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Interaction of substituted cobalt(III) cage complexes with DNA †

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Polycyclic aromatic moieties covalently bound to the inert cationic cobalt(III)–sar cage complex (sar = sarcophagine = 3,6,10,13,16,19-hexaazabicyclo[6.6.6]icosane) intercalate with negatively supercoiled plasmid DNA, as shown by both spectrophotometric and gel electrophoresis studies. Although anthracene itself is not a good intercalating agent, the association constant of the complex between anthracene tied to the cobalt(III)–sar cage and DNA is $\approx 2 \times 10^6$ dm^3 mol^{-1} and the average binding-site size on supercoiled plasmid DNA is ≈2.4 base pairs. The hydrophilic complex cation (a) solubilizes the anthracene moiety in water and (b) enhances DNA binding by its electrostatic interaction with the anionic phosphodiester backbone of DNA. While irradiation of the intercalated complexes at 254 nm led to single-strand cleavage of DNA, irradiation at higher wavelength, 302 and 365 nm, was much less effective. Neither molecular oxygen nor excited singlet or triplet states of anthracene appear to be involved in the cleavage process, and it is likely that the N-radical cation arising from ligand to metal charge or electron transfer in the phosphodiester–complex ion pair gives rise to oxidation of the deoxyribose moieties. Fission of the resulting deoxyribose diphosphate radical cation follows, leading to DNA single-strand cleavage. It also appears that the most easily reduced complex moieties are the most effective cleavage agents. Excited singlet and triplet states of anthracene arising from irradiation at 365 nm are efficiently quenched by DNA and the allowedness of the short-axis in-plane aromatic transitions is also sharply diminished. This in itself is unusual.

Small molecules are able to interact with double-stranded DNA in a number of ways.[1–9] One mode of non-covalent binding involves intercalation of planar aromatic molecules between the base pairs of the DNA helix. Another involves electrostatic binding between the complex cation and the anionic backbone phosphodiester residues. In addition, some metal complexes interact sequence-specifically with DNA,[7] and photoactivation of chromophores bound in either mode can lead to DNA cleavage by various different mechanisms.[1,4,8,9]

In this paper we describe the ability of (a) a series of inert cationic cobalt(III)–sar (sar = sarcophagine = 3,6,10,13,16,19-hexaazabicyclo[6.6.6]icosane) cage complexes covalently linked to polycyclic rings, such as anthracene or phenanthrene, and (b) some 3+ and 4+ monomeric and more highly charged (6+ and 9+) dimeric and trimeric cobalt(III)–sar complexes (Scheme 1), to act as DNA binding and cleaving agents. The hydrophobic polycyclic aromatic groups bound to the hydrophilic cobalt(III)–sar cages are effectively solubilised in water and they have the prospect of intercalating with DNA. The complexes, being cationic, should also associate with the negatively charged phosphodiester backbone of DNA.

In addition to their binding properties, these molecules have the capacity to undergo photoinduced intramolecular electron transfer reactions by at least two mechanisms which are wavelength dependent. Either the singlet or triplet state of anthracene, for example, could reduce the cobalt(III) ion to Co^{II} and the resulting radical cation would be a powerful oxidant able to cleave DNA by an oxidative mechanism. Clearly, this process might be expected to be more effective for the intercalated reagents. Also, the complex cation at higher energy (≈250 nm) undergoes a ligand to metal charge transfer to generate Co^{II} and a radical cation ligand that could be a powerful oxidant. Both aspects are examined in this paper.

Experimental

Reagents

All reagents were of analytical grade used without further purification. The electrophoresis buffer (TAE) contained 0.04 mol dm^{-3} tris(hydroxymethyl)aminomethane (Tris, Sigma), 0.02 mol dm^{-3} acetic acid and 1×10^{-3} mol dm^{-3} ethylenediamine-*N*,*N*,*N*′,*N*′-tetraacetic acid (H_4EDTA, Sigma).[10] All experiments were carried out in 0.03 mol dm^{-3} Tris–HCl buffer, pH 7.6 (Buffer T). Covalently closed circular plasmid pUC9 DNA (2665 base pairs)[11] was prepared as previously described.[12] It was dialysed once for 24 h in 0.01 mol dm^{-3} Tris–HCl (pH 7.6), 1×10^{-3} mol dm^{-3} H_4EDTA and twice for 24 h each in Buffer T. DNA concentrations (in mol of base pairs per dm^3) were determined spectrophotometrically using $\varepsilon_{260} = 13\,400$ dm^3 mol^{-1} cm^{-1}.

