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THE INTERACTION BETWEEN PROTHIDIUM DIBROMIDE AND DNA AT THE MOLECULAR LEVEL

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The interactions in solution and in the fibre state of the trypanocidal drug, prothidium dibromide, with DNA have been investigated using a number of biophysical techniques. The binding parameters at 0.11 ionic strength were determined by spectroscopic means. Sedimentation studies show that the drug is able to unwind closed circular DNA in solution, but X-ray diffraction and linear dichroism experiments indicate that it is uncertain whether this unwinding can be attributed to intercalation in the classical sense. On the basis of our results, we propose that the primary mode of binding is 'sideways' intercalation, supplemented by electrostatic binding along the sugar-phosphate chains and interstrand binding involving hydrogen bonding.

1. Introduction

For a variety of drugs, their mode of binding to DNA has long been envisaged as intercalation [1]. Such an interaction was first proposed on the basis of X-ray diffraction, flow birefringence and small-angle X-ray scattering of DNA-proflavine complexes [2,3]. Since that time, an accumulation of evidence using many different physical techniques (e.g., autoradiography [4], electron microscopy [5], light scattering [6], fluorescence depolarization [7], circular dichroism [8], ESR [9], NMR [10]) has supported this binding scheme, and intercalation is now accepted as the primary binding mode in the interaction with DNA of many drugs containing fused aromatic rings. These include the archetypal intercalator, ethidium bromide [11], the antitumour antibiotics, daunomycin, adriamycin and nogalamycin [12–14], the schistosomicidal drug, miracil D [15], the antimalarial drug, chloroquine [16], as well as many carcinogenic hydrocarbons [17].

All these drugs possess planar aromatic ring systems. The flat ring systems are important, since it is an obvious requirement of the intercalation theory that the drug molecule must possess a reasonably large flat portion (but not too large or bulky) if it is to slip in between the flat base-pairs of the DNA helix. It has in fact been shown [18] that a proflavine derivative, 2,7-di(*t*-butyl)proflavine, does not intercalate, and this had been predicted by molecular model building studies which showed that the steric hindrance involving the bulky side groups should prevent straightforward intercalation of the ring system.

Prothidium dibromide is a trypanocidal phenanthridine, used for the treatment of sleeping sickness in man and nagana in cattle. It has been reported to be more successful than ethidium bromide, since it has prophylactic as well as curative properties [19]. It is thought to have antiviral properties and to interfere with the synthesis of nucleic acids in a variety of organisms.

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The structure of prothidium is interesting, since from molecular models it is difficult to decide whether or not intercalation is likely. It is therefore of interest to compare its binding with that of ethidium bromide, since a comparison of related drugs is essential for the development of new, more specific, chemotherapeutic agents with reduced toxic side effects. Moreover, understanding of the way in which relatively small molecules modify the structure of DNA may shed light on the interaction between DNA and more complex systems, such as enzymes and regulatory proteins.

2. Materials and methods

Prothidium dibromide was obtained from Drs. T.I. Watkins and G. Woolfe, Boots Co. Ltd., Nottingham, U.K. Isometamidium and desaminoisometamidium were obtained from Dr. Woolfe. Some difficulty was experienced in dissolving the crystals, but warming to 35°C was sufficient to permit dissolution at low ionic concentrations. High ionic concentrations were achieved by preparing solutions initially at low ionic strength, and then adding salt to the higher value when completely dissolved. All solutions were stored at 0–4°C, and wrapped in metal foil to minimize photolytic degradation.

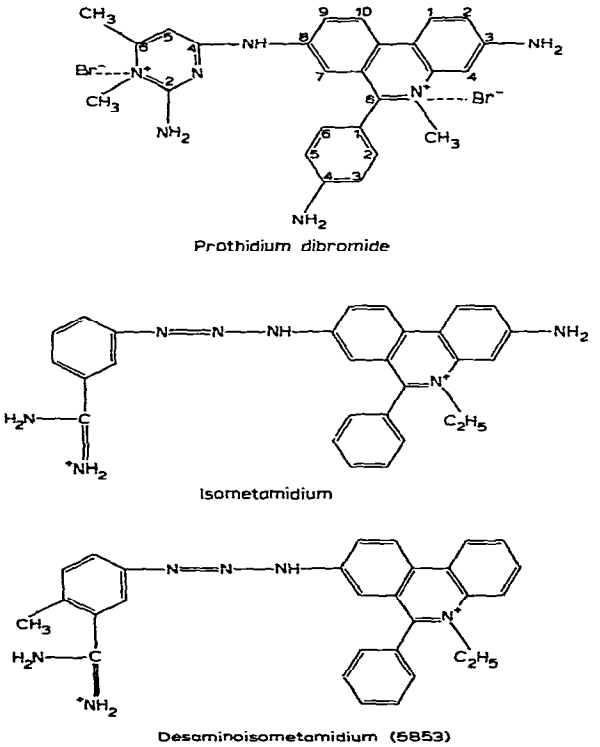


Fig. 1. The structural formulae of prothidium dibromide and two phenanthridine analogues.

The structural formulae of the three drugs are given in fig. 1. The molecular weight of prothidium dibromide was taken as 597.4, and all concentrations calculated taking its peak absorption (at ≈ 460 nm) as $7100\,M^{-1}\,cm^{-1}$. We estimate the precision of this value as $\pm 200\,M^{-1}\,cm^{-1}$.

DNAs from *Micrococcus lysodeikticus*, calf thymus and *Clostridium perfringens* were purchased from Sigma Chemical Co. The shapes of their absorption spectra were similar, though there were slight differences in the wavelength at which maximum absorption was observed (viz., *M. lysodeikticus* 255 nm, calf thymus 258 nm, *Cl. perfringens* 256 nm). All samples showed values of A_{260}/A_{230} greater than 2.2 and of A_{260}/A_{280} greater than 1.9 and were used without further purification. Closed circular (Form I) ϕX 174 RF DNA was prepared as previously described [20] and contained 25–35% nicked circles (Form II). Bacteriophage PM2 DNA, consisting of over 95% closed circular (Form I) molecules, was prepared by the method of Espejo et al. [21]. Samples containing approx. 15% Form II molecules, for the s_{20} experiments, were generated by repeated freeze-thawing [22].

