MOLECULAR PHARMACOLOGY, 9, 544–561

The Unwinding of Circular Deoxyribonucleic Acid by Phenanthridinium Drugs: Structure-Activity Relations for the Intercalation Reaction

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Received November 26, 1973

SUMMARY

WAKELIN, L. P. G., AND WARING, M. J.: The unwinding of circular deoxyribonucleic acid by phenanthridinium drugs: structure-activity relations for the intercalation reaction. *Mol. Pharmacol.* **10**, 544–561 (1974).

The interaction between a series of phenanthridinium drugs and bacteriophage PM2 circular DNA has been studied with the object of investigating how substituents on the phenanthridine chromophore affect its capacity to intercalate between the DNA base pairs. Variations in the quaternising group at position 5 have little or no effect. The presence of a phenyl substituent at position 6 strongly influences external attachment to the DNA helix and affects the unwinding angle. Introduction of a *p*-carboxyl group on the phenyl ring leads to weaker binding. Free primary amino groups at positions 3 and 8 are not mandatory for the intercalation reaction, but their presence adds stability to the complex (approximately 1.7 kcal/mole). Removal of the 3-amino group has little effect other than a small reduction in the unwinding angle, whereas acetylation of the 8-amino group results in weaker binding as well as a lowered unwinding angle. Replacement of both 3- and 8-amino groups by carbethoxyamino substituents leads to markedly weaker binding and an apparent unwinding angle of only 5.1° ± 0.6°, the lowest value observed with any phenanthridine, together with other consequences which suggest a radical alteration in the mechanism of binding to DNA. Introduction of bromine atoms in place of amino groups at positions 3 and 8 yields a heavy-atom derivative which still unwinds the DNA helix by the same angle as ethidium at low ionic strength. Structure-activity correlations for the intercalation reaction deduced from these studies are in agreement with earlier findings relating to antimicrobial activity.

INTRODUCTION

Previous reports from this laboratory have been concerned with the ability of antibiotics and other drugs to remove and reverse the supercoils of closed circular duplex DNAs (1–3). This phenomenon, originally discovered with the intercalating drug ethidium bromide (4, 5), has been shown to provide a sensitive means of monitoring alterations in the winding of the DNA helix associated with drug binding. In particular, it has been adopted as a novel criterion for assessing whether drugs bind by an intercalation process (6–9) or not. The rationale is that all intercalation models demand some alteration in the winding of the helix (6–8), which must, provided that certain experimental requirements are satisfied,

This work was supported by grants from the Royal Society and the Medical Research Council.

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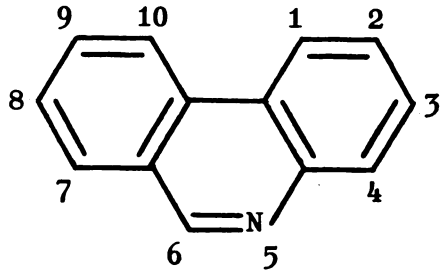
result in measurable variation of the supercoiling in closed circular duplex DNA. Theoretical and practical aspects of the method have been fully documented (1, 3, 10).

To date over 40 drugs have been examined for effects on the supercoiling of circular DNA, among which more than half are acknowledged intercalating agents (see refs. 11 and 12 for a compilation of results). In addition, two groups of substances have been discovered which affect the supercoiling of circular DNA by unknown, probably nonintercalative, mechanisms: steroidal diamines (13) and the triphenylmethane dyes crystal violet and methyl green.[1] Considerable insight has been gained from these experiments as to what constitutes a likely intercalating drug, but more precise conclusions are hampered by the diversity of structure among the drugs tested, most of them having been chosen for their medical or biological importance.