Cobalt(III) complexes

All of the cobalt(III) complexes used (Scheme 1) are soluble in water. The synthesis, physical, and photochemical behaviour of the Co^{III}–sar cage linked anthracene complex **1** have been reported.[13] The synthesis and properties of **2**–**7** have been described in ref. 14, and **8** in ref. 15. The dimer and trimer complexes **9** and **10** are reported in ref. 16, **11** is unpublished at present,[17] and **12** is described in ref. 18.

Electronic absorption spectroscopy

All measurements were carried out with a computer-interfaced UV-visible spectrophotometer (Varian Cary 1E) using a 1 cm quartz cuvette. The sample and reference cell compartments

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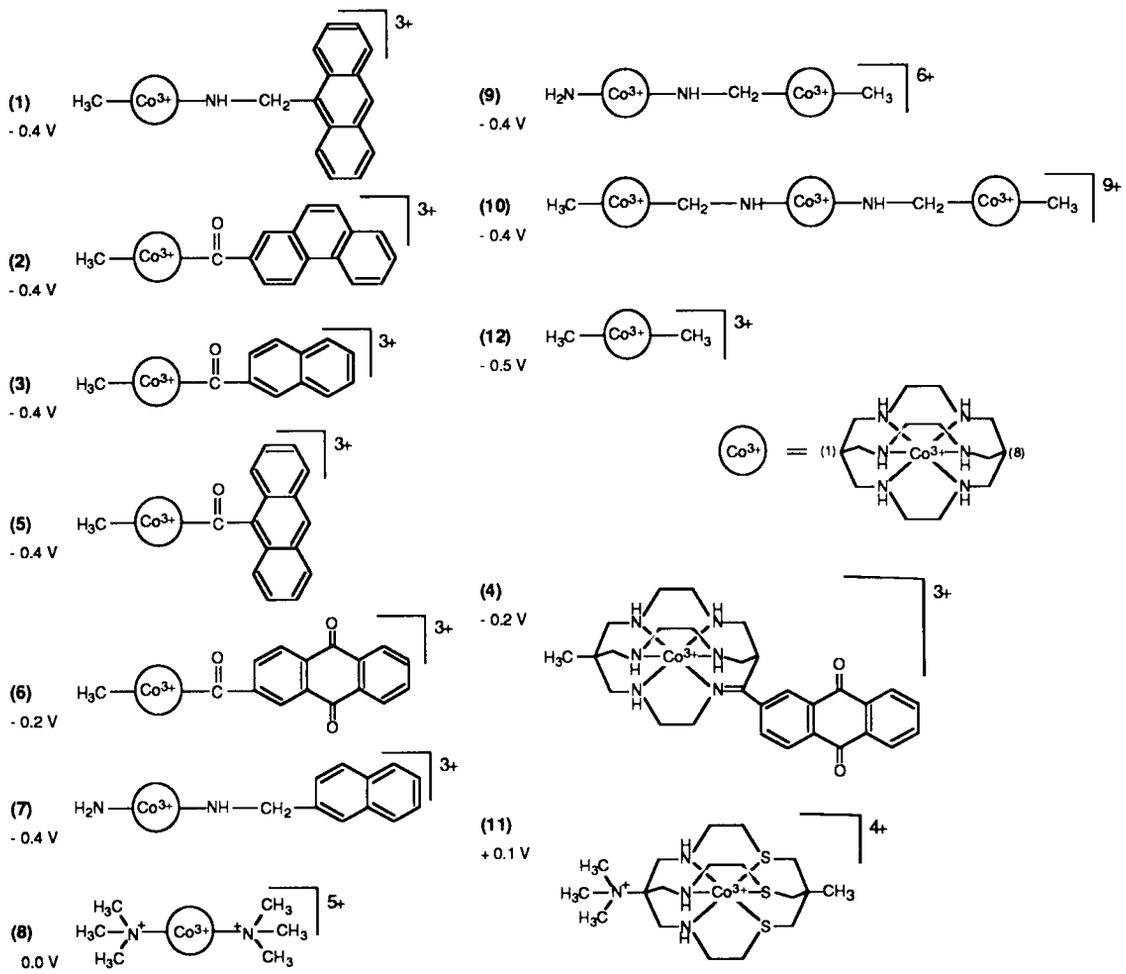


