at the active site. If so, it would contain the i and $i + 1$ subsites and bind to the substrates shown in our Figure 1.

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Spin-Labeled Oxazolopyridocarbazole as a Probe for Studying Nonintercalating DNA Groove Binding Ligands

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ABSTRACT: A spin-label (P-OPC) composed of the nitroxide-containing ring proxyl linked at the C1 position of the intercalating fluorescent chromophore oxazolopyridocarbazole (OPC) has been synthesized. The spin-labeled OPC was found to interact with DNA and polynucleotides according to an external minor groove binding mode with association constant values K_{app} ranging from 10^5 to 10^6 M^{-1}. External binding was obvious from the inability of P-OPC to increase the length of sonicated DNA upon binding, the low unwinding angle (9.6°) of circular PM2 DNA, and the low energy transfer from DNA bases to bound chromophore. Binding of P-OPC to DNA or polynucleotide results in a strong immobilization of the proxyl moiety, resulting in the appearance of an asymmetric and broad ESR spectrum with a maximal hyperfine splitting of 56.5 G. In the equilibrium conditions, the occurrence of superimposed ESR spectra related to the P-OPC fraction undergoing rapid motion and to the P-OPC fraction immobilized allows the estimation of the concentrations of free and DNA-bound spin-label. The external mode of binding to DNA as well as the characteristics of the ESR spectra make P-OPC suitable for the determination of DNA binding parameters of nonintercalating ligands using competition experiments. The measurement of the binding constants of distamycin A to poly[d(A-T)] and poly[d(G-C)] is taken as an example.

The great majority of antitumor and antiviral agents as well as a number of antibiotics exert their pharmacological activities by interfering with DNA. Attention has been recently focused on agents that reversibly bind to DNA in the helical grooves or along the surface of the phosphate backbone of the helix [see Zimmer and Wähnert (1986) for review]. This class of

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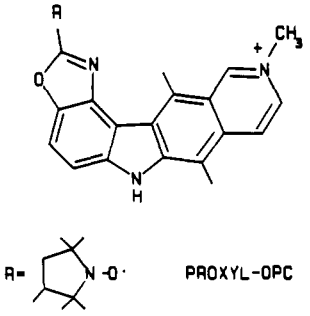

FIGURE 1: Chemical structure of the spin-label proxyl-OPC.

compounds referred to as nonintercalators usually bind specifically to DNA base pair sequences. The prototypes of these compounds are represented by the DNA-binding antibiotics distamycin and netropsin which selectively bind to AT-rich regions of DNA (Kopka et al., 1985a,b). The association constant value (K_{app}) with DNA or synthetic polynucleotides is one of the key parameters required for the study of the specificity of the interaction and leads to the estimation of the thermodynamic parameters of the binding. Because of the extremely weak fluorescence and/or absorbance changes upon DNA binding of many drugs in aqueous medium, the estimation of K_{app} values requires the use of indirect techniques involving mainly competition with the fluorescent intercalating compound ethidium bromide (EB) (Le Pecq & Paoletti, 1967). A simplified method consisting of the measurement of the IC_{50} value (the amount of competitor that decreases the fluorescence of the ethidium–DNA mixture by 50%) (Baguley, 1982), which is supposed to reflect the K_{app} value, is widely used. At the present time the suitability of the EB assay seems to be questionable for the following reasons: Recent reports have clearly shown the occurrence of EB–DNA fluorescence quenching in the presence of molecules having electron-donating moieties such as hydroxy and amino groups in their structure (Baguley & Le Bret, 1984; Davis et al., 1987). This phenomenon, probably involving the formation of a charge transfer between the competing drug and EB, mainly occurs in the AT-rich region of DNA and mimics the exclusion of EB from its binding sites. (ii) The binding sites of EB and nonintercalating ligands are necessarily different, and the competition conditions are restricted to the case where the competing ligand occludes the access to intercalative binding sites.

An alternative competition method can be provided by the use of spin-labeled compounds, since the ESR spectrum of such molecules allows the simultaneous estimation of both free and DNA-bound molecules. Spin-labeled analogues of the intercalating agents 9-aminoacridine and ethidium bromide have already been synthesized in this way and used as probes for studying drug–DNA interaction (Hong & Piette, 1976; Sinha et al., 1976; Robinson et al., 1980). We describe in the present paper the preparation and physicochemical properties of a new spin-label composed of the oxazolopyridocarbazole chromophore (OPC) [see Auclair et al. (1988) for review] linked to the nitroxide-containing compound 2,2,5,5-tetramethylpyrrolidine-*N*-oxyl (proxyl) (Figure 1). The proxyl-OPC derivative was found to act as a DNA outside binder and can be usefully employed for the estimation of K_{app} of nonintercalating molecules.

Materials and Methods

Preparation of Spin-Labeled Oxazolopyridocarbazole (OPC). Spin-labeled OPC was prepared by the covalent addition of 4-(aminoethyl)proxyl to the two-electron-oxidized form of 9-hydroxyellipticinium (NMHE), 9-oxoellipticinium (NMOE), as previously described (Auclair et al., 1984). The oxidation of NMHE to NMOE was achieved in 0.05 M phosphate buffer (pH 7.40) by horseradish peroxidase–H_2O_2 (Auclair & Paoletti, 1981). The adduct so obtained was purified on a hydrophobic XAD2 column using an ammonium acetate/methanol mixture as eluant. In all preparations, the purity of the spin-label as checked by HPLC and TLC was greater than 98%.

Identification of Purified Proxyl-OPC. Mass spectra (FAB): calculated 443.5, observed (m/z) 443.39. Absorption spectrum in water molar extinction coefficient 41 500 (314 nm). Fluorescence spectra in water (uncorrected): exc, 315, 363, and 446 nm; em, 550 nm. ESR spectrum in water: triplet a^N = 15.3 G; g = 2.0056.

