$^1$H NMR study of an ethidium dimer poly(dA-dT) complex: evidence of a transition between bis and monointercalation


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ABSTRACT.

Comparative $^1$H NMR and optical studies of the interaction between poly(dA-dT), ethidium bromide (Et) and ethidium dimer (Et$_2$) in 0.7 M NaCl are reported as a function of the temperature. Denaturation of the complexes followed at both polynucleotide and drug levels leads to a biphasic melting process for poly(dA-dT) complexed with ethidium dimer ($t_{1/2} = 75^\circ$C; $93^\circ$C) but a monophasic one in poly(dA-dT): ethidium bromide complex ($t_{1/2} = 74^\circ$C). In both cases drug signals exhibit monophasic thermal dependance (Et = 81°C; Et$_2$ = 95°C). Evidence is presented showing that the ethidium dimer bisintercalates into poly(dA-dT) in high salt, based on the observation that i) dimer and monomer ring protons exhibit similar upfield shifts upon DNA binding, ii) upfield shifts of DNA sugar protons are twice as large with the dimer than with ethidium bromide. Comparison between native DNA fraction and bound drug fraction indicates that ethidium covers, $n = 2.5$-3 base pairs. The dimer bisintercalates and covers, $n = 5.7$ base pairs when the helix fraction is high but as the number of available sites decreases the binding mode changes and the drug monointercalates ($n = 2.9$).

INTRODUCTION.

Bifunctional intercalators bind strongly to DNA through different modes, one of which being bisintercalation (1-10). Moreover the relative importance of these types of binding depends on the experimental conditions (5,9-12). Because these molecules are potential antitumor agents (8,10) and are reagents for biological research, it is of interest to characterize in detail their interactions with DNA. To date DNA binding of bifunctional intercalators has been extensively studied by viscometry, electron microscopy, fluorescence, circular dichroism ... but, at this time, only one short abstract has appeared which refers to an NMR study of the interaction between DNA and a bifunctional derivative of ethidium(13). Yet NMR is a suitable technique for such studies because it provides a large set of informations both at the drug and at the DNA levels (14).

Here we report a comparative proton NMR study at 270 MHz of the interaction between poly(dA-dT) and ethidium bromide, Et, or ethidium dimer, Et$_2$. 

(Scheme I) as a function of temperature. The alternating copolymer poly(dA-dT) can fold into small hairpins, whose rapid migration along the main backbone of the helix provides a segmental mobility sufficient to narrow the resonances in the duplex state. Patel (15,16) has shown that this synthetic polynucleotide could be advantageously used as a DNA model in NMR studies of drug-DNA interactions.

While crystallographic (17) and NMR (14) informations are available on the intercalation complex of ethidium bromide, Et, with self complementary dinucleosides, DNA binding of the ethidium dimer seems to be more ambiguous. In low salt the ethidium dimer unwinds closed circular DNA as ethidium bromide itself but, however, it lengthens sonicated DNA rods 1.6 times more than the monomer (5). A similar behaviour has been already observed with bismethidium spermine in low salt (9). Because the latter was reported to bisintercalate in 1 M NaCl (11) we expected ethidium dimer Et₂ also to bisintercalate into poly(dA-dT) in high salt.

In the present study we have followed the poly(dA-dT) resonances and most of those of the drug through the duplex to strand transition of the complexes (base pairs/drug = 16) in 0.7 M NaCl. Optical studies monitoring the thermal denaturation of the drug complexes are also reported.

MATERIAL AND METHODS.

Ethidium was purchased from Boots Pure Drug Co. Ltd. (England) and used without further purification. Ethidium dimer was prepared as reported (18,19). Poly(dA-dT) (sedimentation coefficient S20,w = 5-6) was purchased from collaborative Research (USA). Chemicals were dissolved in pure water and the concentration of the solutions was based on an extinction coefficient of ε₄₇₈ = 5.8 x10³ M⁻¹ cm⁻¹ for ethidium bromide, ε₄₉₇ = 12x10³ M⁻¹ cm⁻¹ for ethidium dimer and ε₂₆₂ = 13.2x10³ M⁻¹ cm⁻¹ (in base pairs) for poly(dA-dT). Dilution of samples by the appropriate buffer gave the following final NMR solutions: [poly...
\[(\text{dA-dT})] = 3.9 \text{ mM (base pairs)}, [\text{drug}] = 0 \text{ or } 0.24 \text{ mM (base pairs/drug = 16)}, 0.7 \text{ M NaCl}, 0.1 \text{ M deuteroacetate buffer pH 5.5, 0.8 mM ethylene diamine tetraacetate.}