Scheme 1 Structures of cobalt(III) cage complexes. Approximate reduction potentials of the cobalt(III) centre are as indicated. All except complexes **4** and **11** are 1,8-substituted Co^{III}–sar cages.

were maintained at 25 °C. Titrations were carried out with [Co^{III}] constant at 2.0×10^{-5} mol dm^{-3} and varying the concentration of plasmid pUC9 ([DNA], ranging between 0 and 2.0×10^{-4} mol dm^{-3} in base pairs) in a total volume of 9×10^{-4} dm^3. Following mixing and treatment at 25 °C for 15 min, which was shown by monitoring the absorbance at 386 nm to be sufficient time for equilibrium to be attained, UV-visible spectra were recorded. The intrinsic binding constant K_i and average binding-site size n (in base pairs) were determined by iteratively fitting data to the modified Scatchard equation (1) of

$$r(C_F)^{-1} = K_i(1 - nr)[(1 - nr)(1 - [n - 1]r)^{-1}]^{n-1} \quad (1)$$

McGhee and von Hippel,[5,19] where the concentrations of bound and free ligand (C_B and C_F), and thence $r = C_B[DNA]^{-1}$, were calculated from the observed absorbance (A_{obs}) at 386 nm, using values of the molar absorptivities at 386 nm (ε_{386}) for the "free" and bound ligand, respectively, of 9800 and 2250 dm^3 mol^{-1} cm^{-1}. Values of ε_{obs} at 386 nm were calculated as $A_{obs}(C_T)^{-1}$, where C_T was the total concentration of Co^{III} (2.0×10^{-5} mol dm^{-3}).

DNA Binding assays

Plasmid DNA (3.26×10^{-5} mol dm^{-3}, in base pairs) was treated with cobalt(III) complexes in Buffer T at 37 °C for 15 min. To all reaction mixtures (total volume of 2.5×10^{-5} dm^3) was added 5×10^{-6} dm^3 of 0.2% bromophenol blue in 50% glycerol, then samples were loaded onto a 1% agarose gel in TAE. The gel was then subjected to electrophoresis for 16 h at ≈1 V cm^{-1} in TAE, stained for 1 h in TAE containing 2 mg dm^{-3} ethidium bromide (3,8-diamino-5-ethyl-6-phenylphenanthridinium bromide), and then destained in TAE for 6 to 12 h. Gels were photographed with transmitted 254 nm UV light using Polaroid type 55 positive/negative film. Photographs are presented as the negative images.

DNA Photocleavage assays

The cobalt(III) complexes (2.0×10^{-5} mol dm^{-3}) were mixed with pUC9 DNA (3.26×10^{-5} mol dm^{-3} in base pairs; 430 ng) in Buffer T at 37 °C for 15 min. The reaction mixture (2.5×10^{-5} dm^3) was then transferred into a flat-bottomed quartz vial with 10 mm diameter and placed on transilluminators (UV Products) which irradiated at peak wavelengths 254, 302 or 365 nm (peak intensity of 250 J m^{-2} h^{-1}). The samples were irradiated for 2 min. The reaction mixtures were treated with 5×10^{-6} dm^3 ethidium bromide (2 mg dm^{-3}), then kept at 37 °C for 30 min in the absence of light to allow displacement of bound Co^{III} by ethidium bromide. Products were resolved by electrophoresis in a 1% agarose gel in TAE containing 0.5 mg dm^{-3} ethidium bromide.

Results and discussion

Binding of Co^{III}–sar–anthracene 1 to DNA

Fig. 1 shows electronic absorption spectra of compound **1** at 2.0×10^{-5} mol dm^{-3} in Tris buffer, pH 7.6 in the presence and absence of DNA. This region of the spectrum is dominated by the absorption of the anthracene moiety.[13] The general decrease in absorbance (hypochromicity) and the red shift in the absorption maxima are indicative of intercalative DNA binding.

