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# A study of the interactions of some polypyridylruthenium(II) complexes with DNA using fluorescence spectroscopy, topoisomerisation and thermal denaturation

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ABSTRACT

The nature of binding of $Ru(phen)_3^{2+}$ (I), $Ru(bipy)_3^{2+}$ (II), $Ru(terpy)_2^{2+}$ (III) (phen = 1,10-phenanthroline, bipy = 2,2'-bipyridyl, terpy = 2,2'2,'' - terpyridyl) to DNA, poly[d(G-C)] and poly[d(A-T)] has been compared by absorption, fluorescence, DNA melting and DNA unwinding techniques. I binds intercalatively to DNA in low ionic strength solutions. Topoisomerisation shows that it unwinds DNA by $22^{o}\pm1$ per residue and that it thermally stabilizes poly[d(A-T)] in a manner closely resembling ethidium. Poly[d(A-T)] induces greater spectral changes on I than poly[d(G-C)] and a preference for A-T rich regions is indicated. I binding is very sensitive to $Mg^{2+}$ concentration. In contrast to I the binding of II and III appears to be mainly electrostatic in nature, and causes no unwinding. There is no evidence for the binding of the neutral $Ru(phen)_2(CN)_2$ or $Ru(bipy)_2(CN)_2$ complexes. DNA is cleaved, upon visible irradiation of aerated solutions, in the presence of either I or II.

## INTRODUCTION

As part of a wider programme to find a method for photocleaving DNA sequence-apecifically or base-specifically, we have been studying the interactions of photo-active molecules with DNA and synthetic polynucleotides (1). These compounds may act either by sensitizing the formation of species known to attack DNA (e.g. singlet oxygen), or by direct reaction with the polynucleotide (e.g. photoredox reactions) (2,3). Molecules such as porphyrins (4), and ruthenium polypyridyl complexes (5) shown in Figure 1, are interesting in this regard, as their excited states are excellent singlet oxygen sensitizers and are also powerful oxidizing and reducing agents.

These ruthenium compounds are cationic, and would therefore be expected to interact with DNA. Our initial spectroscopic studies showed this to be the case, and preliminary experiments also showed that $Ru(phen)_3^{2+}$ (I) and $Ru(bipy)_3^{2+}$ (II) cleave pBR322 DNA, in aerated solutions, upon irradiation with visible light (6). Recently Yamagishi (7) and Barton et al. (8) have reported that I binds to DNA stereospecifically, in a manner apparently invol-

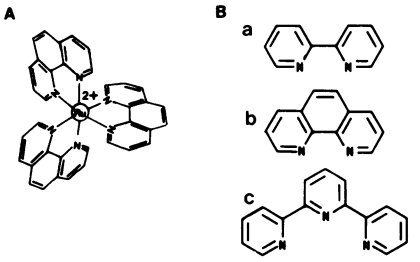



Figure 1. (A) Structural formula of $Ru(phen)_3^{2+}$ . (B) Polypyridyl ligands : a) 2,2'-bipyridyl, b) 1,10-phenanthroline, c) 2,2',2"-terpyridyl.

ving the partial intercalation of one of its three ligands. This work, and that carried out with other metal polypyridyl complexes (10,11), indicated that these might be useful in the study of intercalation.

A large number of polypyridyl and related ligands are known (5). These may be used to test the effect on binding to DNA, of functional groups on the ligands, or heteroatoms in the ligand aromatic ring systems, or ligand size (12,13). Furthermore, square-planar, tetrahedral or octahedral metal complexes (14) are available to study the effect of geometrical structure on binding. The importance of optical isomerism, for example, has already been described by Barton (8,9,10). As metal polypyridyl complexes with different charges are known, the role of electrostatic interactions may also be studied.

These considerations prompted us to extend our investigation of several polypyridylruthenium(II) complexes to include biophysical techniques, so as to determine a) the extent to which they unwind DNA, b) the manner in which they stabilize the DNA duplex, c) the importance of electrostatic interactions to overall binding, and d) whether they show any base or sequence specificity in binding.

## MATERIALS AND METHODS

Materials. $Ru(phen)_3^{2+}$ (I), $Ru(bipy)_3^{2+}$ (II), $Ru(terpy)_2^{2+}$ (III), $Ru(bipy)_2(CN)_2$, and $Ru(phen)_2(CN)_2$ were synthesized from $RuCl_3$ using standard methods (15,16,17). I, II, III were collected as the dichloride salts, reprecipitated as the $PF_6$ salts, and purified on a neutral alumina column with acetone as eluant. The complexes were then reconverted to the water soluble dichloride salts by ion-exchange chromatography on IRA-400(Cl) Amberlite. The cyano complexes were purified on neutral alumina using methanol as eluant.

High molecular weight calf thymus DNA was obtained from Sigma (Cat. No. D4764), purified as described previously (1), and dissolved in 10 mM phosphate buffer ($\mu$ = 0.02, pH = 6.9). An A(260)/A(280) ratio greater than 1.8 indicated a sample substantially free from proteins (11). DNA/dye solutions (dye =

I, II, III, $Ru(phen)_2(CN)_2$, $Ru(bipy)_2(CN)_2$ ) were made up volumetrically and gravimetrically in 10 mM phosphate buffer, or in 100 mM phosphate buffer ($\mu$ = 0.18, pH = 6.9). The concentration of dye stock solutions was determined using extinction coefficients of 19000, 14600, and 16200 $M^{-1}cm^{-1}$ for I, II, and III respectively (18).