In an effort to elucidate which factors may affect the capacity of a potentially suitable chromophore to intercalate, we have undertaken a study of a homologous series of phenanthridines, all closely related to ethidium, which stands as the type-specific example of an intercalating drug. Our primary intention was qualitative: to investigate whether a particular substituted phenanthridine appeared to be capable of intercalating at all, based on the circular DNA-uncoiling test. Given a positive result, measurements of binding were made in order to compare the unwinding angle with that of ethidium. In favourable cases the data permitted calculation of binding parameters which could be compared with those of ethidium, thus providing some assessment of the effect of various substituents on the stability of the intercalated complex. In this regard we were particularly interested to test the suggestion (7) that the ethidium-DNA complex might be stabilised by the formation of hydrogen bonds from the chromophore amino groups to phosphate oxygens in the complementary DNA strands.

The compounds studied have varying substituents in positions 3, 5, 6, and 8 of the phenanthridine ring, and all are analogues of ethidium and its 5-methyl homologue, dimidium. The numbering scheme for the phenanthridine ring system is indicated below; full structural formulae are included in the appropriate sections of RESULTS, where data for each compound are presented.



MATERIALS AND METHODS

Buffers. Experiments were conducted in HEPES[2]-NaOH buffers, pH 7.0, at room temperature, and Tris-HCl buffer, pH 7.9, prepared by addition of HCl to 0.05 M Tris (resultant ionic strength, 0.036). The HEPES-NaOH buffers contained 20 mM HEPES (Calbiochem), 0.1 mM EDTA, and NaCl to yield the required ionic strength (14 mM to give ionic strength 0.02, 94 mM to give ionic strength 0.1, and 494 mM to give ionic strength 0.5). Glass-distilled water was used throughout.

Phenanthridines. Phenidium and RD 16101 were gifts from Dr. G. Woolfe of Boots Pure Drug Company, Nottingham. The other drugs, including ethidium bromide, were kindly provided by Drs. R. Slack and S. S. Berg of May & Baker, Ltd., Dagenham. All were used as supplied, without further purification, the May & Baker compounds having been assayed for solvation and checked for homogeneity by chromatography and electrophoresis in the laboratories of the supplier. In the dry state they were stored in a desiccator in the dark at 0–4°. Table 1 summarises chemical details for each drug together with the molar extinction coefficient at the maximum

[1] L. P. G. Wakelin, M. J. Waring, and R. Lovell, unpublished observations.

[2] The abbreviations used are: HEPES, *N*-2-hydroxyethylpiperazine - *N'* - 2 - ethanesulphonic acid; DMSO, dimethyl sulphoxide.

TABLE 1

Compound	Name or index No.	Molecular formula	Mol wt	Molar extinction coefficient
3,8-Diamino-5-ethyl-6-phenyl-phenanthridinium bromide	Ethidium bromide	$C_{21}H_{20}N_3^+$, Br^-, $0.42H_2O$, $0.32C_2H_5OH$	416	5900 (480 nm)
3,8-Diamino-5-methyl-6-phenyl-phenanthridinium chloride	Dimidium chloride	$C_{20}H_{18}N_3^+$, Cl^-	335.5	6200 (480 nm)
3,8-Diamino-5-methyl-phenanthridinium chloride	M&B 2421	$C_{14}H_{14}N_3^+$, Cl^-, $0.5H_2O$	268.5	4200 (470 nm)
3,8-Diamino-5-methyl-6-(*p*-carboxy-phenyl)phenanthridinium chloride	M&B 3492	$C_{21}H_{18}N_3O_2^+$, Cl^-, HCl, H_2O	434	5930 (485 nm)
3-Amino-5-methyl-6-phenyl-8-acetamidophenanthridinium lactate	M&B 4594	$C_{22}H_{20}N_3O^+$, $C_3H_5O_3^-$, $1.5H_2O$	458	5920 (455 nm)
3,8-Dicarbethoxyamino-5-ethyl-6-phenylphenanthridinium bromide	RD 16101	$C_{27}H_{28}N_3O_4^+$, Br^-	538.5	5800 (430 nm)
5-Methyl-6-(*p*-aminophenyl)-8-aminophenanthridinium chloride	Phenidium chloride	$C_{20}H_{18}N_3^+$, Cl^-	336	6170 (430 nm)
5-Ethyl-6-(*p*-aminophenyl)-8-amino-phenanthridinium chloride	M&B 3016	$C_{21}H_{20}N_3^+$, Cl^-, CH_3OH	381.5	5570 (430 nm)
5-Methyl-6-phenylphenanthridinium bromide	M&B 3427	$C_{20}H_{16}N^+$, Br^-	350	4410 (365 nm)
3,8-Dibromo-5-methyl-6-phenyl-phenanthridinium methyl sulphate	M&B 1765	$C_{20}H_{14}NBr_2^+$, $CH_3SO_4^-$, $0.5H_2O$	548	6530 (400 nm)