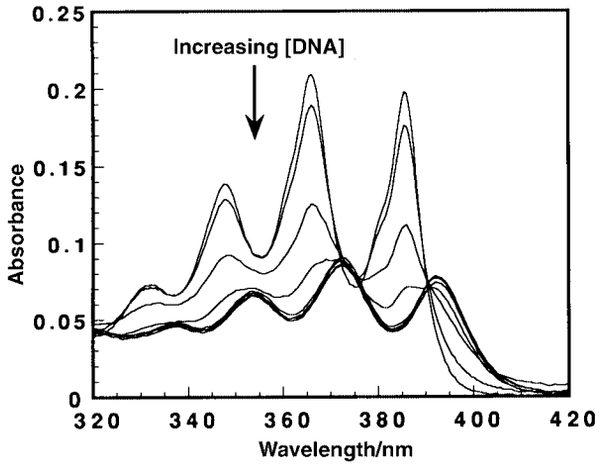


Fig. 1 Spectral changes for the titration of Co^{III}–sar–anthracene **1** at 2.0×10^{-5} mol dm^{-3}, with supercoiled pUC9 plasmid DNA in 0.03 mol dm^{-3} Tris–HCl, pH 7.6 at 25 °C. [DNA] used were (from top to bottom, at 366 nm, for example) 0, 20, 30, 40, 50, 70, 75 and 80 μmol dm^{-3}, in base pairs. Further spectra collected with [DNA] at 90, 100, 150 and 200 μmol dm^{-3} (not shown) were indistinguishable from that at [DNA] = 80 μmol dm^{-3}.

These phenomena have been documented previously for other polycyclic chromophores and their metal complexes.[3–5,8]

Titration of the complex at constant cobalt(III) concentration with varying amounts of DNA showed a continuous decrease in absorbance at the anthracene absorption maxima, and isosbestic points at 372, 376 and 390 nm were observed, as would be expected for a simple equilibrium between DNA-bound and free cobalt(III) complex. Data recorded at 386 nm were fitted graphically by the general equation of McGhee and von Hippel[19] that enables account to be taken of the effect of neighbouring occupied sites on binding of a ligand to an indefinitely long lattice of binding sites. This gave estimates of binding of one cobalt(III) complex per $2.3_5 \pm 0.2$ base pairs of DNA for the average binding site size and 1.8×10^6 dm^3 mol^{-1} for the intrinsic binding constant K_i (Fig. 2). These may be compared with values reported for the analogous (9-anthrylmethyl)ammonium(1+) ion:[5] while the binding-site size is similar, the estimated K_i is some two orders of magnitude higher, which presumably reflects the effect of the expected stronger electrostatic interaction of the 3+ cation **1** with the negatively charged phosphodiester backbone of DNA, over and above the contribution due to intercalative binding common to both compounds. The K_i for binding of **1** to DNA is also one order of magnitude higher than that observed for other cationic (1+) DNA intercalators such as ethidium ion (2×10^5 dm^3 mol^{-1}).[20] The binding site size indicates that, at saturation with **1**, the anionic charge of DNA is more than half neutralised by the intercalating cation.

Binding of compound **1** to DNA by an intercalative mode was also demonstrated by its effects on the electrophoretic mobility of supercoiled plasmid DNA as shown in Fig. 3. The concentration of plasmid DNA was kept constant as [Co^{III}] was increased. Intercalation into the negatively supercoiled closed-circular DNA produces local unwinding of the double helix, resulting in progressive relaxation of the pre-existing supercoils and a consequent decrease in the electrophoretic mobility in agarose gels. As [Co^{III}] ranged from 0.5 to 5×10^{-6} mol dm^{-3}, banding typical of DNA with varying degrees of supercoiling density was observed (data not shown); each band represented plasmid with a particular number of residual negative supercoils. Maximum retardation was observed by [Co^{III}] ≤ 5×10^{-6} mol dm^{-3}, where the Form I (covalently closed) DNA had become completely relaxed (to Form II). The gel showed some smearing of the DNA, implying that the bound cobalt complex partially dissociated during electrophoresis. In addition, a