Poly[d(G-C)], poly[d(A-T)], and calf thymus DNA (42% GC) were purchased from Boehringer Mannheim (Germany). *Escherichia coli* DNA (type VIII, 50% GC) and *Clostridium perfringens* DNA (type XII, 34% GC) were purchased from Sigma Chemical Co. Poly[d(G-m^{5}C)] was purchased from Pharmacia (USA). Nucleic acid concentrations are expressed with respect to nucleotides.

Fluorescence Experiments. Fluorescence spectra were recorded on an SFM 23/B spectrofluorometer (Kontron, Zürich, Switzerland) equipped with a thermostated cell holder. In all cases, the fluorescence experiments were performed in quartz cells (1-cm path length) thermostated at 25 °C. Scattering of excitation radiations was removed by using the appropriate emission filter.

Energy Transfer. The occurrence of an energy transfer from nucleic acids to the bound drug was determined according to Weil and Calvin (1963). The relative quantum yield ratio of the bound drug upon excitation in the UV region vs excitation in the visible region was determined by using the equation:

$$\frac{QI}{Q_{vis}} = \left(\frac{I_{UV}}{I_{vis}}\frac{E_{vis}}{E_{UV}}\right)_{bound}\left(\frac{I_{vis}}{I_{UV}}\frac{E_{UV}}{E_{vis}}\right)_{free} \quad (1)$$

where I and E are respectively the fluorescence intensity and the molar extinction coefficient for exc,vis = 315 nm and exc,vis = 260 nm.

Viscometric Experiments. Viscometric measurements were performed at 25 °C in a semimicrodilution capillary viscometer linked to an IBM XT computer. The capacity of tested compounds to increase the length of sonicated calf thymus DNA and to remove supercoiling of covalently closed circular PM2 DNA was measured under standard operating conditions. The helix unwinding angle was calculated by comparing the equivalence binding ratio with that found for ethidium bromide.

UV Melting Curves. Absorbance versus temperature profiles for DNA and drug-bound DNA duplex were determined by using a thermoelectrically controlled Uvikon spectrophotometer. Samples were heated at a rate of 1 °C/min while both temperature and absorbance were recorded at 265 nm. Best fits of the melting curves dA/dt were obtained by a computed nonlinear regression describing sigmoid equation. The melting temperatures were estimated from the corresponding first derivative of the fitted curve.

Electron Spin Resonance Spectroscopy. ESR spectroscopy was performed at 25 °C by using a Bruker ER100 apparatus operating at 9.8 GHz. All spectra were obtained at 20-mW power with a modulation of 4 G. Samples were placed in a quartz cell and then introduced in the cavity of the spectrometer. Standard experiments were performed in 10 mM cacodylate buffer, pH 7.0, containing 50 mM NaCl. When

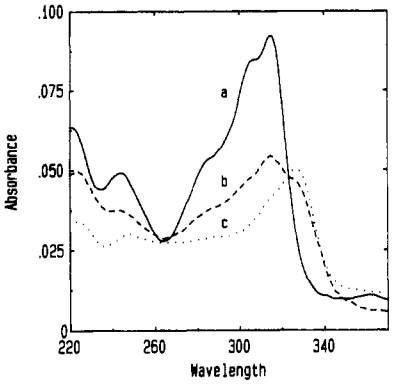

FIGURE 2: Spectral changes of proxyl-OPC upon binding to native DNA from *E. coli*. Assay medium was composed of 0.01 M cacodylate buffer (pH 7.0) containing 3 μM proxyl-OPC. The spectra were recorded as follows: (a) in the absence of DNA, (b) in the presence of 15 μM DNA (expressed in nucleotides), and (c) in the presence of 50 μM DNA. Buffered solutions containing DNA were placed in both sample reference cuvettes (5-cm light path), and P-POC was placed in the sample cuvette.

required, the concentration of P-OPC unbound to DNA was estimated from the height of the high-field peak of the ESR spectrum by using a calibration curve that describes the height of the high-field peak as a function of P-OPC concentration. For competition experiments, the ESR spectra were recorded following the addition of increasing concentrations of the competitor and the concentration of P-OPC unbound to DNA was calculated as described below (see Results). For the estimation of the binding index (BI), the assay medium was composed of the buffer indicated above containing 5 μM P-OPC. The ratio of the height of the central peak versus the height of the high-field peak was determined following the addition of polynucleotide or DNA until a concentration of 50 μM expressed in nucleotide. For the competition experiments involving BI determination, this procedure was repeated in the presence of increasing concentrations of competitor.

RESULTS

Association Constant Values for DNAs and Polynucleotides. The binding of P-OPC to DNA and polynucleotides results in changes in the absorption spectrum characterized by both hypochromic and bathochromic shifts. Figure 2 shows the spectral changes of P-OPC produced by increasing amounts of bacterial DNA from *E. coli*. There is a well-defined isosbestic point near 320 nm, indicating that a limited number of bound species (theoretically one) are in equilibrium with the free molecule. The concentrations of free and bound P-OPC were calculated by the mean of the relations:

$$\epsilon_1 = \epsilon_{1f}C_f + \epsilon_{1b}C_b \quad (2)$$

$$\epsilon_2 = \epsilon_{2f}C_f + \epsilon_{2b}C_b \quad (3)$$

i.e.