of the long wavelength peak in the aqueous buffers used here. The des-amino analogue of dimidium, M&B 3427, is only sparingly soluble in purely aqueous systems: its molar extinction coefficient in HEPES-NaOH buffers was therefore determined by extrapolating to zero ethanol concentration the absorption coefficient measured in various ethanol-buffer mixtures. Between 50% and 2% (v/v) ethanol concentration the molar extinction coefficient was found to be invariant.

Drug solutions were freshly prepared whenever possible and maintained in the dark at 0–4° or stored frozen at −22°.

DNA. Calf thymus DNA (highly polymerised sodium salt, type 1) was obtained from Sigma Chemical Company. Solutions containing 2 mg/ml were prepared by homogenising the DNA into buffer solution and sonicating it for 30 sec, using a medium probe and medium power level on an MSE 150-W ultrasonic disintegrator while cooling the sample in an ice-water bath. The viscosity was drastically lowered by the sonication, and the resulting solutions were clarified by filtration through glass fibre filters; they were stored frozen at −22°.

Bacteriophage PM2 and its host pseudomonad BAL-31, kindly provided by Dr. R. T. Espejo, were used to prepare PM2 DNA as described by Espejo *et al.* (14). All samples used here contained 70–90% covalently closed circles. At no stage was this DNA exposed to ethidium bromide or other DNA-binding drugs before its use in drug binding experiments. PM2 DNA concentrations are expressed in terms of the molar concentration with respect to nucleotides, assuming a molar extinction coefficient at 260 nm of 6600.

Analytical ultracentrifugation. Sedimentation coefficients were measured by boundary sedimentation at 20° and 34,000 rpm in a Beckman model E ultracentrifuge equipped with ultraviolet optics. Details of procedure and computation of S_{20} values were as previously described (1). Drug-DNA complexes were prepared by either method 1 or 2 of Waring (1): in the former a fresh complex solution is prepared for each individual ultracentrifuge run; in the latter successive increments of a drug solution in buffer are added directly to an ultracentrifuge cell containing 0.6 ml of PM2 DNA in buffer having an absorbance at 260 nm of 0.600

(i.e., 91 μM in nucleotides). The latter method is more economical, permitting reuse of the same DNA sample up to four or five times, but in either case the amount of DNA per sample is the same: 54.5 nmoles of nucleotides. As no differences attributable to the use of the different methods were detectable, the latter method was generally employed except when high drug to nucleotide ratios were required with drugs of limited solubility. In all cases the drug and the DNA were in contact for at least 30 min before the commencement of sedimentation, which was assumed to be adequate for attainment of equilibrium, since the spectral changes that occurred on binding appeared to be practically instantaneous. Occasional checks were performed by maintaining samples in the dark at room temperature for a few hours before ultracentrifugation, or by rerunning samples. Sedimentation coefficients are presented as directly determined; they have not been adjusted to correct for viscosity, buoyancy, or DNA concentration.