Spectroscopy and Thermal Denaturation. Absorption spectra were obtained on a Pye-Unicam SP8200 UV/visible spectrophotometer and uncorrected fluorescence spectra were recorded on a Perkin-Elmer MPF-44B fluorimeter having an R928 photomultiplier. Spectra were taken using thermostatted (25°C) 10 mm cuvettes, and solutions with optical densities less than 0.2. An excitation wavelength of 450 nm was used for both I and II.

Thermal denaturation curves were measured by monitoring the increase in absorbance at 260 nm for poly[d(A-T)] and CT-DNA, and 254 nm for poly[d(G-C)]. Temperature was measured with an FS-23D thermistor. The polynucleotide concentration was 3 x $10^{-5}$ M phosphate. The melting temperature ($T_m$) was calculated as specified (19), and the curve width ($\sigma_T$) was taken as the temperature range between which 10% to 90% of the absorption increase occured. The melting curves and temperatures for CT-DNA and the synthetic polynucleotides were consistent with the literature (19,20). Some of the dyes absorb strongly at 260 nm, but this absorption was found to be independent of temperature.

Topoisomerisation. The enzyme Topoisomerase 1 (Calf thymus, Bethesda Research Laboratories, Cat. No. 8042 SA/SB) was used to convert supercoiled ccc pBR322 DNA to its relaxed state. Samples of pBR322 DNA (in excess of 80% cccDNA) containing ca. 0.5 µg DNA in 50 µl of reaction buffer, topoisomerase, and I or II at a known concentration were incubated at 37C for 3 hours. The standard reaction buffer (BS) was 50 mM Tris, 50 mM KCl, 10 mM $MgCl_2$, 0.5 mM DTT, 0.1 mM EDTA and 30 µg/ml bovine serum albumin. Reaction buffers (BL) were also used containing DTT and serum albumin as above, 5 mM Tris, 5 mM KCl, and 0.0, 0.1, 0.2, 0.3, 0.4, 0.75, 0.8, 1.0, 1.2, 1.5 mM $MgCl_2$.

Following incubation, the reactions were stopped, the ruthenium polypyridyl compounds extracted and the plasmid DNA electrophoresed as previously described (1).

Photolysis. Preliminary photolysis experiments were carried out using 6 mm glass tubes containing 10 µl of a solution of pBR322 (0.5 µg), I or II (0 to 10 µM) in the 10 mM phosphate buffer. Solutions were irradiated at wavelengths above 430 nm, using a filtered (Kodak 2E) 150W xenon lamp. Samples were then made up to 20 µl in buffer, and run on agarose gels as described previously (1).

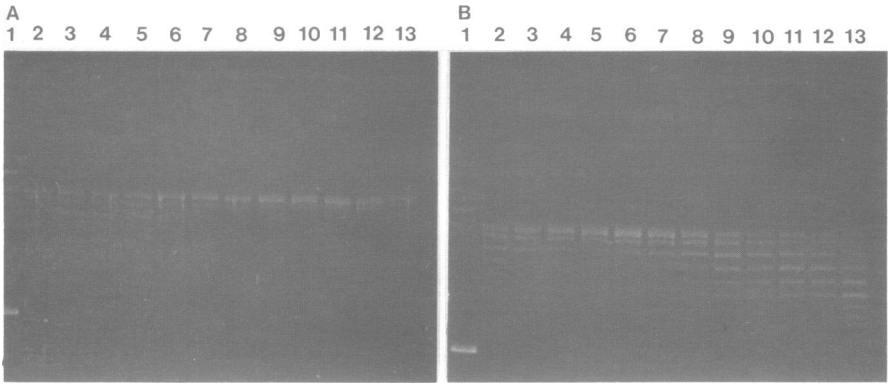



Figure 2. Unwinding of DNA by $Ru(phen)_3^{2+}$. The unwinding of pBR322 plasmid DNA was determined by topoisomerisation in BL buffer, at increasing concentrations of I in A) 1.5 mM $MgCl_2$ and B) 0.75 mM $MgCl_2$. pBR322 not treated with topoisomerase I consists of oc and ccc$^-$ DNA (lane 1) pBR322 treated in the presence of I at concentrations of 0.0, 0.06, 0.12, 0.18, 0.24, 0.30, 0.36, 0.42, 0.48, 0.54, 0.60 0.66 μM is shown in lanes 2 to 13 respectively.

## RESULTS

Topoisomerisation Experiments. The topoisomerisation assay may be used to determine whether a compound unwinds cccDNA (1,21). Intercalating agents unwind DNA and shift the topoisomer distribution when present in a reaction. This shift is used to assay intercalative unwinding. To quantify the effects of I and II, samples relaxed by topoisomerase I in the presence and absence of these compounds were compared using the band counting method (21). The mean (centre) of each topoisomer distribution was estimated, and the difference between means calculated. The ratio of input dye to plasmid DNA was used to calculate the unwinding induced per input dye molecule. In the standard reaction buffer (BS) no detectable shift in the topoisomer distribution was seen on addition of I, even at P/D ratios as low as 4. Since $Mg^{2+}$ ions are known to affect the intercalation of certain compounds (22), the ruthenium polypyridyl compounds were tested for the effect of $Mg^{2+}$ on topoisomerisation under low salt conditions (BL buffer). $Mg^{2+}$ was found to have a significant effect on the interaction of I with DNA. The effect of increasing I concentration on the topoisomer distribution at 1.5 mM and 0.75 mM $Mg^{2+}$ is shown in Figures 2A and 2B. The least squares estimate of unwinding under these conditions is 0.5° and 1.4° respectively per input residue. The conditions of incubation and electrophoresis differ such that in the absence of I the centre of the