Selection of the BP/D ratio value was limited, both by the minimal concentration of intercalator (ethidium bromide and ethidium dimer) required to observe the drug protons and by the low solubility of the poly(dA-dT) : Et \text{ complex in water.}

For the purpose of the optical studies, NMR solutions were diluted tenfold with 0.1 M deuteroacetate buffer, 0.7 M NaCl. For comparison a sample containing poly(dA-dT) and ethidium bromide (base pair/drug = 12.5) was also prepared in 0.1 M phosphate buffer pH 7.4.

NMR spectra were recorded on a Bruker WH 270 interfaced to an Aspect 2000 computer and operating in the Fourier transform mode. The chemical shifts are referenced to an internal standard of 2,2-dimethyl-2-silapentane-5 sulfonate (DSS). Temperature was monitored by a B-ST 100/700 controller unit. Sample size was 0.3 ml.

Optical studies were run over an Unicam SP 8-100 spectrophotometer. Optical densities at 250 nm and 480 nm were read simultaneously using 2 mm and 1 cm path length cuvettes, respectively. Temperature was monitored in the cuvettes by a thermistor probe and regulated by an Haake temperature controller.

**RESULTS.**

**NMR studies.**

At ionic strength lower than 0.7 M melting of the poly(dA-dT) : Et \text{ complex in the temperature range 55-105°C occurred apparently as in the absence of the drug when followed by chemical shift changes. Therefore, the complex denaturation was only seen at high temperature through both an increase and a sharpening of the peaks corresponding to the uncomplexed single-stranded poly(dA-dT). This can be related to a slow exchange process between free poly (dA-dT) and the complexed form in this ionic conditions.}

Consequently, NMR studies were performed at 0.7 M ionic strength, conditions allowing the poly(dA-dT) resonances to shift as average peaks along the entire melting process.

Figure 1 shows the proton NMR spectra of poly(dA-dT), poly(dA-dT) : ethidium bromide and poly(dA-dT) : ethidium dimer complexes recorded at 90°C. The poly(dA-dT) signals have previously been assigned by Patel et al. (15,20). In the spectra of the poly(dA-dT) : drug complexes at high temperature, minor peaks are observed in addition to the DNA resonances and correspond to drugs protons. Ethidium resonances in the poly(dA-dT) : Et complex were readily assigned by comparison with dye alone in the same temperature range. Those of
Figure 1. 270 MHz $^1$H NMR spectra of poly(dA-dT), poly(dA-dT) : ethidium bromide and poly(dA-dT) : ethidium dimer complexes in 0.1 M deuteracetate buffer 0.7 M NaCl, 0.8 mM EDTA pH 5.5 at 90°C (poly(dA-dT) = 3.9 mM base pairs; input ratio = base pairs/drug = 16). * drug resonances.

ethidium dimer (18,19) however, were poorly resolved in the complex, even above 100°C and thus a few of them were assigned. However, these spectra clearly show that protons of the complexed ethidium dimer remain strongly upfield shifted at 90°C whereas those of ethidium monomer have already reached their positions in the uncomplexed form. Such a behaviour further illustrates the already reported higher DNA binding affinity of ethidium dimer (5).

The chemical shifts of the non exchangeable base and sugar protons in poly(dA-dT), poly(dA-dT) : Et and poly(dA-dT) : Et$_2$, are plotted versus temperature in figure 2. Most of the DNA resonances shift downfield as the temperature increases. In both poly(dA-dT) and poly(dA-dT) : Et, the chemical shifts changes display a monophasic cooperative DNA denaturation process, poly(dA-dT) $t_{1/2} = 73.5 \pm 1°C$ ; poly(dA-dT) : Et $t_{1/2} = 74 \pm 1°C$). By contrast, in poly (dA-dT) : Et$_2$, the polynucleotide resonances exhibit biphasic temperature dependencies, clearly evidenced on adenine H$^2$ proton with two successive midpoints (lower, $t_{1/2} = 75 \pm 1°C$ ; upper, $t_{1/2} = 93 \pm 1°C$).