$$C_b = (\epsilon_1\epsilon_{2f} - \epsilon_2\epsilon_{1f})/(\epsilon_{1b}\epsilon_{2f} - \epsilon_{2b}\epsilon_{1f}) \quad (4)$$

$$C_f = (\epsilon_1\epsilon_{2b} - \epsilon_2\epsilon_{1b})/(\epsilon_{1f}\epsilon_{2b} - \epsilon_{2f}\epsilon_{1b}) \quad (5)$$

where ϵ_{1f} and ϵ_{1b} are the extinction coefficients of the free and bound P-OPC at 314 nm (index 1) or 328 nm (index 2). ϵ_1 and ϵ_2 are the extinction coefficients measured at these wavelengths. Binding curves so obtained can be plotted according to Scatchard (1949) and fitted according to Mc Ghee and Von Hippel (1974) by using a nonlinear regression procedure. This leads to the determination of the association constant (K_{app}) values and allows the estimation of the apparent size of the binding site n. Values of K_{app} and n obtained from this treatment are summarized in Table I. P-OPC displays a 6-fold higher K_{app} value for poly[d(G-C)] than for poly[d(A-T)] and an intermediate value for natural DNA purified from *C. perfringens* containing roughly 34% GC. The number of nucleotides covered by P-OPC increases from 2.3 in poly[d(G-C)] to 5 in poly[d(A-T)], suggesting that the geometry of the binding is markedly different in G-C and in A-T base pairs. K_{app} for DNA from *E. coli* was determined as a function of ionic strength. Record plots (log K_{app} versus log [Na^+]) (Record et al., 1976) for these data yield a straight line whose slope is close to 0.8 (data not shown). This is in a good agreement with the involvement of one positive charge in the binding process.

Table I: Binding Parameters of P-OPC to DNAs and Polynucleotides[a]

	poly-[d(G-C)]	poly-[d(A-T)]	*E. coli*	calf thymus	PM2 phage
K_{app} (10^6 M^{-1})	2.0	0.3	1.8	1.6	
r_{max}	0.43	0.2	0.36	0.37	
n	2.3	5.0	2.7	2.7	
slope[b]					
P-OPC				0.20	
OPC				2.38	
unwinding angle[c] (deg)					
P-OPC					9.6
OPC					22.4
energy transfer at 255 nm[d]					
P-OPC				2.5	
OPC				5.8	

[a] Measurement of the association constant values K_{app} of P-OPC to DNA and polynucleotides has been performed in 0.01 M cacodylate buffer (pH 7.00) by using the change in absorbance of OPC chromophore upon DNA binding. Binding curves have been treated according Mc Ghee and Von Hippel (1974), from which K_{app} and r_{max} (P-OPC bound to nucleotide ratio at saturating concentration of P-OPC) have been estimated. n ($1/r_{max}$) indicates the apparent size of the binding site expressed in number of nucleotides. DNA from *E. coli* and *C. perfringens* contain 50% and 70% AT base pairs, respectively. [b] Slope of the straight line given by the equation $\log \eta/\eta_0 = k(1 + 2r)$ which accounts for the increase of the viscosity of sonicated DNA as a function of the amount of drug bound (r). [c] Unwinding angle of PM2 phage DNA upon binding of drugs. [d] Energy-transfer efficiency from DNA bases to bound drug as measured according to Weil and Calvin (1976).

Mode of Binding. (A) Viscometric Studies. Information on the nature of the binding to DNA was provided mainly by the viscosimetric determination of the length increase of sonicated DNA and the unwinding angle of circular PM2 DNA as well as by the occurrence of energy transfer from DNA bases to the bound drug. In the former technique, the theoretical treatment of viscosimetric data has shown that if $\log \eta/\eta_0$ is plotted vs $\log (1 + 2r)$, where η and η_0 are the intrinsic viscosities of sonicated DNA in the presence and in the absence of the tested drug and r is the number of molecules bound per nucleotide, the slope value of the straight line so obtained is expected to be near 2.2 for monointercalating agents (Saucier et al., 1971). In a similar way, the change of the torsion of the DNA helix from phage PM2 caused by intercalation is expected to be in the range of 15–26° (Lerman, 1961). Data indicated in Table I show that binding of P-OPC to sonicated DNA did not result in a length increase of the helix, whereas, accordingly, the unwinding angle of PM2 DNA is lower than 10°. These features are in agreement with the occurrence of external binding. Data related to the OPC chromophore are given in the same table as a positive control for intercalation.

(B) Energy Transfer. Energy transfer from DNA bases to bound chromophores can be revealed by calculation of the ratio Q_λ/Q_v which indicates the relative quantum yield of the bound drug upon excitation in the UV region vs excitation in the

Table II: ΔT_m Values for P-OPC and Distamycin A to Duplex DNA from Phages T4 and T4*[a]

	DNA from			
	phage T4		phage T4*	
r(total)	0.15	0.25	0.15	0.25
ΔT_m				
P-OPC	9.3	12.5	10.0	13.2
distamycin	11.2	15.4	12.1	15.4

[a] ΔT_m indicates T_m (drug-bound duplex) − T_m (drug-free duplex). In each experiment, the assay medium was composed of 8 mM phosphate buffer, pH 7.0, containing 10 mM NaCl and 20 μM DNA. r indicates drug to nucleotide ratio.

visible region (Le Pecq & Paoletti, 1967). If energy transfer occurs, this ratio must be greater than unity. Energy transfer is believed to take place only with compounds in close contact with DNA bases as it occurs for drugs intercalated into nucleic acids (Le Pecq & Paoletti, 1967; Reinhardt et al., 1982). The experimental data shown in Table I correspond to the relative fluorescence quantum yields as measured at an excitation wavelength of 255 nm and obtained with OPC chromophore and P-OPC bound to DNA. Data values indicate that, compared to OPC, P-OPC is subjected to a markedly lower energy transfer from DNA bases, suggesting an unfavorable position of the chromophore of P-OPC with respect to the plane of bases, which is in agreement with a nonintercalative mode of binding.