Spectrophotometry. Ultraviolet-visible absorption spectra of complexes were measured using a Unicam SP 1800 recording spectrophotometer. Where a clear isosbestic point or points occurred, quantitative measurements of drug binding were made by the spectrophotometric method described previously (1, 15), using a Unicam SP 500 series II spectrophotometer. The accuracy of measurement was enhanced by using 40-mm-light-path quartz semimicrocuvettes containing 3.60-ml volumes of (a) buffer, (b) sonicated calf thymus DNA, 2 mg/ml in buffer, or (c) PM2 DNA, 91 μM in buffer. In all other respects the procedure was as described in ref. 1. Wavelengths chosen for binding measurements were as follows: ethidium, dimidium, and M&B 3492, 470 nm; M&B 2421, 460 nm; phenidium and M&B 3016, 425 nm; M&B 3427, 378 nm; M&B 1765, 400 nm.

Equilibrium dialysis. Specially constructed cells (16) having two 3-ml compartments separated by a Visking dialysis membrane were loaded with 91 μM PM2 DNA in one chamber and the appropriate drug solution in the other. They were stirred to equilibrium in a water bath at 20° for 20 hr, after which the drug concentration in each chamber was determined spectrophotometrically, using 40-mm-light-path quartz semimicrocuvettes. For the free drug side the extinction coefficients listed in Table 1 were used; for the other side the complex was dissociated by addition of an equal volume of dimethyl sulphoxide, and the total drug concentration was estimated using the following molar extinction coefficients determined for 50% (v/v) buffer-DMSO mixtures: M&B 4594, 6460 (480 nm); RD 16101, 6390 (440 nm). Controls were performed to verify attainment of equilibrium in 20 hr and complete dissociation of the DNA complexes by DMSO.

Binding curves constructed from the spectrophotometric or equilibrium dialysis measurements were used to determine the binding ratio, ν (drug molecules bound per nucleotide), in the complexes subjected to ultracentrifugal analysis. Whenever possible they were also plotted in the form required by the Scatchard equation (17),

$$\frac{\nu}{c} = Kn - K\nu \qquad (1)$$

where c is the free drug concentration, K is the intrinsic association constant, and n is the number of binding sites per nucleotide. For drugs which bind to DNA such plots are typically curved (especially when circular DNA is used; see DISCUSSION), but when a reasonably linear plot was observed binding parameters were estimated from a least-squares fitted line. In two cases (M&B 3427 and M&B 1765 at ionic strength 0.1) the high ultraviolet absorption by the free drug present in excess precluded determination of ν values above about 0.1 (see Figs. 8B and 9B); here a slight extrapolation was necessary to estimate the binding at high ratios in the ultracentrifugation experiments.

While every effort was made to determine binding ratios under conditions identical with those used in the ultracentrifugation experiments, no account could be taken of possible pressure effects on the equilibrium. Such effects are known to be negligible for ethidium (18). That they are likely to be negligible for the drugs studied here is indicated by the lack of significant curvature in plots of log x vs. time, where x is the

distance from the axis of rotation, in the sedimentation experiments (1, 18).

RESULTS

A summary of the principal results is given in Table 2. It will be found convenient to refer to this table while the experimental findings with each drug are described in turn.

Substituents at position 5 (R_4). The nature of the quaternising group has little or no effect on the helix unwinding angle or on the binding constants. In Fig. 1 the effects of ethidium and dimidium on the sedimentation behaviour of PM2 DNA are compared. It is evident that the interchange of methyl and ethyl groups at position 5 makes no difference; both plots show the characteristic removal and reversal of the DNA supercoiling with an equivalence region, where closed and nicked circles cosediment as a single unresolved boundary, centred around 0.050 (ethidium) or 0.052 (dimidium) drug molecules bound per nucleotide. Thus both qualitative and quantitative aspects of the response of closed circles to the binding of the two drugs are practically indistinguishable. The same applies to the behaviour of the nicked circles, which show the typical steady decrease in S_{20} after the equivalence binding ratio is reached, as noted previously with ethidium (1, 4, 5). Helix unwinding angles for the two drugs may be compared using the equation

$$\nu_{c_1}\phi_1 = \nu_{c_2}\phi_2 \qquad (2)$$

where ν_{c_1} and ν_{c_2} are the equivalence binding ratios for each drug, and ϕ_1 and ϕ_2 are their respective unwinding angles (1). Taking ϕ for ethidium as 12° (7), the unwinding angle per bound dimidium molecule is calculated to be 11.5° ± 2.2° (Table 2), not significantly different.