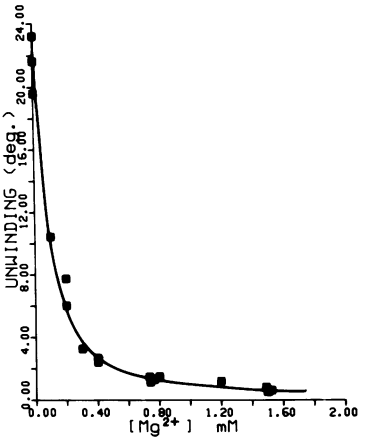
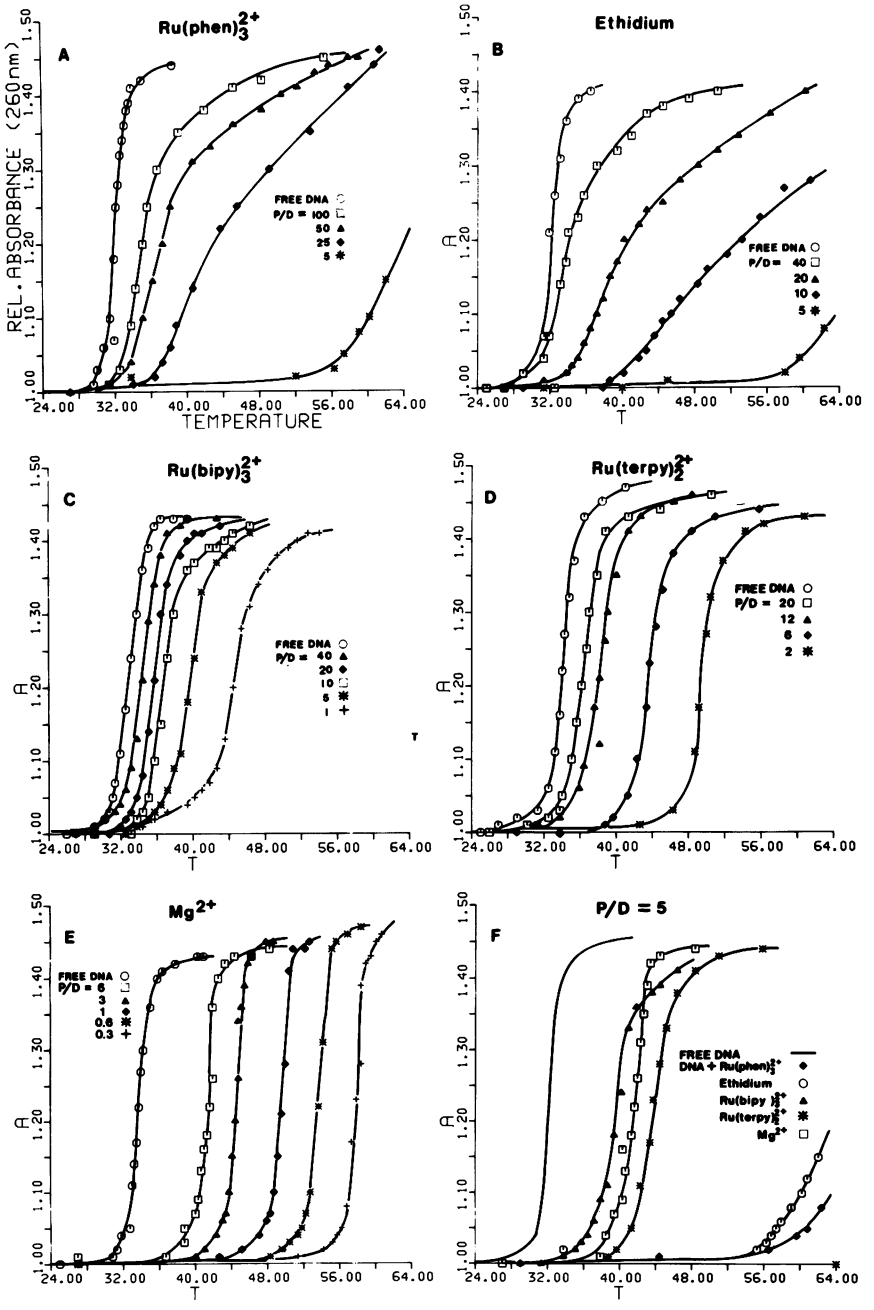



Figure 3. Magnesium dependence of the unwinding of DNA by I. Plot of degrees unwound per input residue of dye, at varying $Mg^{2+}$ concentrations, in the BL buffer, assayed by the topoisomerisation method.

topoisomer distribution is about +2 supercoils in each case. Addition of I initially causes the distribution to centre on zero before negative supercoiling predominates at higher I concentrations. Data for a range of $Mg^{2+}$ concentrations are summarised in Figure 3. Maximum unwinding was obtained in the absence of $Mg^{2+}$ and at 22±1° is close to the 26° estimated for ethidium (23). This unwinding is halved when the reaction buffer contains 0.1mM $Mg^{2+}$, and further reduced with increasing $Mg^{2+}$ concentration such that unwinding per input I residue appears to asymptotically approach 0°. Neither II, III, $Ru(phen)_2(CN)_2$ nor $Ru(bipy)_2(CN)_2$ cause a detectable shift in topoisomer distribution under any of the conditions tested, including P/Ds of 6 in buffer containing no $Mg^{2+}$. The lack of unwinding was shown not to be due to inhibition of topoisomerase 1 by any of these compounds.