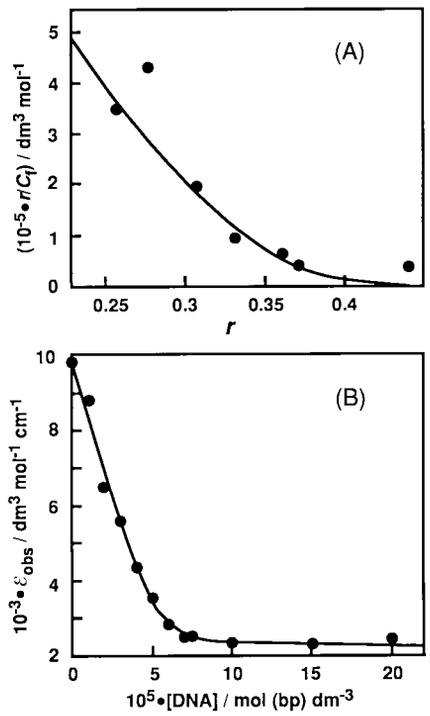


Fig. 2 Plasmid pUC9 DNA binding to the Co^{III}–sar–anthracene complex **1** (2.0×10^{-5} mol dm^{-3}). Data measured at 386 nm (Fig. 1) were analysed using the modified Scatchard equation (1), of McGhee and von Hippel.[19] (A) Plot of $r(C_F)^{-1}$ against r, where r is calculated as C_B[DNA]$^{-1}$ with C_B and C_F representing the concentrations of bound and free cobalt(III) ligand **1**, respectively. The solid curve was calculated using values of 1.8×10^6 dm^3 mol^{-1} for the intrinsic association constant K_i of the Co^{III}–DNA complex and 2.35 base pairs (bp) for the binding-site size n. (B) Effect of [DNA] on the observed apparent molar absorptivity of **1**, ε_{obs} at 386 nm. The solid line was calculated with $K_i = 1.8 \times 10^6$ dm^3 mol^{-1} and $n = 2.35$, using values of 9800 and 2250 dm^3 mol^{-1} cm^{-1} for ε_{386} of the "free" and bound ligand, respectively.

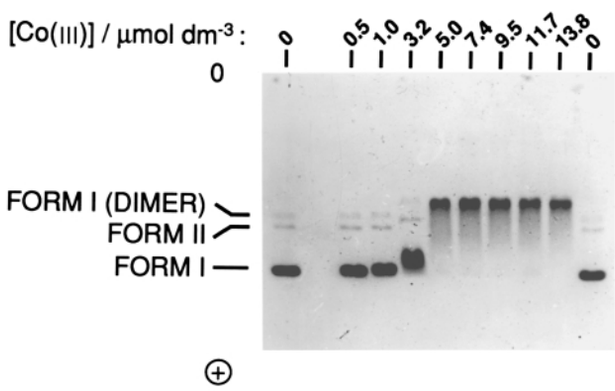


Fig. 3 The effect of the Co^{III}–sar–anthracene complex **1** on the electrophoretic mobility of negatively supercoiled circular plasmid DNA. Plasmid DNA (3.26×10^{-5} mol dm^{-3} in base pairs) was treated with **1** at the indicated concentrations, and DNA species were separated electrophoretically. The gel was stained with ethidium bromide, destained and photographed with UV irradiation; the result is presented as the photographic negative. **O** marks the origin of the electrophoresis. Forms I and II represent covalently closed supercoiled and relaxed circular DNA, respectively. Form I (dimer) corresponds to (initially) supercoiled circular DNA of twice the normal size present in the plasmid preparation. The data indicate that the supercoiled plasmid is fully relaxed at [Co^{III}] ≤ 5 μmol dm^{-3}.

smaller change in mobility of the relaxed Form II DNA was observed in comparison to the nicked Form II DNA in the absence of the complex. This further small change in mobility may be attributed to neutralisation of negative charges on the DNA by the cationic complex. The band assigned to the