As in the case of ethidium, the visible absorption peak of dimidium is depressed and shifted to longer wavelengths on binding to DNA, yielding a clear isosbestic point at 513 nm and thereby enabling spectrophotometric estimation of the binding. Both drugs were practically quantitatively bound to the DNA up to ν values of 0.06–0.07 in this low ionic strength buffer (within the limits of sensitivity imposed by the spectrophotometric assay), so that no estimates of the binding constants are available. However, it can fairly be stated that no alteration in binding due to substitution of 5-methyl for ethyl was detectable.

To these results may be added those previously reported for propidium iodide in a study of drug action on ΦX 174 replicative form DNA (1). This drug bears a quaternised aminopropyl 5-substituent (Table 2), but again no difference in unwinding angle or binding parameters was detectable as compared to ethidium.

Substituents at position 6 (R_3). Removal of the phenyl group leads to an altered unwinding angle which reflects drastic alterations in the behaviour of the drug (M&B 2421). In buffers of low ionic strength ($\leqq 0.1$) a red, gelatinous precipitate is formed when the drug solution is added to DNA, no matter how carefully the mixing is effected, and the precipitation is irreversible. At an ionic strength of 0.2 precipitation still occurs, but at low drug to nucleotide ratios there is a bathochromic and hypochromic shift in the absorption spectrum, but without an isosbestic point. In buffer of ionic strength 0.5 no precipitation occurs with a 6-fold molar excess of drug, and a similar spectral shift occurs, accompanied by an isosbestic point at 504 nm. Experiments with M&B 2421 were therefore conducted at this high ionic strength (Fig. 2).

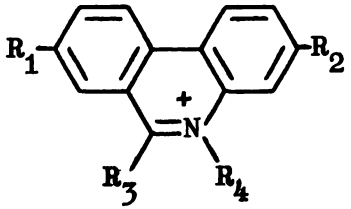
The S_{20} variation of closed circular PM2 DNA shows the fall and rise attributable to removal and reversal of the supercoiling, and comparison of the equivalence point with that for ethidium in the same buffer leads to a calculated unwinding angle of 8.3° ± 0.7° (Table 2). At the same time the S_{20} of the nicked circles exhibits the usual decrease seen with intercalating drugs. The shape of the S_{20} plot for the closed circles differs from that generally observed at low ionic strength (cf. Fig. 1) in two respects: the starting value in the absence of drug is considerably lower, and there is a perceptible rise before the sharp fall towards the equivalence point. This behaviour is entirely normal at moderate to high ionic strength (10) and has been observed before with circular DNA exposed to helix-unwinding drugs (13, 16).

The Scatchard plot for binding of this

TABLE 2

Interaction of phenanthridines with closed circular duplex DNA

The data summarise the results of the experiments with PM2 DNA reported here, together with those for propidium iodide published previously (1). Binding constants for ethidium at ionic strengths of 0.1 and below are taken from earlier work (15, 19) and refer to experiments with linear DNA; they are included for purposes of comparison with those of the other drugs at the same ionic strength. The binding constants quoted are empirical, having been calculated from the slopes and intercepts of the experimentally observed linear Scatchard plots presented in Figs. 2, 5, 8, and 9. Estimates of error in the equivalence binding ratios and apparent unwinding angles are derived from the total span of the equivalence region where closed and nicked circles cosedimented; they should be regarded as limits rather than statistical standard errors.