Three different buffers were used. For the sedimentation studies, 0.05 M Tris-HCl (pH 7.9), was adopted in common with earlier work [20,23]. The other experiments used either a 'low' (0.01 M Tris-HCl, 0.01 M NaCl, pH 7.6) or 'high' (0.01 M Tris-HCl, 0.1 M NaCl, pH 7.6) ionic strength buffer. All DNA concentrations were calculated by taking the molar extinction coefficient at 260 nm, ϵ_{260}, to be $6600\,M^{-1}\,cm^{-1}$.

2.1. Spectroscopic binding

The visible absorption spectra of DNA/prothidium dibromide mixtures, for a series of bind-

ing ratios, were recorded on a Cary 17 spectrophotometer. We used cylindrical cuvettes of 5 cm path length and 15 ml capacity. The drug/DNA ratio was altered successively using a novel mixing scheme [24]. The drug concentration remained constant within each series and isosbestic behaviour could be observed directly, if present. A similar mixing procedure was adopted in parallel with the reference cuvette to compensate for the small effect of light scattering by the DNA in the mixture.

The binding spectra were digitized and analysed as linear combinations of the free drug spectrum and the spectrum of completely bound drug. The latter was taken as the spectrum of a DNA/prothidium mixture of binding ratio, ν (bound drug per nucleotide pair), sufficiently low that a further reduction in its value did not result in any measurable metachromatic shift. A weighted least-squares minimization program was used to find the free and bound drug concentrations for each mixture spectrum, and these data were fitted to the excluded-site binding model [25] using the Simplex quadratic convergence method [26].

2.2. Analytical ultracentrifugation

Sedimentation coefficients were determined by boundary sedimentation in a Beckman model E analytical ultracentrifuge equipped with ultraviolet optics as described previously [20]. They are presented as s_{20} values, determined directly at 20°C, and uncorrected for viscosity, buoyancy or DNA concentration.

2.3. X-ray diffraction

X-ray diffraction photographs from DNA/prothidium fibres were taken using nickel-filtered copper Kα radiation ($\lambda = 1.5418$ Å) from either a Hilger and Watts microfocus generator or a Marconi-Elliott GX6 rotating-anode generator. Sets were operated at 35 kV, with tube currents of 3 and 60 mA, respectively. Exposure times varied with the thickness of the fibre (and, to a lesser extent, with its drug content) but were typically 5–7 days using the Hilger and Watts generator and 6–8 h using the rotating-anode generator. Pinhole cameras with a specimen-to-film distance of about 3 cm were used with the microfocus generators. A focussing camera with toroidal optics and a specimen-to-film distance of 3.5 cm was used with the rotating-anode generator.

In order to minimize scattering of the X-rays by air, the cameras were continuously flushed at a moderate rate with helium gas. The helium was humidified prior to flushing by passing it through water and then through a saturated salt solution, and a pot of the appropriate saturated salt solution was placed inside each camera. At least 1 h was allowed for the fibre to equilibrate to the desired humidity before each exposure.

2.4. Visible absorption spectra of fibres and linear dichroism measurements

Visible absorption spectra of DNA/prothidium fibres were recorded for polarized light in the wavelength range 400–650 nm using a microspectrophotometer system based on a Zeiss Universal microscope. The fibre was placed on the rotating stage of the microscope between the crossed analyser and polarizer and oriented for complete extinction. The analyser was then removed, and

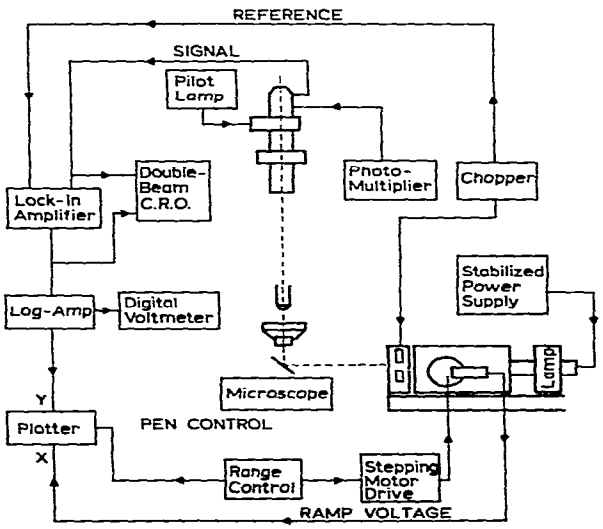


Fig. 2. Schematic illustration of the microspectrophotometer system used to determine the dichroic ratios of fibres.

absorption spectra taken for polarizer orientations perpendicular and parallel to the fibre axis under various conditions of relative humidity.

The full microspectrophotometer system is shown in fig. 2. There is an absolute limit to the absorbance which can be measured of about 3.8 absorbance units for a 2 nm band pass, due to stray light. In fact some flattening of the absorbance peaks becomes noticeable at around 3.5 absorbance units. In order to minimize this effect only thin fibres of low prothidium content could be measured. A minimum diameter of about 80 μm was set by the necessity of obtaining X-ray diffraction patterns of the fibres in a reasonable time [27].

The measured absorbance of a control DNA fibre, which has no absorbance band in this wavelength region, was always between 0.3 and 0.4 at 650 nm depending on the thickness of the fibre. This is the combined result of the cylindrical lens effect of the fibre and of light scattering by the specimen, particularly by salt crystallites and impurities. These effects are also presumably present in the absorption spectra of DNA/drug fibres, and an allowance for them was made by subtracting the measured value of the absorbance at a wavelength where the drug does not absorb (i.e., 650 nm).