The adenine H$^2$ and to a lesser extent, the adenine H$_1$, and thymine CH$_3$ (5) resonances undergo large downfield shifts (1.09 ppm, 0.35 ppm and 0.44 ppm respectively) through poly(dA-dT) fraying and display at 270 MHz, extrabroa-
dening due to moderately fast exchange between the double and single stranded states of the polymer. However, the mono or biphasic melting processes can also be followed from the decrease in linewidth of the adenine H₈ proton with temperature (figure 3) due to the rapid exchange between helix and coil states of the polymer relative to the H₈ chemical shift differences (0.2 ppm).

At high temperature (> 100°C) the chemical shifts of the polynucleotide protons are similar in the presence or in the absence of the dyes but, below 60°C, some of them (thymine H₆, H₁, H₂, and adenine H₄, and H₃) are shifted slightly upfield in the complexes (Table I). Due to the high BP/D ratio, these upfield shifts are small but probably significant especially in the case of

![Figure 3. Thermal dependence of the width of adenine H₈ resonance. •: no drug added; •: + ethidium bromide; *: + ethidium dimer.](image)
TABLE I. Upfield shifts (Δδ in Hz ± 1 Hz) of thymine (TH₆, TH₁, TH₃) and adenine (AH₄, AH₃) protons in poly(dA-dT) : Et and poly(dA-dT) : Etl₂ complexes relative to poly(dA-dT) at 60°C (BP/D = 16).

<table>
<thead>
<tr>
<th></th>
<th>TH₆</th>
<th>TH₁</th>
<th>TH₃</th>
<th>AH₄</th>
<th>AH₃</th>
</tr>
</thead>
<tbody>
<tr>
<td>Poly(dA-dT) : Et</td>
<td>-5</td>
<td>-8</td>
<td>-5</td>
<td>-5</td>
<td>-5</td>
</tr>
<tr>
<td>Poly(dA-dT) : Etl₂</td>
<td>-8</td>
<td>-16</td>
<td>-11</td>
<td>-8</td>
<td>-11</td>
</tr>
</tbody>
</table>

the sugar protons H₃, whose chemical shifts are almost insensitive to the temperature changes.

Unfortunately, the proton H₂ which could elicit the largest upfield shift on ethidium or ethidium dimer intercalation cannot be accurately recorded during the first step of the poly(dA-dT) : Etl₂ complex denaturation because it broadened beyond detection.

The temperature dependence of the chemical shifts of ethidium and ethidium dimer protons in their complexes is shown in figure 4. Relative to the free dyes, the phenanthridinium ring protons undergo, in the two complexes, large upfield shifts on DNA binding (H₂, H₄, H₇, H₉ : 0.9-1.1 ppm) with a similar shielding for corresponding protons below the melting temperature of their complexes. Thermal profiles of ethidium protons are monophasic and

Figure 4. Plots of drugs protons chemical shifts versus temperature. Left : ethidium bromide ; right : ethidium dimer.
obviously reflect the cooperative melting of the bound poly(dA-dT) with a midpoint of 81 ± 1°C. On the other hand, protons of the dimer only shift downfield in the second step of the poly(dA-dT) biphasic melting transition (t_{1/2} = 94 ± 1°C).

Optical studies.

Denaturation of poly(dA-dT) in the absence and in the presence of ethidium bromide or ethidium dimer (base pairs/drug = 16) was monitored by UV-visible spectroscopy using concentrations ten times lower than in the NMR experiments [DNA = 3.9x10^{-4} M (base pairs); 0.1 M deuteroacetate buffer pH 5.5, 0.7 M NaCl, drug = 2.4x10^{-5} M].

Poly(dA-dT) absorption at 250 nm exhibits a single transition in the absence of drug (t_{1/2} = 73.5 ± 0.5°C) or in the presence of ethidium bromide (t_{1/2} = 75.5 ± 0.5°C) (data not shown). In the poly(dA-dT) : Et complex, the temperature dependence of the 480 nm band of the dye (figure 5) also monitors a monophasic denaturation process with a midpoint at 79 ± 0.5°C.

In the presence of ethidium dimer, a biphasic curve is observed at 250 nm (data not shown) but the two successive steps are poorly resolved and an average t_{1/2} value (82 ± 1°C) can only be derived from this curve. Melting of the DNA complexed with Et₂ occurs at higher temperature (t_{1/2} = 95 ± 0.5°C) as clearly shown from the 480 nm absorbance changes with increasing temperature (figure 5).