Thermal Denaturation. The melting behaviour of DNA and synthetic polynucleotides is affected by intercalators (4, 11, 24, 25). We have studied the effects of the ruthenium polypyridyl complexes on the melting of CT-DNA and poly[d(A-T)]; it was not possible to study the effects on poly[d(G-C)] due to its high stability even at very low ionic strengths. A comparison was made between the ruthenium polypyridyl complexes, the known intercalator ethidium, and the electrostatically binding $Mg^{2+}$ ion. In preliminary experiments, using the 10 mM phosphate buffer, the $T_m$ of poly[d(A-T)] was 48°C, and was increased by 5° in the presence of I (P/D = 20), but only slightly increased (< 2°) by II and III, even at very high dye concentrations (P/D <1). In a lower ionic strength buffer (1 mM phosphate, 2 mM NaCl), used in further experiments, the $T_m$s of poly[d(A-T)] and CT-DNA were 31° and 59°. The melting curves for poly[d(A-T)] in this buffer, in the presence of I, II, III,


A
Ru(phen)$_3^{2+}$
REL. ABSORBANCE (260nm)
TEMPERATURE
FREE DNA
P/D = 100
50
25
5
B
Ethidium
A
T
FREE DNA
P/D = 40
20
10
5
C
Ru(bipy)$_3^{2+}$
FREE DNA
P/D = 40
20
10
5
1
D
Ru(terpy)$_2^{2+}$
FREE DNA
P/D = 20
12
6
2
E
Mg$^{2+}$
FREE DNA
P/D = 5
3
1
0.6
0.3
F
P/D = 5
FREE DNA
DNA + Ru(phen)$_3^{2+}$
Ethidium
Ru(bipy )$_3^{2+}$
Ru(terpy)$_2^{2+}$
Mg$^{2+}$
1.00
1.10
1.20
1.30
1.40
1.50
24.00
32.00
40.00
48.00
56.00
64.00

ethidium, and $Mg^{2+}$ are shown in Figures 4A to 4F. It can be seen that the curves fall into two classes. In the first, both the $T_m$ and the curve width ($\sigma_T$) increase with increasing P/D, the curve becoming biphasic. In the second only the $T_m$ changes - the transition remains sharp. I and ethidium fall in the first class, while II, III and $Mg^{2+}$ fall in the second (Figures 5A and 5B). When $Mg^{2+}$ is added to a solution of poly[d(A-T)] and I, it causes the curve to assume the behaviour of the second class (Figure 6). When the concentrations of I and $Mg^{2+}$ are both low, the effect on $T_m$ is additive, but as the concentration of $Mg^{2+}$ is increased, its effect on the curve comes to dominate.

Although the $T_m$ of CT-DNA is considerably higher than that of poly[d(A-T)] the stabilisation resulting from the presence of I is comparable. The $T_m$ is increased by 4°, 10°, 18°, and 20°C at P/Ds of 50, 25, 12, and 6 respectively. The melting curves show an increased $\sigma_T$ at P/Ds of 50, 25 and 12, but a reduction of $\sigma_T$ at a P/D of 6. Presumably, at this P/D, the DNA is saturated with dye, so that a proportion of the dye is left over, to bind electrostatically.

Absorption and Fluorescence Spectra. Both intercalation and electrostatic interaction can induce changes in the electronic spectra of molecules binding to DNA (26,27). Intercalation has the more marked effect. Spectroscopic studies may be used to determine the strength of binding, the effect of ionic strength, and whether binding shows any base-specificity.

Slight changes were observed in the visible spectra of I and II, on addition of CT-DNA, in the 10 mM phosphate buffer. Isosbestic points were observed at 465 and 470 nm respectively, and the points of maximum change at 477 and 480 nm (Figures 7A and 7B). The flourescence spectra of I and II are markedly affected by CT-DNA, at low ionic strength. Emission intensity is enhanced and the fluorescence peaks centred at 595 and 610 nm, in the free dyes, are red shifted and narrowed (Table 1 and Figures 7C and 7D). As the excited state reduction potential of I (0.82 V) (5) is less than the oxidation potentials of guanine (0.85 V) and adenine (0.95 V), quenching of its emission by redox interaction with the purine bases is not expected (28). The observed enhancement may be due to stiffening of I when bound, and protection from solvent molecules and quenchers when in the hydrophobic environment of the DNA (8,29).

Figure 4. Thermal denaturation curves for poly[d(A-T)] in the presence of the ruthenium polypyridyl compounds, ethidium and $MgCl_2$. Figures 4A to 4E show the melting curves in the presence of single compounds, at varying P/Ds. Figure 4F shows the melting curves in the presence of different compounds at a P/D of 5. $3x10^{-5}$ M DNA in 1 mM phosphate, 2 mM NaCl buffer was used in all experiments.

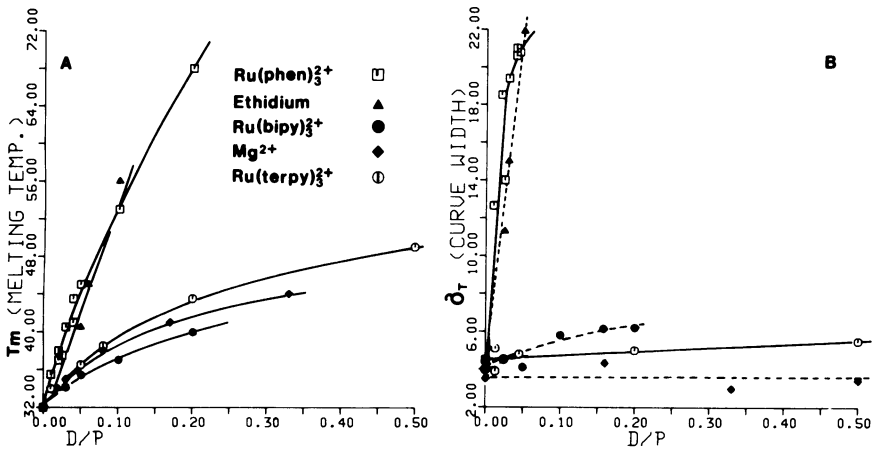



Figure 5. Melting temperatures and curve widths. The $T_m$ and $\sigma_T$ of poly-[d(A-T)] in the presence of the ruthenium polypyridyl compounds, ethidium and $MgCl_2$, at varying P/Ds. $3x10^{-5}$ M DNA in 1 mM phosphate, 2 mM NaCl was used in all experiments.