3. Results

3.1. Binding parameters for linear DNA

The absorbance of prothidium dibromide obeys Beer's Law up to concentrations of at least 0.1 mM, which is well above the highest free drug concentration ($\approx$0.02 mM) used in the spectroscopic series. Above a concentration of 0.5 mM departures from linearity occur due to dimerization of the drug molecules [28], as evidenced by a red shifting of its spectrum with increasing drug concentration. There is little doubt that the large hydrophobic ring surfaces are the main cause of this stacking. Solutions of around 0.05 mM concentration have a maximum absorption at 460 nm, and at a concentration of 0.25 mM the maximum has shifted to around 465 nm. By analogy with ethidium bromide, the red shifting may be explained by Br^- acting as auxochromes (electron donators) at specific sites on the dimer when the concentration is raised [29]. The presence of a negative ion close to a chromophore is, in general, equivalent to enlarging the territory available to the π-electron system with a consequent red shifting of the wavelength of the electronic transition

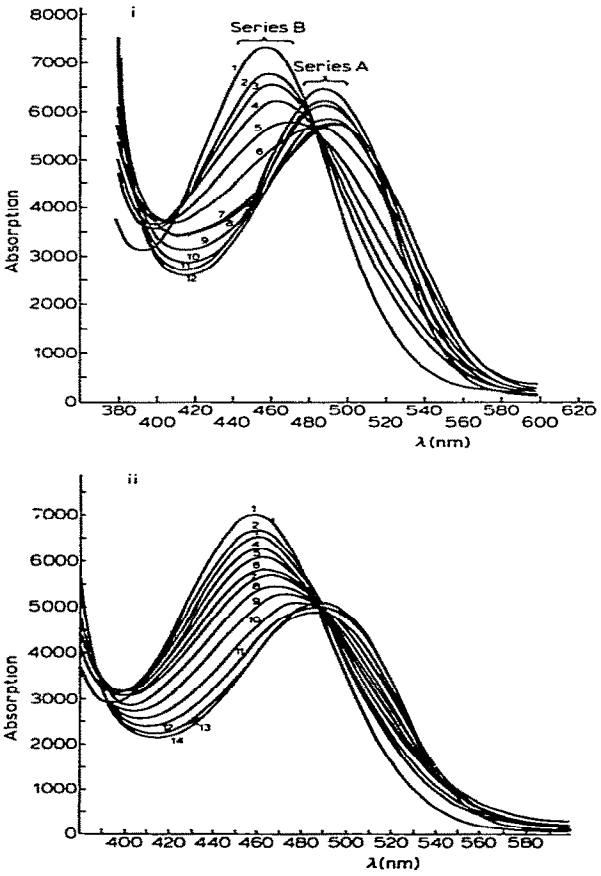


Fig. 3. The absorption spectra for prothidium dibromide binding to calf thymus DNA in (i) low and (ii) high ionic strength buffers (see text). (i) Total drug concentration for each spectrum, 20.6 μM. DNA nucleotide pair concentration, in μM (spectra 1–12, respectively): 0, 2.8, 3.8, 5.1, 6.9, 9.3, 12.5, 16.9, 22.8, 30.6, 56.1, 102.2. (ii) Total drug concentration for each spectrum, 16.6 μM. DNA nucleotide pair concentration, in μM (spectra 1–14, respectively): 0, 3.4, 4.6, 6.2, 8.3, 11.2, 15.2, 20.5, 26.7, 37.4, 50.4, 68.1, 91.9, 124.1.

[30], although evidence of a hypsochromic effect has also been presented [31]. The most probable sites for attachment would be in the positively charged areas around the N_5^+ of the chromophore and the N_1^+ of the pyrimidyl moiety. The N_3 of the pyrimidyl ring has a lone pair of electrons. It could either act as an H-bond acceptor or it could be protonated and electrostatically attract a Br^-. The observation that red shifting of the prothidium spectrum becomes evident at lower concentrations than for ethidium and dimidium may be related to the fact that there are two Br^- for every drug molecule. Increasing ionic strength appears to cause slight red shifting of the spectrum as well, though this effect is slight (1–2 nm). Increased ionic concentration is thought to facilitate dimerization primarily by screening of the positive charges by the anions of the salt, thus diminishing the unfavourable electrostatic repulsion [29].

The spectra for the binding of prothidium to DNA differ from those for ethidium and dimidium [24,32], providing a direct indication that the type of binding may be significantly different. At low ionic strength (fig. 3i), for each DNA type, the spectra show a red shift from the free drug spectrum for all mixtures, but at higher binding ratios ($\nu \gtrsim 1.0$) a hyperchromic effect is evident, accompanied by a slight blue shifting. In fact, each spectral series can be split into two sub-series, one (series A) comprising the spectra with binding ratios less than or about unity and the other (series B) consisting of the spectra with binding ratios greater than or about unity. Each series shows isosbestic behaviour within itself. Series A has isosbestic points at about 455 nm and around 520 nm, and series B has isosbestic points at 410–415 nm and 485–490 nm. At higher ionic strength (fig. 3ii) this rather complicated behaviour is less apparent and all the spectra appear to belong to a single series, with near-isosbestic behaviour at 400–405 nm and 485 nm. It seems likely that the binding operating at high salt concentration is similar to the type predominating at large binding ratios (viz., series B) when the salt concentration is low. There is little variation in the spectra when different DNA types are used.

Since fairly good isosbestic behaviour was obtained at high ionic strength, these results were analysed in terms of a single binding species using the excluded-site model. The resulting Scatchard plots for binding to the three DNA species tested are shown in fig. 4. This analysis was not carried out on the binding spectra obtained at low salt concentration, since it is inappropriate to a case that does not show isosbestic behaviour throughout the range of mixing ratios.