DISCUSSION

Shifts of DNA and drug protons as well as absorbance changes at 250 nm
TABLE II. Melting point ($t_{1/2}$ °C) of poly(dA-dT) and poly(dA-dT) complexes in 0.1 M deuteroacetate buffer, 0.7 M NaCl pH 5.5 determined by proton NMR and UV visible spectroscopy (input ratio base pairs/drug = 16).

<table>
<thead>
<tr>
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<th>1H NMR</th>
<th>UV visible spectroscopy</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>DNA</td>
<td>Drug</td>
</tr>
<tr>
<td>Poly(dA-dT)</td>
<td>73.5±1°C</td>
<td>-</td>
</tr>
<tr>
<td>Poly(dA-dT) : Et</td>
<td>74.0±1°C</td>
<td>81±1°C</td>
</tr>
<tr>
<td>Poly(dA-dT) : Et$_2$</td>
<td>75.5±1°C</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>93.0±1°C</td>
<td>94±1°C</td>
</tr>
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$^a$) DNA midpoints are average of values measured on $H^2$ (when observable), $H^5$, $H^6$ and adenine $H_1$.

and 480 nm were used to monitor the thermal denaturation of the poly(dA-dT) : Et and poly(dA-dT) : Et$_2$ complexes (figure 5). Shifts towards higher temperatures of the melting points of the complexes (Table II) relative to the polymer alone indicate that both Et and Et$_2$ are helix stabilizers (21). Drugs always show monophasic thermal profiles with upper midpoints (Table II). In the Et$_2$ complex, thermal dependence of the phenanthridinium protons is closely parallel to the higher phase of the DNA melting. Therefore the poly(dA-dT) proton shifts and the 250 nm hyperchromicity induced by increasing temperature reflect denaturation of both free and bound DNA while only melting of the complexes is monitored by the thermal dependence of the visible 480 nm band and downfield shifts of the drug protons. Biphasic melting curves are usually observed when drugs bind tightly to DNA or polynucleotides either by intercalation or otherwise (2,16,22). Such biphasic processes cannot be accounted for by cooperative binding of the drug to DNA (2,33) but are related to the bound drug redistribution along the remaining polymer double stranded region during the thermal denaturation (21,24,14). Moreover, for a given value of the DNA/dye ratio, transition from a biphasic to a monophasic process only depends on the lowering of the drug affinity for DNA. Thus according to the decrease of electrostatic interactions on increasing the ionic strength, the poly(dA-dT) : Et complex exhibits biphasic and monophasic melting profiles, in 0.1 M phosphate buffer pH 7.4 (23) and in 0.1 M sodium acetate, 0.7 M NaCl respectively. In the case of Et$_2$, its affinity for poly(dA-dT) in 0.7 M NaCl is higher (> 10$^6$ M$^{-1}$) (5) than that of the monomer allo-
wing to follow the entire complex denaturation which occurs in this ionic conditions below 100°C through a biphasic process.

In poly(dA-dT) : Et, the phenanthridinium protons undergo large upfield shifts on poly(dA-dT) binding due to the ring current contributions from superimposed DNA base pairs. Moreover, some DNA base and sugar protons are slightly upfield shifted (thymine $H_6$, $H_1$, $H_3$, and adenine, $H_1$, $H_3$) (Table I). Similar observations have been reported by Patel et al., in the poly(dA-dT) : Et complex in low salt (23) and in the poly(dA-dT) : proflavine complex in 1 M NaCl (25). These features seem to be characteristic of intercalation of aromatic ligands into DNA. Indeed, non-intercalative drugs like bleomycin (26) or steroid diamine dipyrandium (27) mostly induce downfield shifts of polynucleotide protons on complex formation.

Three modes of DNA binding may be assumed for the ethidium dimer : external binding, monointercalation, bisintercalation. The former must be ruled out because phenanthridinium protons undergo large upfield shifts on poly (dA-dT) binding.

Discrimination between mono and bisintercalation modes is more difficult. In high salt conditions, the electrostatic interactions between positively charged compounds and DNA backbone are strongly weakened (28). Therefore, the rate of exchange between dangling and intercalated ring in the poly(dA-dT) : Et$_2$ complex is expected to be roughly similar to that of ethidium between free and intercalated states in poly(dA-dT) : Et complex. Assuming an upper limit of 340 Hz (1.25 ppm at 270 MHz) between free and intercalated ring protons, a slow exchange on the NMR time scale requires a life time in either state widely superior to 15 ms. Since a lifetime value of 1 ms was reported for ethidium : calf thymus DNA complex in 0.1 M NaCl at 40°C (29) the rate of exchange free ring $\leftrightarrow$ intercalated rings in the poly(dA-dT) : Et$_2$ complex should remain fast on the NMR time scale.