Compound	R_1	R_2	R_3	R_4	Ionic strength	Association constant	No. of sites/nucleotide	Equivalence binding ratio	Apparent unwinding angle
Ethidium	NH_2	NH_2	C_6H_5	C_2H_5	0.02	[2.5×10^6]		0.050 ± 0.007	[12°]
	NH_2	NH_2	C_6H_5	C_2H_5	0.036	[2.0×10^6]		0.051 ± 0.006	[12°]
	NH_2	NH_2	C_6H_5	C_2H_5	0.10	[6.6×10^5]		0.055 ± 0.005	[12°]
	NH_2	NH_2	C_6H_5	C_2H_5	0.50	1.33×10^5	0.11	0.048 ± 0.009	[12°]
Dimidium	NH_2	NH_2	C_6H_5	CH_3	0.02			0.052 ± 0.010	11.5° ± 2.2°
Propidium	NH_2	NH_2	C_6H_5	$(CH_2)_3$—N(+)(C_2H_5)—CH_3 (C_2H_5)	0.036				12.0° ± 3.4°
M&B 2421	NH_2	NH_2	H	CH_3	0.50	1.28×10^5	0.11	0.069 ± 0.006	8.3° ± 0.7°
M&B 3492	NH_2	NH_2	C_6H_4—COOH(*p*)	CH_3	0.02			0.051 ± 0.006	11.8° ± 1.4°
M&B 4594	$NHCOCH_3$	NH_2	C_6H_5	CH_3	0.02			0.074 ± 0.007	8.1° ± 0.8°
RD 16101	$NHCOOC_2H_5$	$NHCOOC_2H_5$	C_6H_5	C_2H_5	0.02	1.8×10^5	0.33	0.118 ± 0.014	5.1° ± 0.6°
Phenidium	NH_2	H	C_6H_4—NH_2(*p*)	CH_3	0.036			0.070 ± 0.006	8.6° ± 0.7°
M&B 3016	NH_2	H	C_6H_4—NH_2(*p*)	C_2H_5	0.036			0.0565 ± 0.008	10.6° ± 1.5°
M&B 3427	H	H	C_6H_5	CH_3	0.02	2.83×10^5	0.14	0.078 ± 0.013	7.7° ± 1.3°
	H	H	C_6H_5	CH_3	0.10	3.32×10^4	0.28	<0.145	>4.6°
M&B 1765	Br	Br	C_6H_5	CH_3	0.02			0.050 ± 0.009	12.0° ± 2.2°
	Br	Br	C_6H_5	CH_3	0.10	2.01×10^4	0.33	<0.092	>7.2°