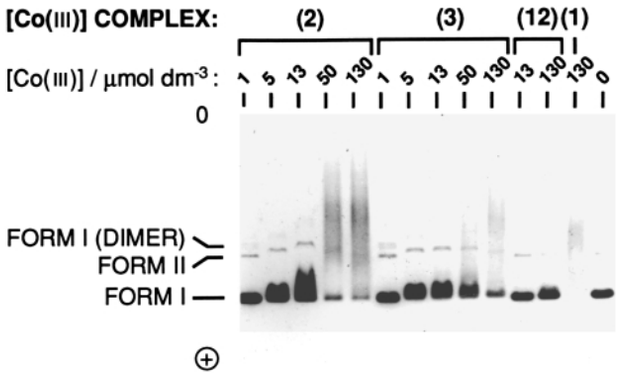


Fig. 4 The effects of complexes **1**–**3** and **12** at indicated concentrations on the electrophoretic mobility of plasmid DNA (3.26×10^{-5} mol dm^{-3} in base pairs) in 0.03 mol dm^{-3} Tris–HCl, pH 7.6. Gel electrophoresis was as described in the legend of Fig. 3.

dimeric plasmid behaves like the monomeric Form I DNA, as expected.

Given that complete relaxation of the Form I DNA (3.26×10^{-5} mol dm^{-3}) was observed at [Co^{III}] $\leq 5 \times 10^{-6}$ mol dm^{-3}, we can estimate a lower limit for the DNA unwinding angle φ_e associated with intercalation of each molecule of the complex cation. With knowledge of the K_i (evaluated above), the concentration of bound cobalt(III) complex at equilibrium may be calculated, which enables an estimation of the density of sites on DNA that must be occupied to produce complete relaxation. For the Co^{III}–sar–anthracene complex **1**, the calculation indicates that one molecule of the intercalator per 7 bp of DNA is sufficient. Assuming an initial supercoiling density (σ) of -0.07, this corresponds to $\varphi_e \approx 16°$, which is within the range of 13 to 26° observed with other intercalators.[3,21]

Fig. 4 shows the effect of treatment of plasmid DNA with increasing concentrations of the Co^{III}–sar cage complexes **1**, **2**, **3**, and **12** (Scheme 1), as determined by electrophoresis in the absence of ethidium bromide. There are diverse effects among the four complexes indicative of stable intercalative binding to DNA by **1**, **2**, and **3**, which contain polycyclic aromatic groups, but not by **12**, which does not. However, the binding affinities of the phenanthrene and naphthalene complexes appear to be somewhat lower than that of the anthracene conjugate (*cf.* Fig. 3).

DNA binding by complexes **4**–**7** was examined in a similar manner (data not shown). The DNA-binding affinity and/or stability of the DNA–Co^{III} complex of **1**, **2**, and the anthraquinone complexes **4** and **6** was observed to be higher than that of **3**, **5** and **7**. The variation in the shape and geometry of the complex relative to the helix, its charge distribution, and the ability of the intercalating moiety to fit in between the base pairs of DNA appear to be important in influencing DNA binding, and this is in accord with expectations based on the work of others.[2–4,8,9] For example, the polarity of the anthraquinone moiety probably enhances its intercalation in a manner akin to that of acridines and flavins. This difference is reflected, for example, in the reported affinities for DNA of the (9-anthrylmethyl)ammonium(1+) ion ($K_i = 2 \times 10^4$ dm^3 mol^{-1})[5] and protonated (1+) anthraquinone-based 9,10-$O_2C_{14}H_7$-[$S(O)_2NH(CH_2)_3NH_3^+$]-2 ($K_i = 2 \times 10^5$ dm^3 mol^{-1}).[8]

Photochemical DNA cleavage

Photochemical cleavage of DNA following binding of complexes **1**–**12** (Scheme 1) was examined (Fig. 5). Samples of DNA (3.26×10^{-5} mol dm^{-3} in base pairs) were treated with 2.0×10^{-5} mol dm^{-3} Co^{III}–sar cage complexes at 37 °C for 15 min. The mixtures were irradiated at 254, 302, or 365 nm for 2 min. These wavelengths span the ligand to metal charge transfer region and the singlet and triplet $\pi \longrightarrow \pi$ transitions of the polyaromatic moieties. The irradiated samples were then treated