If the ethidium dimer monointercalates, protons of the intercalated ring would be shifted as ethidium bound to poly(dA-dT) while protons of the dangling ring should be less shielded. Therefore, the averaged resonances would be less upfield shifted than those observed for ethidium in poly(dA-dT). Nevertheless, if monointercalation occurs together with external stacking between dangling rings of two different dimers, the averaged signals would be shifted to almost the same extent as in the bisintercalation mode. However, the external stacking seems very unlikely because self-association of ethidium is low [$K_{ass} \sim 100$ M$^{-1}$ (30)]. Moreover, at BP/D = 16 (1/4 saturation by Et$_2$), such an interaction should imply a cooperative DNA binding of
the drug what is not observed (5). Therefore, monointercalation with or without stacking of dangling chromophores can be ruled out.

By contrast, chemical shifts of the phenanthridinium protons at 70°C (native complex) being similar in both the ethidium and ethidium dimer complexes (e.g. H: Et complex, 5.41 ppm ; Et: complex, 5.37 ppm) it can be concluded that, ethidium dimer bisintercalates into poly(dA-dT) in high salt conditions. This conclusion is reinforced by the upfield shifts of thymine H, H_, H, and adenine H, and H, protons on drug binding, which in the native complex are twice as large in the presence of the ethidium dimer than in the presence of the ethidium monomer (Table I).

A simplified analysis of the melting curves leads to the determination of the average number n of DNA base pairs protected against thermal denaturation by a drug molecule. Let f' and f be, respectively, the molar fractions of bound drug and non denatured poly(dA-dT).

\[ f' = \frac{[\text{drug}]_b}{[\text{drug}]_t} \quad [1] \]

\[ f = \frac{[\text{DNA}]_n,f + [\text{DNA}]_n,b}{[\text{DNA}]_t} \quad [2] \]

Subscripts n, f, b, t, refer to native DNA and free, bound and total concentration of the species. Assuming the drug does not bind to single stranded DNA, free and bound native DNA concentrations [DNA]_n,f and [DNA]_n,b can be evaluated from relationships:

\[ [\text{DNA}]_n,b = n [\text{drug}]_b \quad [3] \]

\[ [\text{DNA}]_n,f = \frac{1}{K} \frac{[\text{drug}]_b}{[\text{drug}]_f} = \frac{1}{K} \frac{[\text{drug}]_b}{[\text{drug}]_t - [\text{drug}]_b} \quad [4] \]

Here, K is the DNA affinity constant of the drug. The assumption about exclusive double stranded DNA binding of the drug is legitimate since, at high temperature, chemical shifts of corresponding protons are similar for isolated species [poly(dA-dT) or drug] and for mixture [poly(dA-dT) + drug]. Combination of equations [1], [2], [3] and [4] give relation [5] between f and f'.

\[ f = \frac{f'}{f} + n f' \frac{[\text{drug}]}{[\text{DNA}]} \quad [5] \]

f' and f were independently estimated from experimental chemical shifts of drug and poly(dA-dT) protons. \( \delta_T \), \( \delta_f \), \( \delta_u \) are the chemical shifts of a proton of the DNA or the drug, respectively at the temperature T, below (l) and above (u) the transition. Then f (respectively f') is equal to:

\[ \frac{\delta_u - \delta_T}{\delta_u - \delta_f} \]

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In biphasic processes (Et$_2$ complex or Et complex in low salt) the calculation of \( f' \) can only be performed using the second phase of the duplex to strand transition, since the drug is entirely DNA bound (\( f' = 1 \)) at a lower temperature. If, for a given proton, the difference between the chemical shifts at low (bound state) and high temperature (free state) is greater than 1 ppm (the condition is verified for the adenine H$^2$ and several phenanthridinium ring protons) then the absolute error in \( f \) or \( f' \) is less than 0.03 (assuming that the chemical shifts are reliable to ± 0.003 ppm). We do note that within the experimental error range and for \([\text{DNA}] = 3.9 \times 10^{-3} \text{ M (base pairs)}\) the linear term in \( 5 \) dominates as soon as \( K \) exceeds \( 2.5 \times 10^5 \text{ M}^{-1} \).