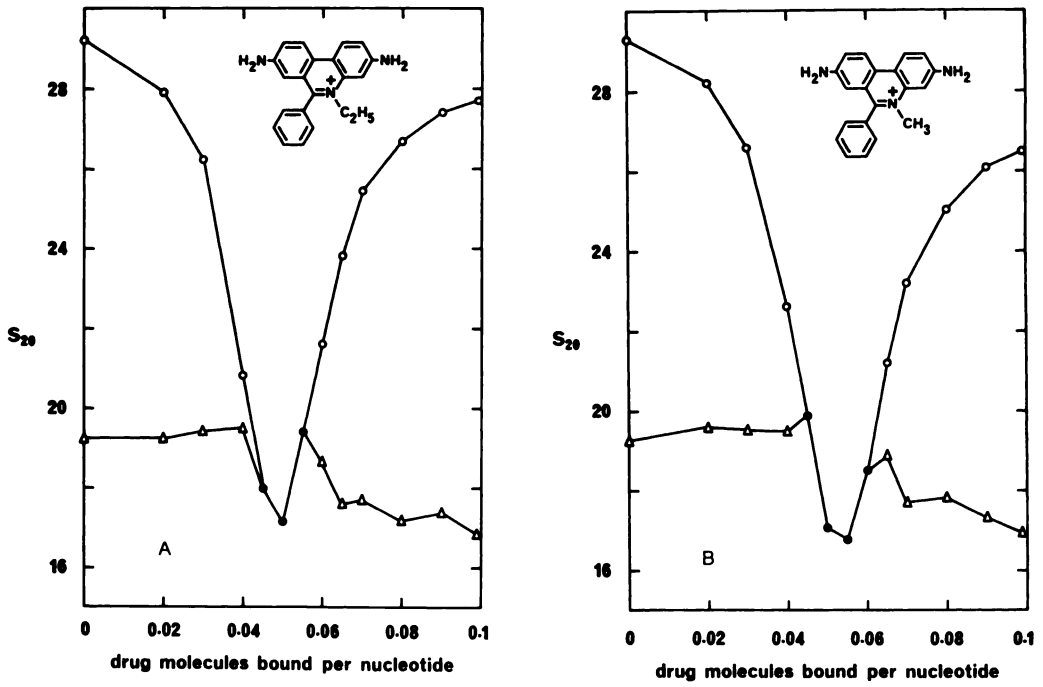


FIG. 1. *Effects of ethidium (A) and dimidium (B) on sedimentation coefficient of PM2 DNA*
The solvent was HEPES-NaOH buffer, ionic strength 0.02. The DNA preparation contained 90% closed circular molecules, allowing unambiguous identification of closed and nicked components in resolved boundaries. The abscissa shows the average level of drug binding to the two DNA components together, determined by the spectrophotometric method. ○ and △, results from resolved boundaries of closed and nicked circles, respectively; ●, weight average sedimentation coefficients from nonresolved boundaries.

drug to PM2 DNA is satisfactorily linear (Fig. 2B) and yields binding parameters almost identical with those of ethidium under the same conditions (Table 2). Clearly, then, no significant stabilisation of the intercalated complex is afforded by the presence of the 6-phenyl ring in ethidium.

Introduction of a *p*-carboxylate grouping on the 6-phenyl ring yields an amphoteric neutral molecule (M&B 3492) with consequences which are practically the converse of those just described: the unwinding angle is not affected (Fig. 3A; Table 2) but the strength of binding is substantially reduced. The binding was measured by the spectrophotometric method, because a clear spectral shift was found with an isosbestic point at 515 nm. Although the resulting Scatchard plot is decidedly curved (Fig. 3B), the binding is manifestly less tight than that of ethidium, since only 70% of the added drug was bound at the equivalence point. In fact, if the two (least accurate) points at low values of ν are neglected, a least-squares fitted line yields an approximate binding constant of 5×10^5 M^{-1}, suggesting a 5-fold lower affinity than that of ethidium. Having regard to the dipolar ionic character of the molecule, this observation accords well with earlier conclusions about the dominant role of electrostatic forces in the establishment of the intercalated complex (11, 15, 20).

Substituents at positions 8 and 3 (R_1 and R_2). Particular attention was paid to analogues possessing modifications at these positions, because of the suggestion of Fuller and Waring (7) that the 3,8-diamino groups of ethidium might form hydrogen bonds to charged oxygens of the phosphate groups of both polynucleotide strands, helping to stabilise the complex. Blocking the 8-amino