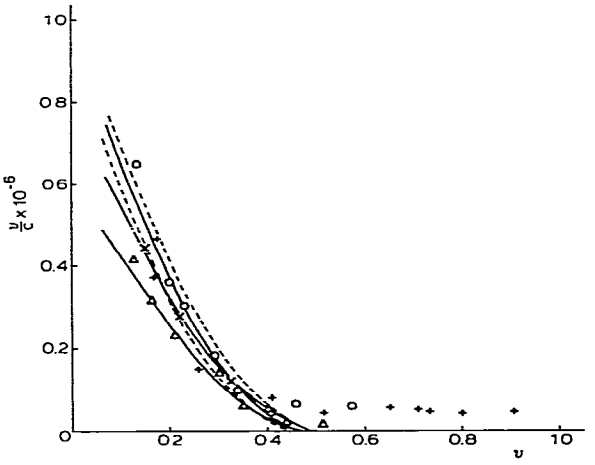


Fig. 4. Scatchard plot for the binding of prothidium dibromide to various DNAs at high ionic concentration. ⊙, calf thymus; △, *Cl. perfringens*; ×, *M. lysodeikticus*. (Note: supplementary data points marked + were obtained by equilibrium dialysis.) The dashed lines on either side of the best-fit line for calf thymus DNA indicate the effect of 5% deviations in the n and k values.

The Scatchard plots show that the data for binding to *M. lysodeikticus* DNA can be fitted fairly well to the single binding species, excluded-site model. For binding to *Cl. perfringens* and calf thymus DNA the fitting is inadequate at large values of ν, and this is probably due to the onset of other modes of binding. The constants for binding to *M. lysodeikticus* DNA, and the constants for the binding at low ν values to the *Cl. perfringens* and calf thymus DNA, are listed in table 1. There is no evidence of any G-C specificity in the binding. The values for n, the binding site size, are similar for the three DNA types tested, and there is very little variation amongst the values of the association constants, k.

Table 1

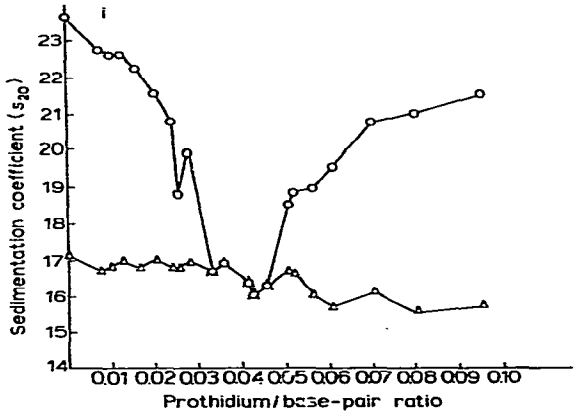
Best-fit parameters for prothidium dibromide binding to DNA at high ionic concentration

The goodness-of-fit parameter is a measure of how closely the data points approach the best fitted curve. Larger values of this parameter indicate a better correspondence between the points and the fitted curve.

DNA type	G-C content (%)	Binding site size (n)	Association constant (k) ($\times 10^{-5}$)	Goodness-of-fit parameter
Cl. perfringens	30	2.03	6.06	0.606
Calf thymus	42	2.11	9.72	1.508
M. lysodeikticus	72	2.01	7.24	0.059

3.2. Analytical ultracentrifugation

Fig. 5 shows the variation of the sedimentation coefficient, s_{20}, of closed circular DNAs as increasing amounts of the phenanthridine drugs prothidium, isometamidium and desaminoisometamidium, are added. It should be noted that the abscissae of fig. 5 show the total drug/base-pair ratios, and not the bound drug/base-pair ratio, ν. However, calculations based on our binding measurements reveal that these values must be similar to within 5% or better because of the high association constants of these drugs. Each of the drugs causes the typical fall and rise in the s_{20} value of the closed circles, which is diagnostic of unwinding agents. With increasing drug concentrations, the number of duplex helical turns (β) decreases with a consequent change in the number of superhelical



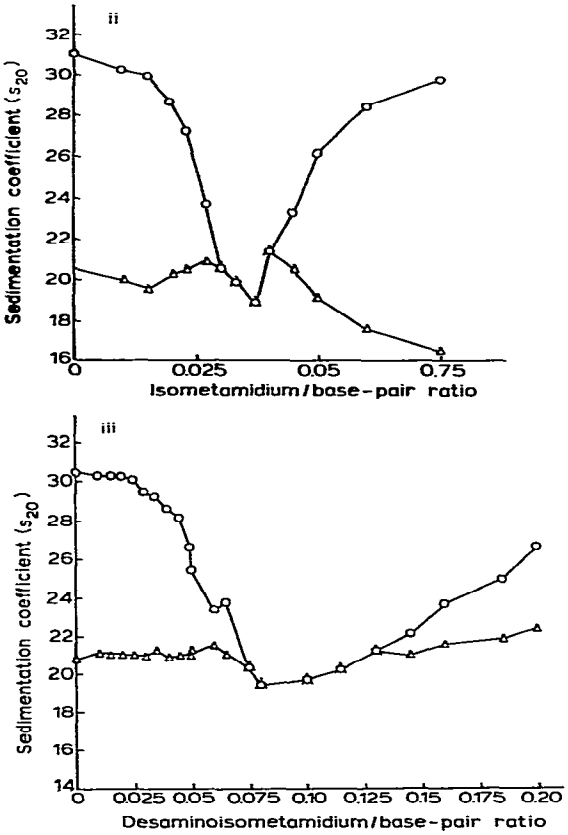


Fig. 5. The effect of binding phenanthridine drugs to circular DNA. (⊙———⊙) Sedimentation behaviour of closed circular (Form I) DNA; △———△ sedimentation of nicked circular (Form II) DNA. (i) Prothidium dibromide with ϕX 174 RF DNA; (ii) isometamidium with PM2 DNA; (iii) desaminoisometamidium with PM2 DNA. The buffer in each case was 0.05 M Tris-HCl, pH 7.9.

turns (τ). As the initially negatively supercoiled DNA molecules are relaxed, their sedimentation coefficient falls, reaching a minimum at $\tau = 0$ (completely relaxed, Form Ir). A further increase in τ causes positive supercoiling, accompanied by an increase in s_{20}. The behaviour of the nicked (Form II) DNA in the preparations provides a useful internal control, which monitors second-order effects caused by the binding of drugs, other than those due to changes in the supercoiled tertiary structure.