In figure 6 the molar fraction of double stranded DNA \( f \) weighted by the input ratio \([\text{DNA}] / [\text{drug}] \) is plotted versus the molar fraction of bound drug \( f' \) for the poly(dA-dT) : Et complexes (low and high salt) and poly(dA-dT) : Et$_2$ complex (0.7 M NaCl). As expected, because ethidium bromide in low salt binds tightly to DNA (\( K \approx 5.10^5 \text{ M}^{-1} \)) we observe a linear dependence of \( f \times [\text{DNA}] / [\text{drug}] \) versus \( f' \) for the Et complex in 0.1 M phosphate buffer.

In 0.7 M NaCl the curve deviates from linear behaviour for \( f' \) greater than 0.5. A least square analysis of experimental data gave a \( K \) value of \( 2.38 \pm 0.12 \times 10^4 \text{ M}^{-1} \) in good agreement with the results reported in the literature (31,32) and extrapolated to 73°C (\( f' = 0.82 \)) (Table III).

Both in high and in low salt, ethidium bromide protects the same average number (\( n = 2.5-2.9 \)) of base pairs. This value is compatible with intercalation of ethidium into DNA following the neighbour exclusion model. The over-estimation of \( n \) on the actual value (\( n = 2 \)) is probably due the over-simplification of the Scatchard like modelling of the binding relative to a statistical model (34). In the case of the ethidium dimer complex \( f \times [\text{DNA}] / [\text{drug}] \) follows a sigmoidal curve whose asymptotes have slopes of 5.75 (\( f' \to 1 \)) and 2.9 (\( f' \to 0 \)). This unusual \( f \) pattern is interpreted assuming the drug to
TABLE III. Intercalation site size n (in base pairs) and poly(dA-dT) binding constant for ethidium (Et) and ethidium dimer (Et₂) determined following equation [5].

<table>
<thead>
<tr>
<th>Na⁺ (M)</th>
<th>n ± SE</th>
<th>K M⁻¹ ± SE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Et</td>
<td>0.1</td>
<td>2.50 ± 0.05</td>
</tr>
<tr>
<td></td>
<td>0.7</td>
<td>2.80 ± 0.20</td>
</tr>
<tr>
<td>Et₂</td>
<td>0.7</td>
<td>5.75 ± 0.05</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2.90 ± 0.50</td>
</tr>
</tbody>
</table>

be redistributed along the DNA helix during the thermal denaturation following the simplified scheme below (scheme II).

1. At the beginning of the melting transition the remaining DNA helix provides sufficient sites to allow all the heterocycles to be intercalated, which result in n = 5.75. The number of protected bases is roughly in agreement with the value n = 4 found for bisintercalation following the nearest neighbour exclusion model.

2. The length of DNA remaining double stranded shortens upon melting so that not all the dimer molecules can bisintercalate, some have to monointercalate.

3. Finally the DNA double stranded region is too short to allow any other binding mode for the dimer than monointercalation. At this level ethidium bromide and ethidium dimer exhibit similar behaviour (n = 2.9).

Transition from bis to monointercalation for bifunctional ligands seems not to be unusual when the DNA becomes saturated with drug that is to say when free ligand concentration becomes large (5,35). Actually at low values of free ligand concentration (f'→1) bisintercalation is shown to dominate the binding process while at high value of free ligand concentration (f'→0) monointercalation becomes significant due to the unavailability of contiguous
free sites required for bisintercalation.

Moreover viscosimetric measurements were performed with sonicated calf thymus DNA in strictly similar conditions (0.7 M NaCl, 0.1 M acetate buffer, pH 5.5). A slope of 5.16 was found for the function of log(\eta/\eta_0) vs. log(1+2r) where \eta and \eta_0 are respectively the intrinsic viscosity of sonicated DNA in presence and in absence of drug and r is the molar ratio of bound drug to DNA base pairs. This value clearly shows the DNA bisintercalation of Et_2 since such process leads to slopes between 4 and 6. So at low [drug]/[DNA] ratio and high ionic strength Et_2 bisintercalates into poly(dA-dT) and DNA. Thus it appears that in low salt the interactions between DNA backbone and the positively charged chains precludes bisintercalation (5). A similar situation is encountered with bismethidium spermine dimer (9,11).