(C) Groove Location. There is much supporting evidence for the selective binding of nonintercalative ligands within the minor groove of B-DNA [see Zimmer (1986) for review]. However, CPK (molecular modeling) binding studies also suggested that P-OPC may be as well accommodated in the major groove in either the monomeric or autoassociated dimeric stacked form. In order to rule out the latter possibility, we have performed comparative binding experiments using, on the one hand, DNA from T4 phage, which displays glycosylated bases in the major groove resulting in restricted accessibility, and, on the other hand, nonglycosylated DNA from the mutant T4* phage, which displays free accessibility. The occurrence of an equivalent number of binding sites in both DNAs can be investigated by measuring the elevation of the melting temperature upon P-OPC binding at nonsaturating concentration (D/P = 0.25) (D/P is the drug to nucleotide ratio). In these operating conditions, the change in T_m is linear with respect to the degree of binding (Crothers, 1971), which in turn depends on the number of accessible binding sites. Results in Table II show that the change of the T_m (dT_m) value resulting from the binding of P-OPC is equivalent with T4 and T4*, indicating that the number of P-OPC molecules bound and hence the number of binding sites are similar in these two DNAs. As expected, distamycin A displays a similar behavior. These results strongly suggest that P-OPC binds to DNA exclusively in the minor groove.

P-OPC Binding to DNA Studied by ESR Spectroscopy. The ESR spectrum of P-OPC in aqueous buffer recorded at room temperature is shown as spectrum a in Figure 3. The three-line spectrum (splitting 15.8 G) results from the anisotropic hyperfine interaction between the unpaired electron and the nuclear spin of the nitrogen atom. This spectrum is characteristic of a nitroxide radical undergoing rapid motion. The relative peak heights indicate a rotational correlation time close to 4×10^{-11} s. This spectrum is referred to that of P-OPC free in solution. In the presence of DNA, the ESR spectrum of P-OPC becames broad and asymmetric with a maximum hyperfine splitting ($2T_{\parallel}$) of 56.8 G (spectrum b in Figure 5).

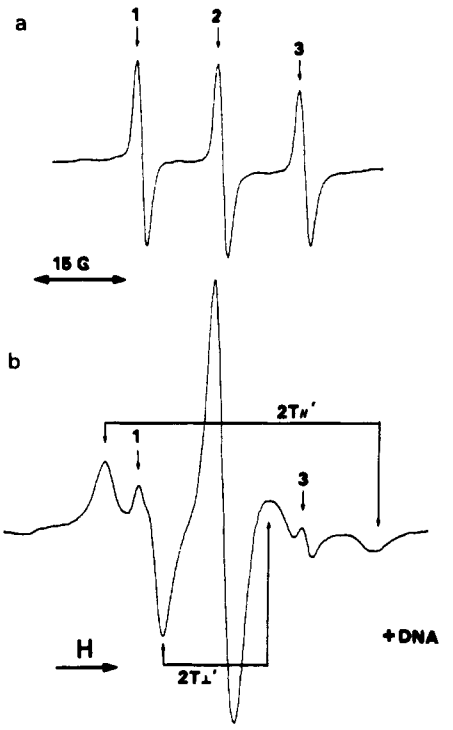

FIGURE 3: ESR spectra of proxyl-OPC in 0.01 M cacodylate buffer, pH 7.0, containing 50 μM NaCl. (a) 15 μM proxyl-OPC free in solution and (b) in the presence of 72 μM DNA (r = 0.21). Microwave frequency was 9.76 GHz, field modulation 2 G, and microwave power 20 mW. $T_{\parallel}'$ and $T_{\perp}'$ represent the outer and inner hyperfine splitting, respectively, of the immobilized nitroxide.

This "powderlike" spectrum is characteristic of a nitroxide whose molecular motion is highly restricted ($T_c \gg 10^{-7}$ s). This spectrum is referred to as P-OPC bound to DNA. The immobilization of P-OPC upon DNA binding suggests that the free rotation of the nitroxide-containing cycle with respect to OPC chromophore is very restricted. In the equilibrium conditions (D/P = 1) as in Figure 3, the resulting spectrum obtained is the superposition of both free and bound spectra in which the heights of the low-field and high-field peaks corresponding, respectively, to bound and free P-OPC fractions (arrows in fig 3b) might be estimated. Panels a, b, c, and d of Figure 4 show typical spectra of P-OPC in cacodylate buffer and in the presence of poly[d(G-C)], poly[d(A-T)], poly[d(G-m^{5}C)] in the B conformation, and poly[d(G-m^{5}C)] in the Z conformation. From the characteristics of these spectra, it can be seen that P-OPC binds to poly[d(A-T), poly[d(G-C)], and poly[d(G-m^{5}C)]. Compared to AT, binding to GC-containing polynucleotides appears more efficient as indicated by the lower concentration in free P-OPC molecules (low high-field peak intensity in spectra a and c compared to spectrum b). This is consistent with the results obtained with UV absorbance spectroscopy (Table I). In the presence of poly[d(G-m^{5}C)] in the Z conformation, P-OPC appears to be only weakly immobilized, suggesting that the single groove present in the Z form is not fully accessible.