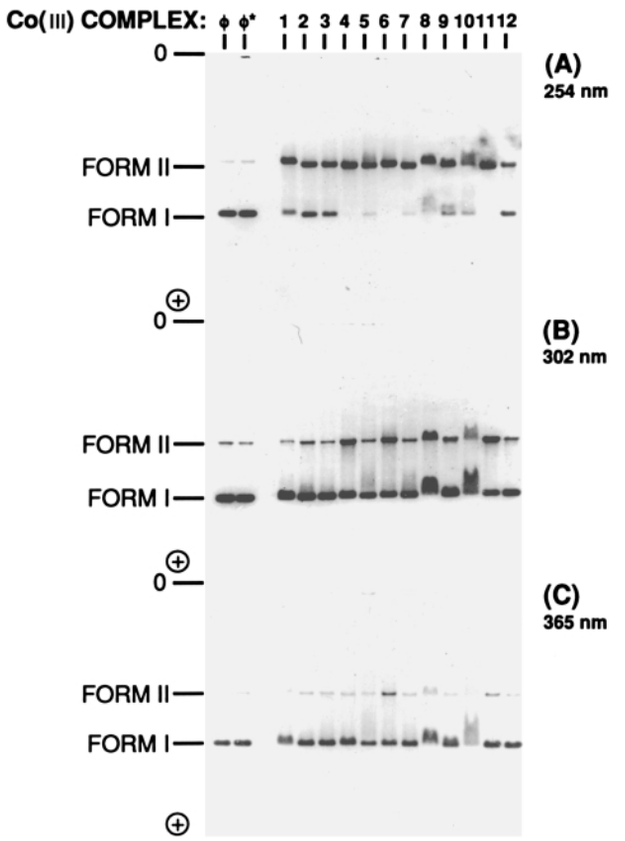


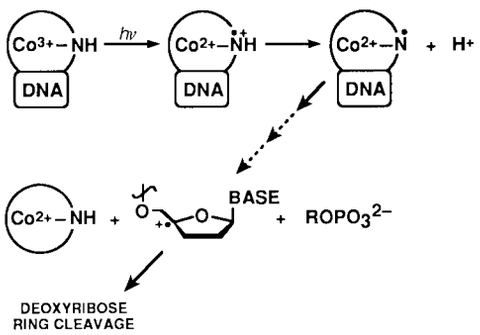
Fig. 5 Photochemical cleavage of DNA (3.26×10^{-5} mol dm^{-3} in base pairs) in the presence of complexes **1**–**12** (each at 2.0×10^{-5} mol dm^{-3}) in 0.03 mol dm^{-3} Tris–HCl, pH 7.6, following irradiation at 254 (A), 302 (B), and 365 nm (C). Bands marked Forms I and II correspond to covalently closed supercoiled and relaxed (nicked) plasmid DNA, respectively. Lanes marked φ contain DNA alone (unirradiated) and lanes φ* correspond to DNA that had been irradiated alone. DNA samples were treated after irradiation with an excess of ethidium to displace bound Co^{III}, and the gels and buffers used for electrophoresis contained ethidium bromide, to detect plasmid cleavage rather than binding of Co^{III}.

with an excess of ethidium bromide in the dark to displace the bound Co^{III}, prior to their electrophoresis in a gel containing ethidium bromide. DNA cleavage, minimally one nick in one strand, is detected by the difference in electrophoretic mobility of the supercoiled (Form I) and nicked (relaxed; Form II) plasmids. No significant DNA cleavage was evident during irradiation of DNA at these wavelengths in the absence of the cobalt complexes (Fig. 5, lanes φ and φ*).

Irradiation at 254 nm following pretreatment of DNA with all of the complexes led to enhanced ultraviolet light-induced DNA cleavage. This photochemical effect was especially apparent with **4**, **6** and **11**, which completely cleaved the DNA during the time of irradiation. To determine if cleavage was due to light absorption by the aromatic chromophores (*e.g.* by the anthracenyl moiety in **1**; Fig. 1), the effects of irradiation at 302 and 365 nm were also examined (Fig. 5B and C). Interestingly, following irradiation at 365 nm, only **6** and **11** elicited perceptible photocleavage of DNA, while at 302 nm the pattern of DNA photocleavage was intermediate between that observed at 254 and 365 nm.