Since phenanthridinium protons H_2, H_4, H_7, H_9 in ethidium bromide and apparently in the ethidium dimer are affected to the same extent (0.9 ppm - 1.1 ppm) on poly(dA-dT) binding, it may be concluded that these drugs intercalate between the DNA base pairs with their long axis parallel to the direction of the Watson-Crick hydrogen bonds. Phenyl ring protons are almost unperturbed because this group lies in either of the grooves. It is difficult from our results to conclude about a definitive intercalation geometry as the DNA base protons are almost unperturbed. Nevertheless, Patel and Canuel (23) have previously reported that the geometry proposed by Tsai et al. (17) for the intercalation of ethidium bromide at UpA site could account for upfield shifts that the phenanthridinium ring protons undergo.

The exchange between single and double stranded forms of poly(dA-dT) is fast relative to the adenine H^8 chemical shift difference (0.2 ppm) but only moderately fast relative to adenine H^2 chemical shift difference (1.09 ppm). Melting of the complexes and particularly the biphasic melting of the poly(dA-dT) : Et_2 complex, is reflected in the decreasing line width of the adenine H^8 proton (figure 3). Moreover the rate of exchange between helix and coil of poly(dA-dT) can be estimated from equation [6] if we assume that H^8 and H^2 are subject to similar relaxation processes in each state (25).

\[
\frac{1}{(\pi T_2)_H^2} - \frac{1}{(\pi T_2)_H^8} = 4\pi (\nu_c - \nu_h)^2 f^2 (1-f)^2 (\tau_h + \tau_c) \quad [6]
\]

1/\pi T_2 is the observed linewidth of either adenine H^8 or adenine H^2 at a given temperature. Subscripts c and h refer to coil and helix DNA. \nu_i and \tau_i are respectively the frequency (in Hz) and the lifetime of H^2 in state i. The definition of f is that given above (molar fraction of double stranded
DNA. We calculate for the same fraction (20%) of native poly(dA-dT) denaturation rates $k_d = 1/T_h$ of $1.16 \times 10^4$ s$^{-1}$ for poly(dA-dT) ($t = 75^\circ$C), $6.9 \times 10^3$ s$^{-1}$ for poly(dA-dT) : Et ($t = 92^\circ$C). Obviously at 78°C the fraying of the poly(dA-dT) helix (37%) in the poly(dA-dT) : Et$_2$ complex occurs even more slowly ($2.5 \times 10^3$ s$^{-1}$).

The Arrhenius plot of $k_d$ is shown in figure 7. For poly(dA-dT) and the poly(dA-dT) : Et complex, we observe a linear dependence of $\ln k_d$ with $1/T$, giving an energy of activation ($\sim 200$ KJ M$^{-1}$) in perfect agreement with data reported elsewhere (31,36). In the case of the poly(dA-dT) : Et$_2$ complex $\ln k_d$ (determined along the second step of the denaturation process) exhibits a biphasic profile which could be due to the simplified two states analysis given by [6]. However the limits of the plateau correspond to the bisintercalation-monointercalation transition (corresponding zones in figures 6 and 7 are indicated by curved arrows). As the slope is very small in this region, it appears that the energetics of the DNA melting and the drug rearrangement cancel each other, resulting in an entropically driven process. The slope of the second step in figure 7 affords an energy of activation $E_a \sim 180$ KJ M$^{-1}$ as in poly(dA-dT) and poly(dA-dT) : Et, whereas the first step leads to $E_a \sim 60$ KJ M$^{-1}$.

We have reported comparative $^1$H NMR and optical studies of the interactions between poly(dA-dT), ethidium bromide and ethidium dimer in high salt as a function of the temperature. The melting of the complexes and the double stranded DNA, free and bound, was monitored through the thermal dependence of the drug and the poly(dA-dT) signals, respectively. Both dyes stabi-
lize the DNA helix, but as compared to ethidium, Et₂ induces a 3 fold increase in the melting point of the complex with poly(dA-dT). Two results strongly suggest that the ethidium dimer bisintercalates into poly(dA-dT) in high salt; at low temperature it induces, on some protons of the polynucleotide, upfield shifts twice as large than the monomer when both the drugs are at the same DNA input ratio; on the other hand the dimer protects about six base pairs against thermal denaturation while ethidium bromide protects only three. It is interesting to note, however, that the DNA binding mode of the dimer changes from bis to monointercalation during the helix to coil transition.

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REFERENCES.