The binding ratio at the minimum or equivalence point, ν_c, can be used to find the unwinding angle per bound drug molecule, ϕ, if the original number of superhelical turns, τ_0, present in the species is known [33]. If N is the number of base-pairs, then

$$\tau_0 = -\frac{N\phi\nu_c}{360} \quad (1)$$

Unfortunately, values for τ_0 are not known accurately, principally because the conformation of DNA under the measuring conditions has frequently been overlooked and the effects of temperature, counterion type and concentration [34–37] have not been considered rigorously enough. Recently, accurate and precise values of τ_0 for the plasmid DNA, pBRβG, have been obtained [38].

Fortunately, however, for this method τ_0 need not be known as long as the equivalence point, ν_c', for ethidium bromide under identical conditions is obtained. If α' and α are the respective fractions of ethidium and the drug under test which are bound in such a way as to cause unwinding, then

$$\alpha\nu_c\phi = \alpha'\nu_c'\phi' \quad (2)$$

There is strong evidence that the unwinding angle for ethidium bromide, $\phi' = 26°$ [39–41], and α' can be conveniently assigned the value of unity. If we assume that $\alpha = 1$ for the test drugs also, i.e., that their unwinding effect on DNA is a direct reflection of their sole mode of interaction with the polymer, then the calculated values of ϕ fall well below that of ethidium (table 2). Alternatively, we might imagine that the true unwinding angle of the other phenanthridines under test here actually lies close to 26°, since they contain the planar, triple aromatic phenanthridinium ring in common with ethidium bromide. If this were the case, values of α could be calculated for each drug; these too are tabulated in table 2. Allowance for the values of n and k, if known precisely under identical conditions, would result in only slightly higher values of ϕ (and α).

3.3. The X-ray diffraction patterns

The X-ray diffraction patterns from DNA/prothidium fibres (fig. 6) are noticeably poorer in quality than those from fibres containing either ethidium or dimidium at corresponding drug/DNA ratios [11,24]. A 3.4 Å meridional reflection was observed in all the patterns, indicating that at least some B-type character was retained even at 75% relative humidity. For fibres of high drug content, the centres of the diffraction patterns were very vague and there were no resolvable layer lines. This is evidence of an irregular structure, but the gross features of the patterns indicate that they remain based on the B-conformation. The patterns display significantly more detail as the proportion of drug is reduced. A fibre with $\nu = 0.083$ is shown in fig. 6i and ii. At 75% relative humidity it exhibits a mixture of A- and B-type conforma-

Table 2

Values of α and ϕ for the three phenanthridine drugs tested (the symbols are explained in the text)

Values of ν_c' are taken from ref. 20 and ref. 42.

Drug	DNA	ν_c	ν_c'	α	ϕ (°)
Prothidium	ϕX 174 RF	0.0425	0.02	0.47	12.2
Isometamidium	PM2	0.0375	0.0255	0.68	17.7
Desaminoisometamidium	PM2	0.105	0.0255	0.24	6.3

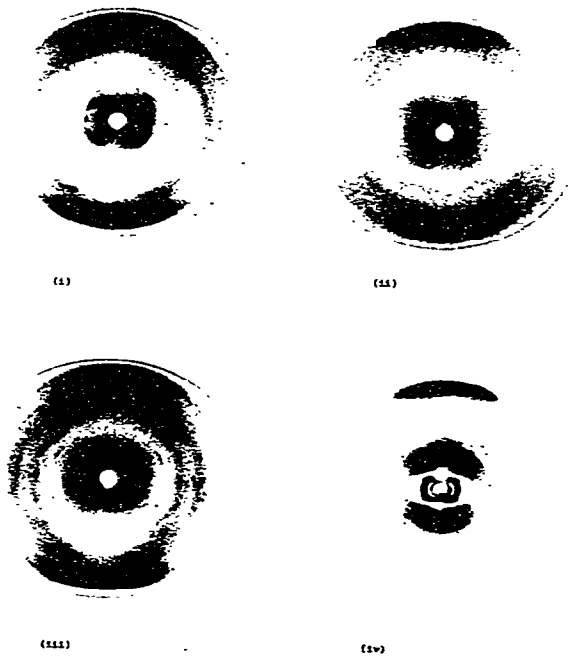


Fig. 6. X-ray diffraction patterns from DNA/prothidium fibres (0.01 M NaCl). (i) $r=0.083$, 75% relative humidity; (ii) $r=0.083$, 92% relative humidity; (iii) $r=0.059$, 75% relative humidity; (iv) $r=0.059$, 92% relative humidity.

tions, whilst at 92% relative humidity it shows the B-conformation only. Fibres of very low drug content often retained some A-type conformation at humidities of 92% and above.

Patterns taken from different prothidium fibres of the same drug content were often variable in quality. This is thought to be largely due to the variability in the drying down of the gel. Some fibres may dry down quickly on the outside to form a skin containing a less orientated interior. Prothidium gels are very viscous, and the proportion of fibres which collapsed on drying down was about 30%. On occasions, particular fibres did not display reversible conformational changes as the humidity was changed.