Enhancement was found to vary with excitation wavelength but this was solely due to DNA induced changes in the absorption spectra, and when corrected for these was independent of excitation wavelength in the 300-500 nm range studied.

In the higher ionic strength (100 mM phosphate) buffer, changes in the electronic spectra of I, in the presence of DNA, were considerably reduced, and no changes at all were observed for II. From Figure 8 it can be seen that the emission intensity is sensitive to ionic strength, returning to that of

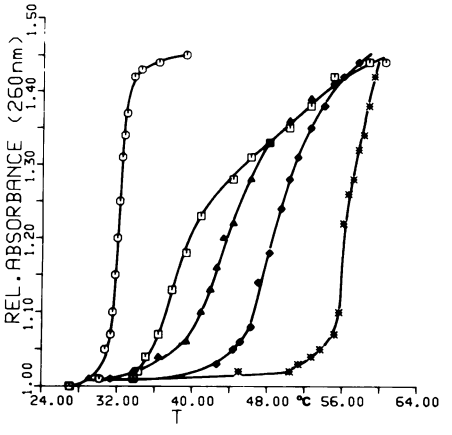



Figure 6. The effect of magnesium on the melting behaviour of poly[d(A-T)] in the presence of $Ru(phen)_3^{2+}$.

—○— Free DNA ($3x10^{-5}$M)
—□— DNA + I (P/D=25) (†).
—▲— (†) + $MgCl_2$ (P/D=25).
—■— (†) + $MgCl_2$ (P/D=10).
—✱— (†) + $MgCl_2$ (P/D=0.5).
in 1 mM phosphate, 2 mM NaCl.

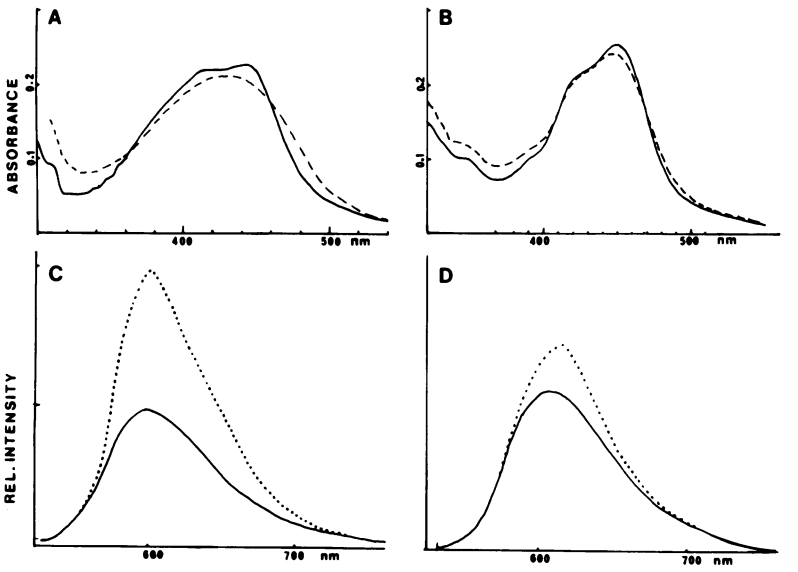



Figure 7. The effect on the emission and absorption spectra of I and II of CT-DNA. The absorption spectra of A) I and B) II and the emission spectra of C) I and B) II in the presence (- - - - -) and absence (——) of CT-DNA (P/D = 40). Dye concentrations of 1-2 x $10^{-5}$ M, 10 mM phosphate buffer, and a $\lambda_{ex}$ of 450 nm were used

the free complexes at $Na^+$ concentrations above 200 and 70 mM respectively for I and II. Emission intensity enhancement was found to be far more sensitive to the presence of $Mg^{2+}$ than would be expected from a simple ionic strength effect (Figure 9). $Mg^{2+}$ concentrations of less than 0.15 mM reduced the int-

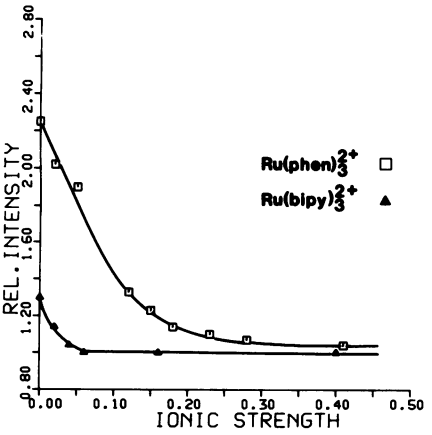



Figure 8. The effect of ionic strength on the emission intensity of I and II in the presence of CT-DNA. Ionic strength was increased by adding solid NaCl to 1x$10^{-5}$ M dye solutions in 10 mM phosphate buffer, containing CT-DNA at a P/D of 20.