The possibility that DNA strand cleavage involved singlet oxygen was examined in experiments using D_2O as solvent. If $^1O_2$ species were involved, cleavage at neutral pH would be observed to be enhanced in D_2O as a result of their longer lifetime.[1,22] However we observed that irradiation at 254 nm of the DNA adduct formed with the anthracene complex **1** in 70 to 90% D_2O resulted in no enhancement in cleavage (data not shown).

Two alternative mechanisms were considered. First, light absorption at the longer wavelengths could generate the singlet or triplet states of the intercalated polyaromatic moiety, leading to reduction of Co^{III} to Co^{II} and the production of an organic radical cation which would be a powerful oxidant.[13] Secondly, irradiation at the shortest wavelength could initiate a ligand to metal electron transfer generating the cobalt(II) state and a nitrogen radical cation which would also be a powerful oxidant. Both types of product could be expected to oxidise the deoxyribose residues and cleave a DNA strand in analogy to chemistry already described [1,4] (as shown in Scheme 2).



Scheme 2

Although the aromatic singlet state process is effective in other contexts,[23] it is apparent here that intercalation efficiently quenches this path. There is little or no sign of such singlet or triplet states leading to oxidative cleavage. Moreover, it is apparent from the spectroscopic binding experiment with **1** (Fig. 1) that the anthracene absorption envelope (330–385 nm) is effectively reduced in intensity and slightly red-shifted. These short axis (in-plane) $\pi \longrightarrow \pi$ transitions[24] presumably lose intensity to other absorption bands in the overall envelope.

The quenching of the singlet and triplet excited states is not so surprising since complex **1** displays that behaviour in aqueous solution by both back electron transfer and energy transfer pathways.[13] However, in some circumstances with polyaromatic ligands, fluorescence is activated by intercalation.[20] Both sets of phenomena diminish access to a radical cation aromatic moiety capable of oxidising the deoxyribose.

However, all the results are consistent with the involvement of a ligand to metal electron transfer from a co-ordinated N atom (Scheme 2). The resulting radical could oxidise the deoxyribose ring and cleave a DNA strand, in keeping with earlier experiments using $[Co(NH_3)_6]^{3+}$ at low wavelengths.[1] Such a process could also be valid for both intercalated and non-intercalated reagents. Proximity of the reagents, as reflected in their binding ability, would enhance the efficiency of the process.

In this context, another feasible path is photoactivated electron transfer within an ion pair between the deoxyribose phosphate and the complex cation leading to phosphate ester cleavage and a deoxyribosyl radical and consequent strand cleavage. Ion-pair photoredox reactions with anions and cobalt(III) amine cations have been postulated earlier and proven for halide ion.[25] Ion-pair charge transfer spectra were recognised early, and later Stirling and Stranks[26] showed that chiral $[Co^{III}(en)_3]^{3+}$ ion racemised in the presence of iodide ion when irradiated at ≈320 nm. The latter process was attributed to electron transfer in the ion pair and subsequent fast racemisation of the $[Co^{II}(en)_3]^{2+}$ ion produced. Although the back electron transfer was fast, the labile cobalt(II) complex had a lifetime (>1 μs) sufficient for it to racemise, at least.

This mechanism is also feasible for all of the reagents used here, whether they are intercalated or not, at least in the short wavelength region required to elicit efficient DNA strand cleavage. It could lead to both phosphate ester cleavage and deoxyribose radical cleavage and further oxidation.

Both pathways are more likely than an $HO^{\bullet}$ radical path, which has often been invoked but for which there is no conclusive evidence. Moreover, $HO^{\bullet}$ radical paths for oxidation are relatively unselective for the substrate. In this set of data one aspect of selectivity is clear: the reagents which are most easily reduced, namely the anthraquinone reagents **4** and **6** and the Co^{III}–N_3S_3 cage **11** (Scheme 1), become the most effective oxidants after photoxidation. This selectivity is most consistent with the photoredox process being the major path. Nevertheless, there are clearly aspects of the radical cleavage pathway which remain to be resolved and established with more certainty.

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