3.4. Linear dichroism

The absorption spectra of the fibres are similar to those obtained from the same complexes in solu-

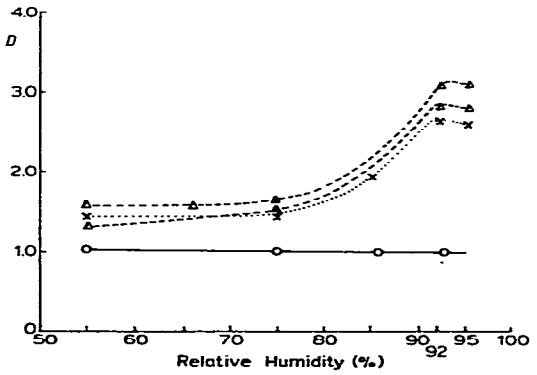


Fig. 7. Representative traces of the dichroic ratios of DNA/phenanthridine fibres as a function of relative humidity. (The dichroic ratio, D, is the ratio of the absorbances for light polarized perpendicular and parallel, respectively, to the fibre axis.) (△) DNA/ethidium, (×) DNA/dimidium, (⊙) DNA/prothidium (binding ratios: 5.3×10^{-2}, 4.2×10^{-2}, 4.3×10^{-2}, respectively).

tion, and at the low ν values used ($\lesssim 0.05$) the positions of the absorbance maxima indicate that most of the drug is in the bound state. The dichroic ratio of prothidium fibres is very different from that obtained with ethidium or dimidium bromide (fig. 7). The dichroic ratio stayed very close to unity over the complete range of relative humidities.

4. Discussion of results

4.1. Possible binding schemes

The binding constants for the three linear DNA species tested are all significantly higher than those obtained with ethidium bromide [27,43], indicating that the DNA-prothidium interaction is stronger. This could be related to the doubly charged nature of prothidium, which renders it strongly attracted to the negative phosphate ions of the DNA backbone. The charge separation in prothidium is 8.0 Å [44], which is the approximate distance separating phosphate groups along either strand of the DNA double helix. Each prothidium molecule has three amino groups and a lone-pair nitrogen, all of which are available to form hydrogen bonds—the amino groups as donors, and the N_3 of the pyrimidyl moiety as an acceptor. The distance between the amino groups is such that hydrogen bonds could be formed to phosphate oxygens located along the same strand, or on opposite strands (across either a narrow or a wide groove). The hydrogen bonding would be expected to be less strong than the electrostatic binding, although it may be mediated by a strong electrostatic character due to charge delocalization around the aromatic rings.

Since prothidium possesses so many groups capable of providing strong interactions, in several different ways, with DNA it is likely that some or all of these binding forces will act simultaneously. The spectra of drug molecules bound primarily by different mechanisms may be quite similar, and the binding parameters describing the different species may not be very different. This would account for the fairly good isosbestic behaviour observed, and the finding that the Scatchard plots indicate more than one binding species.

The initial binding mode appears to be more or less saturated at a binding ratio of about 0.5, i.e., at one bound drug molecule for every two base-pairs. This results in values of n of around 2.0, which are insensitive to the G-C content. Further binding to *Cl. perfringens* and calf thymus DNA can occur, approaching ν values of unity with calf thymus DNA. The saturation of the initial stronger binding occurs at around the value for total binding saturation with ethidium and dimidium, under these conditions. Prothidium is a significantly larger molecule than ethidium and dimidium; thus, it is not unreasonable that the initial binding mode for prothidium should differ from the binding operating with the other two drugs. It is well established that ethidium and dimidium bind primarily by intercalation [1]. If this mode were considered for prothidium, the increased steric hindrance due to its larger size might be expected to result in a lower value for binding saturation and therefore in a larger value for n for the initial binding. No such effect is observed.

High k values are a perennial source of problems in the analysis of DNA/phenanthridine spectra, since they mean that data at low free drug concentrations are difficult to obtain. At low ionic strength the association constants are always larger, because of the reduced competition with countercations, and the analysis is even more difficult than at high salt concentration. For prothidium at low salt concentrations, we believe that the same binding schemes are operating as at high salt concentration, although the electrostatic character of the binding will be enhanced because the phosphates will be less well shielded by Na^+.

4.2. Intercalation or not?

Our sedimentation results (fig. 5) provide conclusive evidence of a prothidium binding mode which causes unwinding of the double helix. While the s_{20} results are diagnostic for unwinding, this may be due to either intercalative or non-intercalative binding [42]. Indeed, irehdiamine A —a steroidal diamine which cannot intercalate in the usual sense because of steric restrictions—has been shown to unwind the double helix by about

half the angle seen with ethidium [45] and other steroidal diamines unwind the helix by a variety of angles [42]. The prothidium results may be interpreted as showing that each bound prothidium molecule causes an unwinding of about 12° (table 2). Alternatively, the unwinding may be 26° with only about half the bound prothidium molecules causing unwinding (table 2). Or, indeed, a situation intermediate between these two may pertain.

It must be realized, however, that if one assumes an unwinding of 26°, such that $\alpha = 0.5$, the practice of fitting a Scatchard plot, based on a strict neighbour-exclusion principle, to a calculated binding isotherm may be of dubious validity. In this case, the magnitude of the derived binding constants is not likely to be greatly affected, but they should not be regarded as rigorously defined, accurate parameters of interaction.

The reduced unwinding effect, compared to that observed with ethidium bromide, could be due to intercalation with a geometry different from that of classical intercalation. It would be sterically possible for the prothidium to intercalate sideways by insertion of part of the triple ring between adjacent base-pairs approximately perpendicular to the helix axis. Indeed, sideways intercalation has been proposed for another bulky phenanthridine, RD 16101, to explain its binding parameters [46]. This is more likely to occur from the narrow groove of the DNA, since there is then the possibility of some additional stabilization by the formation of a hydrogen bond to an amino group of a base-pair. The poor overlap with the base-pairs makes it energetically less favourable a position than the position adopted by ethidium and dimidium.