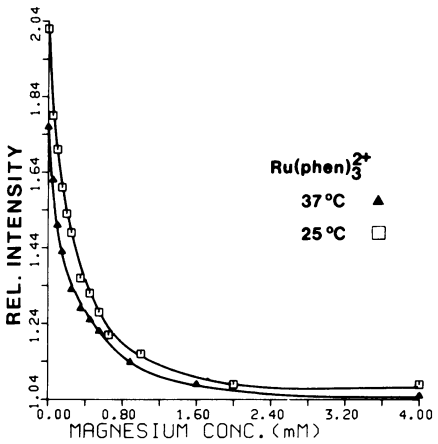



Figure 9. The effect of magnesium on the emission intensity of I in the presence of CT-DNA. Solutions of CT-DNA and I ( $3 \times 10^{-6}$ M, P/D=15) were made up in 5 mM Tris, 5 mM KCl buffer, and small quantities of 0.1 M $MgCl_2$ added.

encity enhancement of I by half in 5 mM Tris, 5 mM KCl buffer, while 2 mM $Mg^{2+}$ was required for the same effect in the 10 mM phosphate buffer. The emission properties of the DNA-dye complexes were unaffected by the pH of solutions in the pH range 5-9.

The enhancement of I emission intensity was dependent on the base content of the DNA used (Table 1). For P/D ratios of less than 50, intensity enhancement was found to increase in the order poly[d(G-C)] < CT-DNA (42% GC) < poly[d(A-T)] (Figure 10). It is interesting that while I shows a red shift in the presence of poly[d(A-T)] similar to that caused by CT-DNA, poly[d(G-C)] causes a blue shift. The visible absorption band of I is also less affected by poly[d(A-T)] than poly[d(G-C)]. The fact that CT-DNA behaves more like poly[d(A-T)] than poly[d(G-C)] at high P/Ds raised the possibility that I binds preferentially to AT rich DNA. To test for any such preference, the enhancement of I in the presence of a 50% poly[d(A-T)] / 50% poly[d(G-C)] mixture was measured. At low P/D values (< 10), this enhancement was the mean of the enhancements produced by each polynucleotide on its own, but at higher P/D values (> 50), it converged on the enhancement caused by pure poly[d(A-T)] (Figure 10). This indicates that given a large number of binding sites to choose from, I prefers A-T:T-A to G-C:C-G binding sites. A similar behaviour was shown in the presence of CT-DNA, enhancement converging on that shown by the poly[d(A-T)] bound complex, at high P/Ds (> 50).

Scatchard analysis of the binding of I to poly[d(A-T)], in 12mM phosphate 3mM NaCl buffer, was carried out using fluorescence data (30). An accurate calculation of the binding constant was not possible, due to the inherent inaccuracies in this method, but it allowed us to determine that the constant

Table 1. The effect of DNA on the emission properties of I and II.

| DYE | DNA | P/D | $\lambda_{EMISSION}$ (nm) | RELATIVE INTENSITY | BAND WIDTH(nm) |
|---|---|---|---|---|---|
| I | --- | -- | 595 | 1.0 | 84 |
| | CT-DNA* | 40 | 600 | 2.0 | 74 |
| | CT-DNA | 10 | 596 | 1.3 | 82 |
| | " | 50 | 600 | 1.85 | 74 |
| | poly[d(A-T)] | 10 | 598 | 1.6 | 80 |
| | " " | 50 | 602 | 1.9 | 70 |
| | poly[d(G-C)] | 10 | 595 | 1.17 | 84 |
| | " " | 50 | 590 | 1.3 | 77 |
| | 50% poly[d(A-T)]<br>50% poly[d(G-C)] | 10 | 597 | 1.45 | 80 |
| | " " | 50 | 600 | 1.7 | 75 |
| II | --- | -- | 610 | 1.0 | 90 |
| | CT-DNA* | 40 | 614 | 1.3 | 85 |
| | CT-DNA | 50 | 612 | 1.05 | 87 |
| | poly[d(A-T)] | 50 | 612 | 1.06 | 87 |
| | poly[d(G-C)] | 50 | 612 | 1.04 | 87 |

* $1 \times 10^{-5}$ M dye in 10 mM phosphate buffer. All others are $1 \times 10^{-6}$ M dye in 12 mM phosphate, 3 mM NaCl buffer. Relative intensities were measured at the wavelength of maximum emission indicated, at 25°C, using an excitation wavelength of 450 nm. Band width was measured at half height.

was of the order of $10^{6}$ $M^{-1}$. The calculated binding number was 6 ± 1 base-pairs per bound dye at saturation.

It was found that neither I nor II showed any spectral changes in the presence of heat denatured DNA, regardless of ionic strength or P/D.

The terpyridyl complex (III) showed no changes in its visible absorption or (its very weak) fluorescence bands in the presence of DNA. Similarly the uncharged complexes $Ru(phen)_2(CN)_2$ and $Ru(bipy)_2(CN)_2$ showed no spectral changes in the presence of DNA.

Photolysis. The irradiation ($\lambda$ > 430 nm) of ccc pBR322 in aerated solutions containing I or II, caused cleavage of the DNA. Short irradiation periods (< 5 min.) produced ocDNA, while longer exposures resulted in linear pBR322 and eventually the fragmentation of the plasmid. For equal irradiation times, higher dye concentrations caused more extensive damage to the DNA. pBR322 was not damaged by I or II if kept in the dark, nor by irradiation if the dyes were not present.

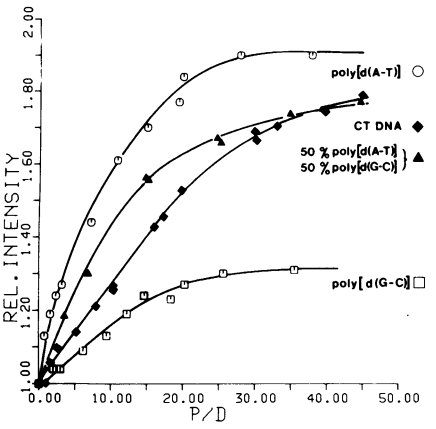



Figure 10. Effect of base composition on the emission properties of I in presence of DNA. The relative intensity of I in the presence of the specified DNAs was measured at the wavelength of maximum emission, using a $\lambda_{max}$ of 450 nm. An I concentration of $1 \times 10^{-6}$ M in 1 mM phosphate, 2 mM NaCl buffer was used.