The quantitative estimation of the concentrations of free and bound P-OPC can be achieved by using as parameter the ratio (a in eqs 6 and 7) of the spin intensity of the low-field peak versus the spin intensity of the high-field peak. At the equilibrium, the concentrations of bound and free proxyl-OPC can be calculated by using the relations:

$$C_f = [1/(1 + a/A)]C_t \quad (6)$$

$$C_b = [1/(1 + A/a)]C_t \quad (7)$$

where C_t, C_f, and C_b are the concentrations of total, free, and

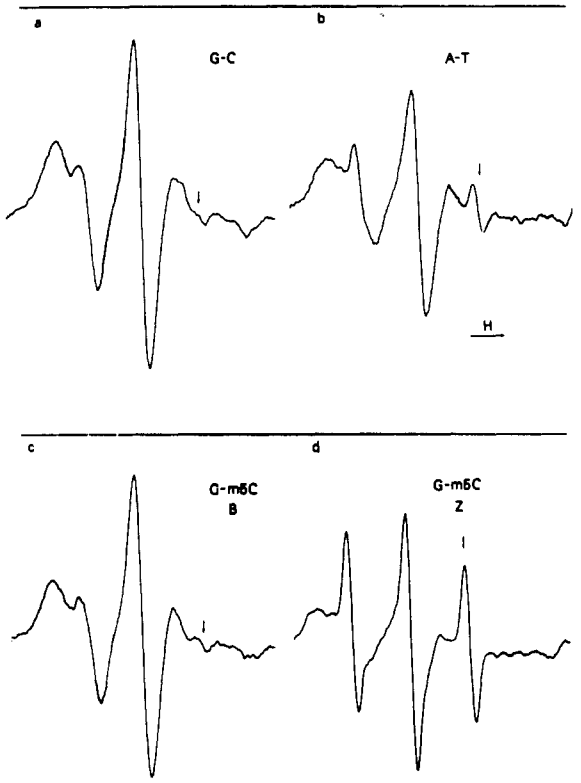

FIGURE 4: ESR spectra of 5 μM proxyl-OPC in the presence of synthetic polynucleotides. (a) 50 μM poly[d(G-C)]; (b) 50 μM poly[d(A-T)]; (c and d) 50 μM poly[d(G-m^{5}C)]. In (a), (b), and (c), polynucleotides were dissolved in 0.01 M cacodylate buffer, pH 7.0, containing 50 mM NaCl. In (d), the assay medium was composed of 0.01 M cacodylate buffer containing 50 mM NaCl and 10 mM $MgCl_2$, and poly[d(G-m^{5}C)] was in the Z conformation as evidenced by circular dichroism.

bound P-OPC, respectively. A represents the ratio of the spin intensity of the low-field peak of totally bound drug (measured in the presence of a saturating concentration of DNA or polynucleotide) versus the high-field peak intensity of free drug (measured in the absence of DNA). This ratio does not depends on the operating conditions of the spectrometer and was found to be 0.5 ± 0.07.

However, in various circumstances (i.e., for low concentrations of bound P-OPC), the spin intensity of the low-field peak often appears quite difficult to evaluate. In contrast, the peak-to-peak spin intensities of the central peak (CP) and high-field peak (HP) of the spectrum can be accurately measured. For a given proxyl-OPC concentration, the ratio (CP/HP = b) of the spin intensity of the central peak versus that of the high-field peak is a function of the percentage of bound proxyl-OPC. However, the ratio CP/HP cannot be directly used for the establishment of competition curves since the central peak intensity corresponds to the superimposed signals of free and bound drug. In an attempt to establish a suitable relation involving CP/CH as a P-OPC binding parameter, we have performed the titration of a given concentration of P-OPC by increasing concentrations of polynucleotides. The variation of the CP/HP value as a function of poly[d(A-T)] and poly[d(G-C)] concentration (C_n) is shown in Figure 5a. Nonlinear regression procedure indicates that the best fit to data points describing the relation CP/CH = $f(C_n)$ is obtained with the equation:

$$\mathrm{CP/HP} = A \exp(BC_n) \quad (8)$$

which can be linearized and expressed as

$$\log(\mathrm{CP/HP}) = L_0 + (\mathrm{BI})(C_n) \quad (9)$$

where L_0 is log (CP/HP) measured for the free drug (C_n = 0) and BI (binding index) is a constant that characterizes the binding efficiency of the spin-label with the polynucleotides. Figure 5b shows the titration curves of P-OPC by increasing concentrations of poly[d(A-T)] and poly[d(G-C)] as defined in eq 9. The slope values of the straight lines obtained correspond to the binding index. BI values determined in standard operating conditions (0.01 M cacodylate buffer, pH 7.0, 0.05 M NaCl) for DNA and polynucleotides are summarized in Table III. BI directly depends on both number of binding sites and association constant K_{app}. As shown in Figure 6, the

Table III: Binding Index (BI) ± SD of P-OPC for Synthetic Polynucleotides and DNA As Measured by ESR Spectroscopy[a]

	BI (mM^{-1})		BI (mM^{-1})
poly[d(G-C)]	27.0 ± 1.7	poly[d(I-C)]	1.8
poly[d(A-T)]	9.4 ± 3.1	DNA from *E. coli*	26.0 ± 2.4
poly[d(G-m^{5}C)]	34.9 ± 2.1		

[a] Measured under standard operating conditions (pH 7.0, 50 mM NaCl) according to the procedure indicated in Figure 5b.