The generally poor resolution of the DNA/prothidium X-ray diffraction patterns is an early indication that the DNA is seriously interfered with by binding prothidium to the helix, although some of the loss in detail is undoubtedly due to the poor orientation of many of the prothidium fibres. Some loss of resolution may be expected due to the greater size of prothidium, resulting in a larger perturbation of the DNA, but the rather drastic worsening of the patterns compared with those from ethidium and dimidium suggests a much less regular character to the binding than that operating with the other two drugs. The patterns which showed a significant proportion of A-type diffraction showed less distinct reflections than those obtained in the control A-DNA patterns at angles corresponding to distances in real space of 15 Å and above. This indicates a limited crystallinity within the DNA-drug structure, probably due to an increase in screw disorder as more drug is bound. There is very little detail remaining in these patterns at 3–10 Å resolution, indicating a severe loss of regularity in the molecules at the nucleotide level.

The B-type patterns all give rise to pitch values less than or about 34 Å at 75% relative humidity and about 34 Å at 92% relative humidity, which indicates no detectable unwinding of the helix compared with the control B-DNA pattern. The intermolecular separation measured from these patterns increases by 2–3 Å as prothidium is bound up to $r = 0.083$, after which it is difficult to resolve the equatorial reflections. The increase indicates that binding by prothidium may result in a bulkier structure, and this could be accounted for by an external mode of binding—either along a strand of the double helix, or across from one strand to the next. Both of these binding schemes could occur to binding levels greater than one drug for every two base-pairs (cf. the spectroscopic results). If both of these binding mechanisms were operating simultaneously, a substantial degradation in the regularity of the structure, as revealed by X-ray diffraction, would result even at much lower binding levels. This is because the electron density of the drug, in an external binding mode, is at a radius equal to or greater than that of the sugar-phosphate backbone.

All the vaguely A-type patterns exhibit a pitch close to 28.1 Å, the pitch of the control A-DNA fibre, irrespective of the humidity at which the patterns were taken. The observation that an A-type character may persist, even when the humidity is raised to 92% relative humidity, may be due to drug binding across the strands tending to hold them in their A-conformation and resisting a conformational change to the B-type structure.

A substantial increase in pitch with increased amounts of bound drug has generally been taken

as presumptive evidence of intercalation [11,47]. On this basis, our X-ray diffraction results exclude the possibility of substantial intercalation in the classical sense in the fibre state, for humidities up to 92% relative humidity. It is possible, however, that classical intercalation occurs at even higher humidities (cf. the acridines [48]), or in solution. Some sideways intercalation may be present, but at lower levels in the fibre than in solution. The most reasonable interpretation of the prothidium X-ray diffraction patterns is that the drug binds to DNA by a mechanism that does not involve a substantial pitch increase, arguing against classical intercalation as the major binding mode. The increased intermolecular separation at high levels of binding suggests that there is external attachment to the sugar-phosphate backbone.

In another series of experiments attempts were made to monitor the extension of the helix associated with binding, as expected from the intercalation model. Unfortunately, viscometric measurements with sonicated rod-like DNA fragments [49,50] were frustrated by erratic precipitation of the drug-DNA complex, often at relatively low binding ratios, so that no reliable relationship between effective contour length and level of drug binding could be determined.

The experimental values of the dichroic ratio for DNA/ethidium and DNA/dimidium fibres are greater than unity (fig. 7), indicating that the drug molecules have a preferential orientation in the fibre. In particular, they are consistent with the drug being orientated essentially perpendicular to the helix axis, as in intercalation. At low humidities binding is probably primarily external, with a fraction of these drugs intercalating at high humidities to give an increased dichroic ratio. In conjunction with X-ray diffraction data [24], and a novel analytic formulation of the dichroic ratio in terms of tilt angles [51], this fraction is thought to be about 52%. This is substantially lower than the level of intercalation (viz., almost 100% at these binding ratios) thought to pertain in solution [52].

The DNA/prothidium fibres show a constant dichroic ratio of about unity, irrespective of relative humidity. This indicates that a number of different binding modes are present, at least some of which involve binding at large angles to the plane perpendicular to the helix axis [51]. The small observed values of the dichroic ratio exclude the possibility of predominant intercalative binding of the classical type, in agreement with the X-ray diffraction results. However, sideways intercalation, involving some tilting or twisting of the chromophore, could be present.

4.3. Summary

Prothidium binds very tightly to DNA, as evidenced by its large association constant. The attachment is considered to be strongly electrostatic, since the binding is strongly affected by the ionic concentration of the environment. Strong electrostatic binding would be expected to show no base-pair specificity, since the environment of all the DNA phosphates is similar. Our spectroscopic results confirm this expected lack of specificity.

In the prothidium molecule both ring nitrogens are quaternised, so that there is a strong charge localization. The pyrimidyl moiety is fairly free to rotate about the bond to the phenanthridine triple ring. Nevertheless, the preferred conformation of the drug is likely to be when it is fully extended such that the pyrimidyl moiety makes an angle of about 120° with the triple aromatic ring, and lies on the same side of the triple ring as the main part of the aminated phenyl substituent. With this geometry the bulky pyrimidyl group would sterically hinder classical intercalation, but sideways intercalation would still be possible.

Sideways intercalation is more likely to occur from the narrow groove of the DNA (fig. 8i), since there is then the possibility of some additional stabilization by the formation of a hydrogen bond to an amino group of a base-pair. This mode of binding would result in an unwinding angle smaller than that for ethidium bromide, which is consistent with the results from our sedimentation and X-ray diffraction studies. Stereochemically, this mode of binding might continue to fairly high binding levels, since it need not be so restricted by neighbour-exclusion effects as are the more classical intercalation positions.