## DISCUSSION

Analysis of the interaction between DNA and ruthenium polypyridyl compounds using topoisomerisation, spectroscopy and DNA melting, has shown that the compounds fall into three classes: (i) Non-binders; (ii) External binders; (iii) Intercalators.

The non-binders are the uncharged compounds, $Ru(phen)_2(CN)_2$ and $Ru(bipy)_2(CN)_2$, neither of which has any effects on the topoisomerisation or melting of DNA, nor do they show any change in the absorption or fluorescence spectra on the addition of DNA. This is evidence that electrostatic interactions play an important role in the binding of the ruthenium polypyridyl compounds to DNA - all of the compounds which bind are charged and their binding is sensitive to the ionic strength of the solution.

The external binders are the compounds $Ru(bipy)_3^{2+}$ (II) and $Ru(terpy)_2^{2+}$ (III). They do not unwind DNA in the topoisomerisation assay even in the absence of $Mg^{2+}$, but they do stabilise poly[d(A-T)] against melting, and II does show changes in its fluorescence and absorption spectra on addition of DNA. III does not fluoresce, and its absorption spectrum is not affected by DNA. However, the effects on the melting curves of DNA make it clear that both compounds do bind to DNA. These effects are quite characteristic, and virtually identical to the effect that $Mg^{2+}$ has on the melting of DNA. The shift in $T_m$ is small (relative to intercalators as discussed below), and the curve width is hardly changed. The fact that there is a very close parallel between these compounds and $Mg^{2+}$ in the effect on the melting of DNA, and the fact that they do not unwind DNA, show that these ruthenium compounds bind mainly through ionic interactions on the outside of the DNA helix. This

binding is eliminated at lower concentrations of $Mg^{2+}$ than the binding of the intercalator $Ru(phen)_3^{2+}$. The tertiary structure of DNA is important for the interaction since there is no change in the fluorescence of II on addition of single stranded DNA. This suggests either that the spacing of the negative charges on the double stranded DNA affects the binding of the doubly charged ruthenium, or that there are some other presumably weak interactions (perhaps in the major groove) with the polypyridyl ligands which can only occur when the DNA is double stranded.

The only ruthenium compound shown in this study to intercalate is $Ru(phen)_3^{2+}$ (I). It unwinds DNA in the topoisomerisation assay as effectively as the classical intercalator ethidium bromide. In the absence of $Mg^{2+}$, I unwinds DNA by 22 ± 1° per added residue, in comparison with ethidium which unwinds DNA by 26° under the same conditions. In view of the difficulty in establishing whether compounds intercalate (31), this data gives a strong indication that I binds in this manner. The binding constants of I and ethidium to poly[d(A-T)] (this study, 32), are comparable at low ionic strengths, but substantially different at higher ionic strengths. The unwinding angle measured for I suggests that both the optical isomers of I are effective intercalators. Otherwise, if only one isomer intercalates it would have to unwind DNA by 44°, and no other monointercalating compounds have been found that unwind DNA by more than 26° (31). This is not inconsistent with the results reported by Barton et al. (8) on the stereospecificity of the binding of I to the B form of DNA. They showed that while there is a preference in binding for the Δ isomer, the Λ isomer also binds. The steric effect of the two non-intercalated phenanthroline ligands is not large enough to cause the complete exclusion of the Λ isomer. When the ligands are bulkier, as for example 4,7-diphenylphenanthroline, complete exclusion is observed (9,29).

The difference in the interactions of the ruthenium compounds with DNA is explainable in terms of the three-dimensional structure of these compounds, their charge, and the size of their ligands. I binds by inserting only one of its three ligands between the base-pairs of DNA. This ligand is prevented from full insertion by the steric effect of the two outer ligands (8). There is sufficient overlap of the inserted ligand's π-orbitals with those of the DNA bases to allow intercalative binding to occur, but the added stabilizing effect of electrostatic interaction is required. Without it binding does not occur, as is seen for the neutral cyano compounds. II has smaller ligands than I, and molecular models show that a considerably smaller degree of overlap of the inserted ligand occurs. Also, this compound is a weaker ionic binder than

I (29) so that both electrostatic and intercalative components to binding are reduced. III has two large ligands, but steric hindrance from the outer ligand prevents sufficient penetration of the other ligand into the DNA for intercalation to occur. If this steric restraint is removed, as in the case of $Pt(terpy)S(CH_2)_2OH$, then the insertion of this ligand results in strong intercalative binding (11,12).

The marked effect on the intercalation of I of low concentrations of $Mg^{2+}$ was quite unexpected and seems to be a novel observation,not so far reported for other intercalators. It has indeed been known for many years that intercalation is inhibited at high $Mg^{2+}$ concentrations (22,23), but these effects are not seen until the $Mg^{2+}$ concentration is two orders of magnitude greater than that required to affect the binding of I. For example, in our own hands 10 mM $Mg^{2+}$ had no effect on the unwinding of DNA by ethidium in topoisomerisation assays (1), though the unwinding of DNA by I was halved by 0.1 mM $Mg^{2+}$. It is now clear that other intercalators are also extremely sensitive to $Mg^{2+}$. In a preliminary study it was found that unwinding by chloroquine, methylene blue and the zinc complex of 5,10,15,20-tetrakis(N-methylpyridinium-4yl)porphyrin (ZnTMPyP) was very sensitive, while the unwinding of acridine orange, cresyl violet, proflavine, acriflavine, 2-nitrosofluorene and the free base 5,10,15,20-tetrakis(N-methylpyridinium-4yl)porphyrin was not (33).