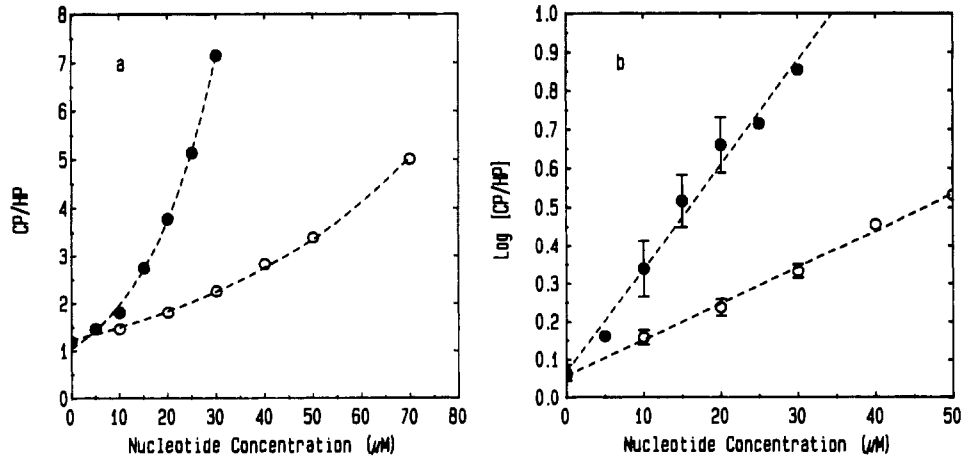

FIGURE 5: Variation of the central peak versus the high-field peak (CP/HP) of the P-OPC ESR spectrum as a function of polynucleotide concentration. (●) poly[d(G-C)]; (○) poly[d(A-T)]. Assay media were composed of 0.01 M cacodylate buffer, 0.05 M NaCl, and 5 μM proxyl-OPC. In panel a, curves in dotted lines are the best fits to data points obtained from the nonlinear regression procedure. Curves correspond to the following equations: poly[d(A-T)], $y = 1.22 \exp(0.02x)$ ($r^2 = 0.999$), and poly[d(G-C)], $y = 1.04 \exp(0.06x)$ ($r^2 = 0.998$). In panel b, each point represents the mean ± SD of four different experiments. The value of the slope of the straight line obtained from linear regression is referred to the binding index (BI) of P-OPC to the corresponding nucleotide.

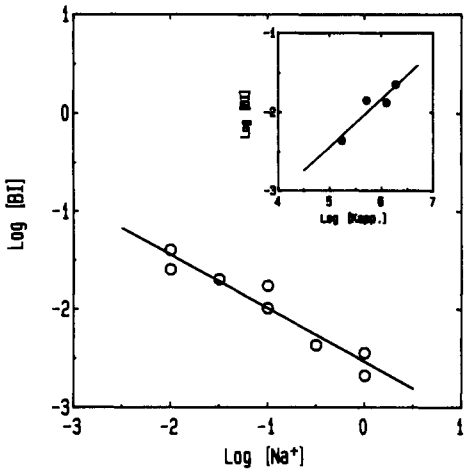

FIGURE 6: Variation of the binding index of proxyl-OPC to DNA from *E. coli* as a function of ionic strength. The slope of the straight line is 0.45. Insert: log (BI) plotted versus log K_{app} yields a straight line whose slope is 0.6. DNA is from *E. coli*; buffer is 0.01 M cacodylate, pH 7.0.

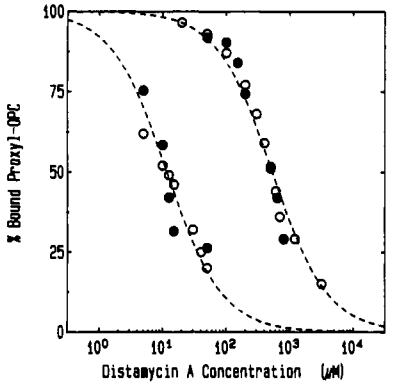

FIGURE 7: Displacement of proxyl-OPC by distamycin A from its binding sites on (a) poly[d(A-T)] and (b) poly[d(G-C)], as estimated by ESR spectroscopy. The assay media were composed of 0.01 M cacodylate buffer, pH 7.0, 20 μM proxyl-OPC, 57.5 μM poly[d(G-C)], or 180 μM poly[d(A-T)]. Data shown represent two different experiments. Curves in dotted lines were obtained from the best fits to data points given by the nonlinear regression procedure.

plot of log (BI) versus log [Na^+] (Record et al., 1976) yields a straight line, the slope of which is 0.45. Accordingly, there exists a linear relation between the variation of BI and K_{app} as a function of ionic strength (insert in Figure 6).

Competition Experiments. The addition of a competing ligand to a solution containing DNA–P-OPC complex should result in the displacement of P-OPC from its binding sites on DNA. Such a displacement can be quantified according to the procedure indicated above and consists in the determination of the concentrations of either free or bound proxyl-OPC after each addition of competing ligand. A competition curve [percent of P-OPC displaced from its binding sites, expressed as a function of the logarithm of the concentration of ligand added (X); see Figure 7] can therefore be established, which should be described by a sigmoidal equation:

$$Y = T + (B - T)/[1 + (10^C/10^X)] \quad (10)$$

where B is the bottom of the curve (usually 0) and T the top of the curve (usually 100). The data points can be fit by nonlinear regression procedure using the Marquart algorithm. The IC_{50} (C in the sigmoidal equation) is then defined as the ligand concentration that displaces 50% of C_d. C_d is defined as the total amount of proxyl-OPC that could be displaced by the competing ligand. It is estimated to be the "bottom" of the sigmoidal curve that we obtain by fitting the data. If proxyl-OPC and the ligand are competing for the same sites on DNA, then C_d is equal to C_{bi}, which is the initial amount of bound P-OPC. Then, the association constant $K_{app}(D)$ for the competitor can be estimated by using the equation:

$$K_{app}(D) = K(\text{P-OPC})[(C_t - C_{bi} + C_d/2)/(IC_{50} - C_d/2)] \quad (11)$$

where C_t is the total concentration of P-OPC, K(P-OPC) is the binding constant of the spin-label, and C_d, C_{bi}, and IC_{50} are as defined above. Therefore, $C_t - C_{bi} + C_d/2$ and $IC_{50} - C_d/2$ respectively represent the amounts of free P-OPC and free competing ligand when the total amount of competitor corresponds to the IC_{50} value. We have used this method to determine the K_{app} of distamycin A for poly[d(A-T)] and poly[d(G-C)]. Figure 7 shows data points and the fitted competition curves obtained by using distamycin A as an example of an external DNA binder competitive ligand.