It would be possible for prothidium to bind from the large groove into an intercalation position similar to that adopted by ethidium bromide,

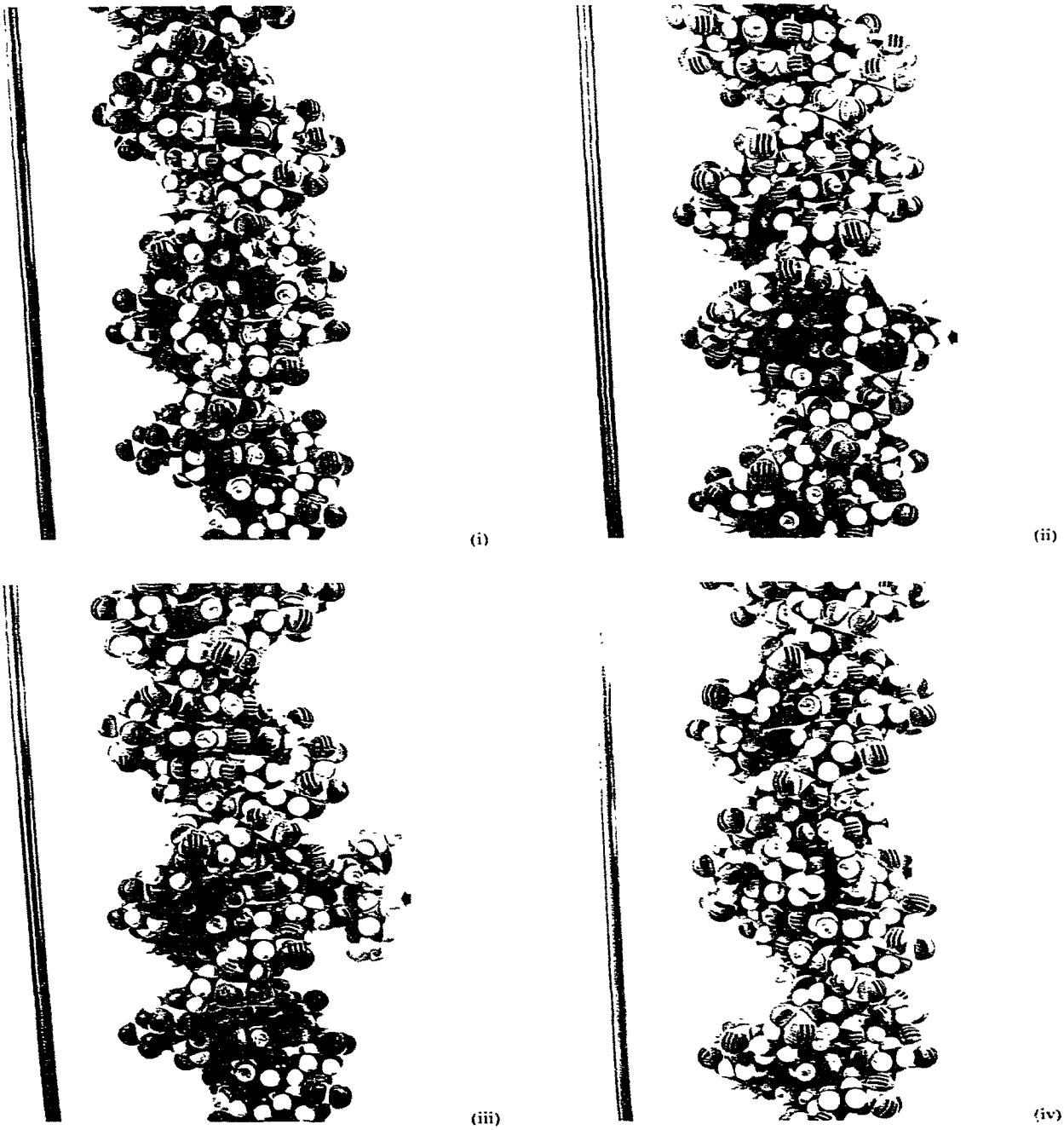


Fig. 8. Possible binding positions of prothidium dibromide to B-DNA. The arrow indicates the position of the prothidium molecule in each case. (i) Prothidium molecule intercalated sideways from the minor groove. (ii) Classical intercalation of prothidium from the major groove. (iii) Electrostatic binding along a sugar-phosphate chain. (iv) Interstrand binding across the major groove.

but the pyrimidyl moiety would need to be rotated close to the phenyl substituent (fig. 8ii). This is not the preferred conformation of the prothidium molecule, and it results in the close approach of the amino groups on the pyrimidyl and phenyl moieties. We can effectively exclude this possibility, since our X-ray diffraction studies do not show the required substantial increase in pitch, and our sedimentation results show a lower unwinding effect than that obtained with ethidium bromide.

In addition to the predominant sideways intercalation, the X-ray diffraction results suggest that there are external modes of binding, certainly at low and moderate ionic concentrations. This would not contribute to any unwinding of the DNA helix. Prothidium could bind electrostatically along the sugar-phosphate chain to two adjacent phosphate groups (fig. 8iii), or across the strands in DNA by forming hydrogen bonds (such as in fig. 8iv). It is possible that the interstrand binding scheme would resist the conformational change of DNA that occurs as the humidity is varied. This would account for the significant amount of A-type diffraction occurring at 92% relative humidity, and is consistent with the dichroism results.

Our sedimentation studies show that prothidium, isometamidium and desaminoisometamidium all unwind closed circular DNA. This suggests that some form of intercalative binding is present with all three drugs. Our X-ray diffraction and dichroism studies are consistent with a sideways intercalation scheme for prothidium. The similarity in structure between all three drugs prompts the inference that they may all bind to DNA in fundamentally similar fashion, which we tentatively attribute to sideways intercalation for the isometamidium drugs as well.

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