Although the nature of intercalation is well understood in a general way and the original proposal by Lerman (34) has been amply verified, it has become apparent that there is considerable variation between the mechanisms of intercalation of different compounds, distinguished mainly by kinetic and thermodynamic studies (31,35). The mechanisms are complex, and no case is fully understood. It now seems that the degree of sensitivity to $Mg^{2+}$ can be used as another factor in distinguishing different modes of intercalation. Most intercalators are cationic compounds with planar aromatic elements, and their binding is due to both stacking and electrostatic interactions. The stacking interactions involve the overlap of $\Pi$-orbitals of the intercalator and DNA bases, while the electrostatic interactions occur between the positive charge or charges on the intercalator and either the DNA phosphates on the outside of the helix, or the electron-rich region between the base pairs. The stacking component in binding is strong in the case of the classical intercalators and so considerable binding survives even at high salt concentrations when the DNA phosphates are shielded and the electrostatic component is reduced. For example ethidium bromide still binds at 2.5M salt (25). In contrast I and the other compounds mentioned above require an electrostatic component if

binding is to occur at all. Presumably this is so because the stacking interaction is weak. In the cases of I and the ZnTMPyP only part of their aromatic ring systems can be inserted into the DNA for steric reasons (8, 9, 29, 36), while in the case of chloroquine the ring system is small with only two rings (36) and it may be that full intercalation is prevented by its bulky substituent group. From the structures of these three compounds the charges most likely will be located so that they interact with DNA phosphates, where the electrostatic bonding would be exposed to competition with other positive ions. It is not possible at the present to explain the sensitivity to $Mg^{2+}$ of intercalation of methylene blue which has a very similar structure to proflavine and acridine orange, two compounds which intercalate in reactions which are much less sensitive to $Mg^{2+}$.

The poly[d(A-T)] melting curves show an interesting difference between the effects of intercalators and external binders (Figures 4A to 4F). The intercalators ethidium bromide and I, have a larger effect on the $T_m$, and the width of the melting curve is substantially increased by them. The effects of the two compounds are virtually identical, further evidence that the ruthenium compound is an intercalator. In contrast, as already mentioned, the external binders have smaller effects on both parameters, especially on the breadth of the curve. In view of the difficulty in distinguishing between intercalative and external binding (31, 37) the analysis and comparison of melting curves may prove a useful diagnostic tool.

It is of considerable interest to establish whether intercalation is base or sequence specific (31, 35, 38). Results showed that many intercalators prefer to bind in the doublet, pyrimidine(3'-5')purine, with a further preferance for DNA which is rich in GC. The evidence suggested that when intercalators were base specific they prefered GC-rich DNA whereas a number of external binders were found to prefer AT-rich DNA (39). In view of these generalisations it is of particular interest that some intercalators have now been identified which bind preferentially to AT-rich DNA. The first to be described was the case of daunomycin (40,41), while the data in this paper shows that I also prefers AT-rich DNA. The evidence that I prefers AT-rich DNA comes from the possibility of distinguishing between the effects of poly-[d(A-T)] and poly[d(G-C)] on the fluorescence of I (Figure 10). At high P/D values (> 40), where the fluorescence enhancement is close to its maximum, the relative intensity caused by poly[d(A-T)] is much greater than that caused by poly[d(G-C)]. At a P/D of 50 the relative intensity is 1.9 for poly[d(A-T)] but only 1.3 for poly[d(G-C)]. When equimolar amounts of the

two polynucleotides are used the relative intensity at the same P/D is 1.75. If I interacted equally with both polynucleotides the expected value is 1.6. Thus it appears that about 75% of the dye molecules bind to poly[d(A-T)]. The preference for A-T sequences is also indicated by the relative intensity caused by calf thymus DNA (42% GC) which is 1.85 at a P/D of 50. In this case the situation is obviously more complicated because of the variety of sequences in natural DNA but a bias in the direction of AT-rich regions is apparent.

Both I and II sensitise DNA to photolysis by visible light in aerated solution. The two compounds are equally effective, showing that intercalation is not required for photolysis. It is possible that the DNA is cleaved by singlet oxygen produced in its vicinity, or as a result of direct photoredox reaction between the ruthenium compounds and the DNA, but the mechanism is not known at present. A further point of major interest is whether the photolysis is base or sequence specific. It has already been shown that the photolysis induced by methylene blue is specific for G (42). As I appears to interact preferentially to AT rich sequences, it is also possible that it will induce photocleavage of DNA preferentially in these regions. The specificity may be increased using combinations of compounds with different specificities, or perhaps by adjusting the ionic conditions. Since there is a large number of compounds known to mediate photolysis of DNA (1, 4, 33, 42, 43), there are excellent prospects for developing a set of photochemical reactions to be used in sequencing and otherwise probing DNA.

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Abbreviations

P/D: Molar concentration of DNA phosphates to dye or $Mg^{2+}$ as appropriate
ccc: covalently closed circular. oc: open circular.
I: $Ru(phen)_3^{2+}$ II: $Ru(bipy)_3^{2+}$ III: $Ru(terpy)_2^{2+}$. phen, bipy, terpy: see Figure 1. BS: standard topoisomerisation buffer. BT: low ionic strength topoisomerisation buffer. DTT: dithiothreitol. EDTA: ethylenediaminetetraacetic acid.

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