The other way to perform competition experiments involves the use of BI as binding parameter. The construction of binding curves, log (CP/HP) = $f(C_n)$ (as indicated above), in the presence of increasing concentrations of competing ligand results in the appearance of different straight lines whose slopes (corresponding to BI values) decrease as a function of the concentration of the ligand. A typical example of such a procedure using distamycin A as competing ligand

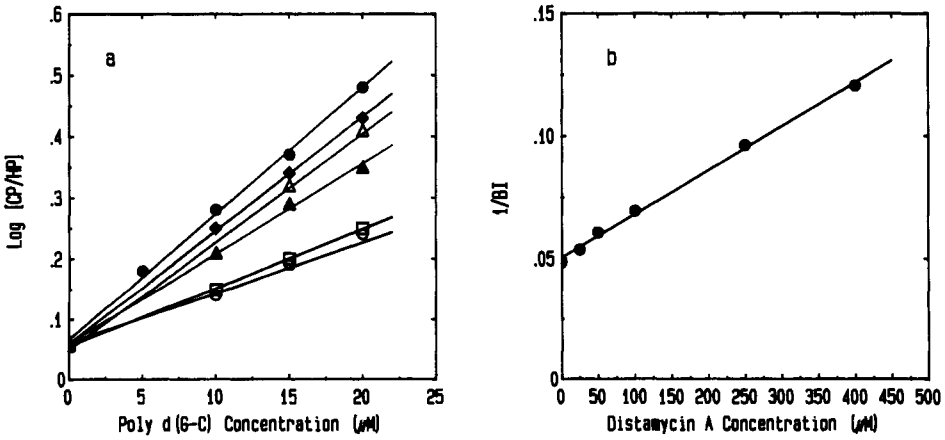

FIGURE 8: (a) Changes in BI value of proxyl-OPC for poly[d(G-C)] occurring upon the addition of the outside binder competing ligand distamycin A. Assay medium was composed of 0.01 M cacodylate buffer, pH 7.0, containing 50 mM NaCl and 5 μM proxyl-OPC. Distamycin A concentrations were as follows: (●) 0; (♦) 25 μM; (△) 50 μM; (▲) 100 μM; (□) 250 μM; and (○) 400 μM. (b) Plot of 1/BI versus the concentration of the competing ligand. The straight line obtained allows us to calculate the IC_{50} value of the ligand for the polynucleotide (see text).

Table IV: Binding Parameters of Distamycin A to Poly[d(A-T)] and Poly[d(G-C)]

	poly[d(A-T)]	poly[d(G-C)]	ratio
IC_{50}[a]	11.7 μM	529 μM	45.2
K_{app}[a]	1.3×10^6 M^{-1}	6×10^4 M^{-1}	22
IC_{50}[b]	9.2 μM	256 μM	28
IC_{50}[c]	0.07 μM	36 μM	514

[a] IC_{50} and K_{app} values were estimated from the competition curves shown in Figure 7. [b] IC_{50} values were estimated by using BI according to the procedure shown in Figure 8. [c] IC_{50} values were estimated from ethidium bromide exclusion technique; data were taken from Baguley (1982).

and poly[d(G-C)] as polynucleotide is shown in Figure 8a. The concentration of distamycin A that decreases the BI value by 50% can be estimated from the straight line describing the variation of BI (1/BI) as a function of the concentration of distamycin A (Figure 8b). This value corresponds to the concentration of competing ligand that decreases by 50% the apparent concentration of binding sites and is therefore related to the IC_{50} obtained by using competition curves. In the standard operating conditions, IC_{50} values of distamycin A for poly[d(A-T)] and poly[d(G-C)] were found to be 5 and 256 μM, respectively. It should be pointed out, however, that according to this procedure the experimental data available did not allow estimation of the concentration of free P-OPC. Consequently, the K_{app} of the competitive ligand cannot be simply estimated.

Discussion

The aim of the present work was to demonstrate the feasibility of the spin-label technique involving competitive binding as a tool for measuring binding to DNA and polynucleotides of nonintercalating molecules. This method can be proposed instead of ethidium bromide assay when direct measurement of the drug binding involving either spectral modifications or equilibrium dialysis cannot be performed. The spin-label proxyl-OPC has been selected for such a purpose for the following reasons: (i) P-OPC interacts with DNA through a nonintercalative mode in the minor groove which is the DNA binding region of most nonintercalating molecules. (ii) The binding in the minor groove results in the complete immobilization of the nitroxide moiety, allowing determination of the actual concentrations of free and bound P-OPC from the ESR spectra parameters. (iii) The association constant values K_{app} of P-OPC for DNA and polynucleotides are in the range of 10^5–10^6 M^{-1}, allowing one to perform competition experiments with most of the DNA binders. The first problem related to the competition method using ethidium bromide as probe arises from the possible binding of EB to different types of binding sites, namely, intercalating and external sites. In contrast, the superimposed ESR spectra corresponding to immobilized and free P-OPC account for a single equilibrium between P-OPC bound inside the minor groove of DNA and P-OPC free in solution. This assumption is based on the following observations: (i) viscometric and energy-transfer experiments clearly show that P-OPC is not able to reach intercalating sites, (ii) the number of binding sites is quite similar in DNAs isolated from phages T4 and T4*, which display an occluded or freely accessible major groove, respectively, and (iii) there exists a well-defined isosbestic point on P-OPC absorbance spectra recorded at various concentrations of DNA. The second major problem arises from the occurrence of EB–DNA fluorescence quenching in the presence of molecules bearing electron-donating moieties, such as hydroxy and amino groups. This feature, resulting from the occurrence of a charge-transfer complex at the DNA level (especially in AT-rich regions) between EB and the competing drug, mimics the exclusion of EB from its binding sites (Baguley & Le Bret, 1984; Davis et al., 1987). In contrast, ESR spectra allow the direct measurement of the actual amounts of P-OPC bound to DNA and of the free drug in solution. In competition experiments, the increase in the high-field peak reflecting P-OPC undergoing rapid motion directly corresponds to the displacement of P-OPC from its binding sites whereas the decrease of the low-field peak corresponds to the concomitant decrease of the amount of bound P-OPC. In order to minimize the fluctuations in the intensity of the ESR spectrum which may arise from setting the ESR apparatus for each point, the displacement of P-OPC can be evaluated by measuring the ratio of the low-field peak versus the high-field peak intensities. Using this procedure, competition curves have been satisfactory, thus establishing use of this method as a probe in the external DNA binding of distamycin A. The best fit to data points of the competition curves (Figure 7) given by the nonlinear regression procedure is obtained for the expected sigmoid equation (eq 10). This supports the validity of the procedure, which therefore allows the estimation of IC_{50} and K_{app} from these competition curves. However, in such a competition experiment, the limitation of the technique comes from difficulty in the estimation of the amount of bound P-OPC from the weak intensity of the broad peak in the low field of the ESR spectra, especially in the presence of high concentrations of competitive ligand. Normalization of the spin intensity using the high-field peak to central peak ratio appears to be the best alternative in the quantification of the P-OPC binding. Titration of P-OPC by increasing concentrations of DNA or polynucleotides gives access to the binding parameter BI, which is directly related to the association constant value K_{app} of P-OPC to the polynucleotide. Determination of BI as a function of the competitive ligand concentration allows us to evaluate the concentration of ligand that decreases the BI value by 50%. This concentration corresponds to the one that apparently decreases by 50% the number of accessible P-OPC binding sites and is therefore related to IC_{50}, which is defined as the competitive ligand concentration that displaces 50% of P-OPC from its binding sites. In any cases, the selectivity of the binding of any DNA ligand to either A-T- or G-C-containing nucleotides can be expressed as the ratio of their respective ID_{50} or K_{app} to these polynucleotides. Table IV summarizes the data obtained with distamycin A by using ESR spectroscopy and using ethidium bromide exclusion (Baguley, 1982). The main feature is that the ratio value that reflects the extent of selective binding to A-T base pairs appears roughly 1 order of magnitude higher with data provided by the ethidium bromide assay compared to that obtained by ESR spectroscopy assays. This may be explained by the fluorescence quenching which may occur upon distamycin A binding to A-T base pairs, resulting in the overestimation of the binding efficiency of the drug to poly[d(A-T)].

Registry No. P-OPC, 127183-95-3; poly[d(A-T)], 26966-61-0; poly[d(G-C)], 36786-90-0; poly[d(G-m^{5}C)], 51853-63-5; distamycin A, 636-47-5.

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DNA Ligases from Rat Liver. Purification and Partial Characterization of Two Molecular Forms†

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ABSTRACT: The differential ability of mammalian DNA ligases to use oligo(dT)·poly(rA) as a substrate has been used to detect, and thereby extensively purify, two immunologically distinct forms of DNA ligase from rat liver. The activity of DNA ligase I, which is unable to use this template, is uniquely increased during liver regeneration, while that of DNA ligase II remains at a low level. Both enzymes require ATP and Mg^{2+} for activity and form an adenylylated intermediate which is stable and reactive. After SDS–PAGE, such radiolabeled complexes correspond to polypeptides of 130 000 and 80 000 Da for DNA ligase I and to 100 000 Da for DNA ligase II. That these labeled polypeptides do indeed correspond to active polypeptides of two different forms of DNA ligase is shown by the removal of the radiolabeled AMP, only when the intermediate is incubated with an appropriate substrate. In contrast to other eukaryotic DNA ligases, rat liver DNA ligase II has a lower K_m for ATP (1.2×10^{-5} M) than DNA ligase I (6×10^{-5} M). Also, DNA ligase II can use ATPαS as a cofactor in the ligation reaction much more efficiently than DNA ligase I, further discriminating the ATP binding sites of these enzymes. Finally, antibodies raised against the 130 000-Da polypeptide of DNA ligase I specifically recognize this species in an immunoblot and inhibit only the activity of DNA ligase I.

DNA ligase catalyzes the formation of a phosphodiester bond between adjacent 5′-phosphoryl and 3′-hydroxyl termini at single-strand nicks in double-stranded DNA (Lehman, 1974). It is therefore an essential element of the coordinated multienzyme processes of semiconservative DNA replication and DNA excision repair, both of which involve the joining of single-stranded breaks.

In mammalian cells, two forms of DNA ligase have been reported in a number of tissues including calf thymus, rat liver, and human placenta (Soderhall & Lindahl, 1975; Teraoka et al., 1986; Bhat & Grossman, 1986). DNA ligase I is the predominant activity in rapidly growing cells and is generally isolated as a 130-kDa polypeptide. DNA ligase II was first described from calf thymus as a late eluting activity during hydroxyapatite chromatography (Soderhall & Lindahl, 1973). The activity of this enzyme is similar in quiescent and dividing cells and is associated with a 68–80-kDa polypeptide (Teraoka et al., 1986; Arrand et al., 1986; Lindahl et al., 1989). Furthermore, it has been shown that these two enzymic forms are immunologically distinct: antibodies against DNA ligase I do not cross-react with DNA ligase II, and vice versa (Soderhall & Lindahl, 1975; Teraoka & Tsukada, 1986).

Earlier reports on DNA ligase from rat liver showed that the total level of DNA ligase activity increased in conjunction with nuclear DNA synthesis during liver regeneration (Tsukada & Ichimura, 1971). Using a rabbit antiserum raised against calf thymus DNA ligase I, Soderhall (1976) showed that although DNA ligase II (obtained by hydroxyapatite chromatography) was the major activity in normal liver, it was the DNA ligase I component which was uniquely increased during liver regeneration. About the same time, the purification of a DNA ligase from rat liver nuclei was described (Zimmerman & Levin, 1975). When compared with contemporary data, this enzyme exhibited many of the properties

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