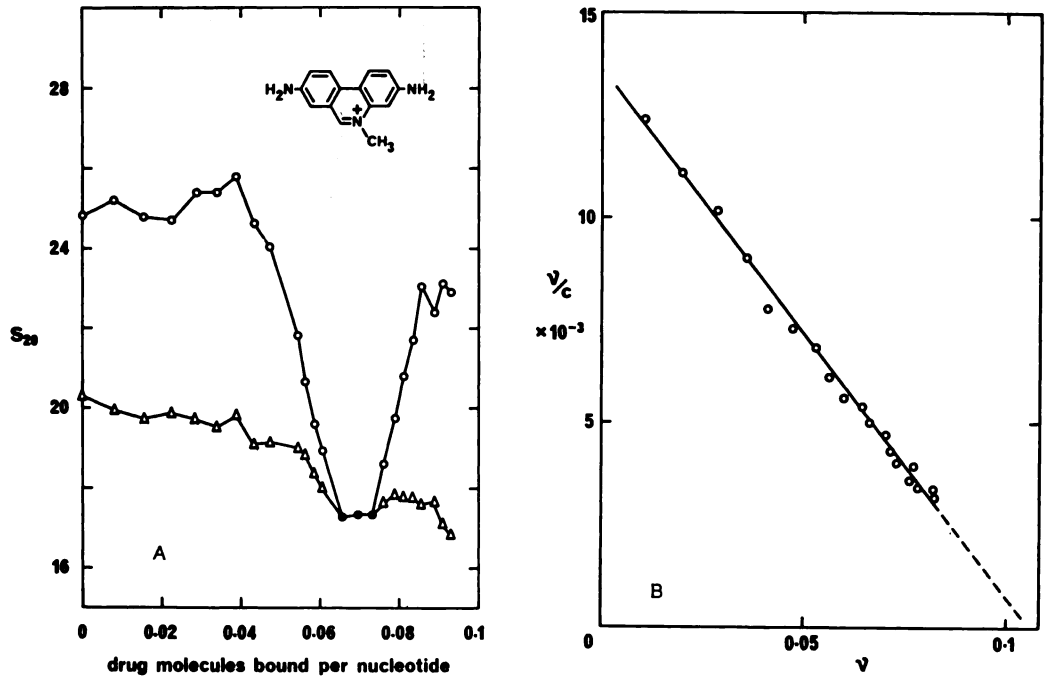


FIG. 2. *Interaction between M&B 2421 (des-phenyldimidium) and PM2 DNA*
The solvent was HEPES-NaOH buffer, ionic strength 0.50. The DNA preparation contained 85% closed circular molecules. A. Effect on the sedimentation coefficient, with symbols as described in the legend to Fig. 1. The abscissa represents the average level of drug binding to the two DNA components together, taken from spectrophotometric measurements presented in the form of a Scatchard plot (B).

group by acetylation (M&B 4594) results in a lowered unwinding angle (Fig. 4), and the binding constant falls somewhat. Although this derivative shows a bathochromic and hypochromic shift in the long wavelength peak of the drug spectrum on binding to DNA, there is no evidence of an isosbestic point in buffer of ionic strength 0.02. The binding was therefore determined by equilibrium dialysis. While insufficient data were available to construct a satisfactory Scatchard plot, an approximate binding constant of 1.3×10^6 M^{-1} was calculated, indicating about half the binding affinity of ethidium.

When both amino groups are blocked (RD 16101, having carbethoxyamino groups at both positions 3 and 8) the unwinding angle is reduced to 5.1°, the lowest value observed for any of the phenanthridines, and the binding constant falls more drastically, to a value 14-fold lower than that of ethidium (Fig. 5; Table 2). Like the monoacetyl derivative just mentioned, this drug also shows a bathochromic and hypochromic spectral shift on binding to DNA, but no isosbestic point appears. Equilibrium dialysis was therefore employed to determine the binding (Fig. 5B). Over the range covered by the sedimentation experiments the Scatchard plot is reasonably linear, although a few points at higher drug to nucleotide ratios (not shown) indicated the existence of secondary binding sites which become populated at ν values above about 0.3. It is noteworthy that the linear portion of this Scatchard plot extrapolates to the ν-axis, yielding an apparent number of "strong" binding sites of 0.33/nucleotide. While a precise number for ethidium binding to PM2 DNA in this buffer cannot be given, it is certain that there are more sites available to the dicarbethoxy derivative, probably 2–3 times as many.

Removal of the 3-amino group appears to reduce the unwinding angle somewhat, although the binding remains quite strong.

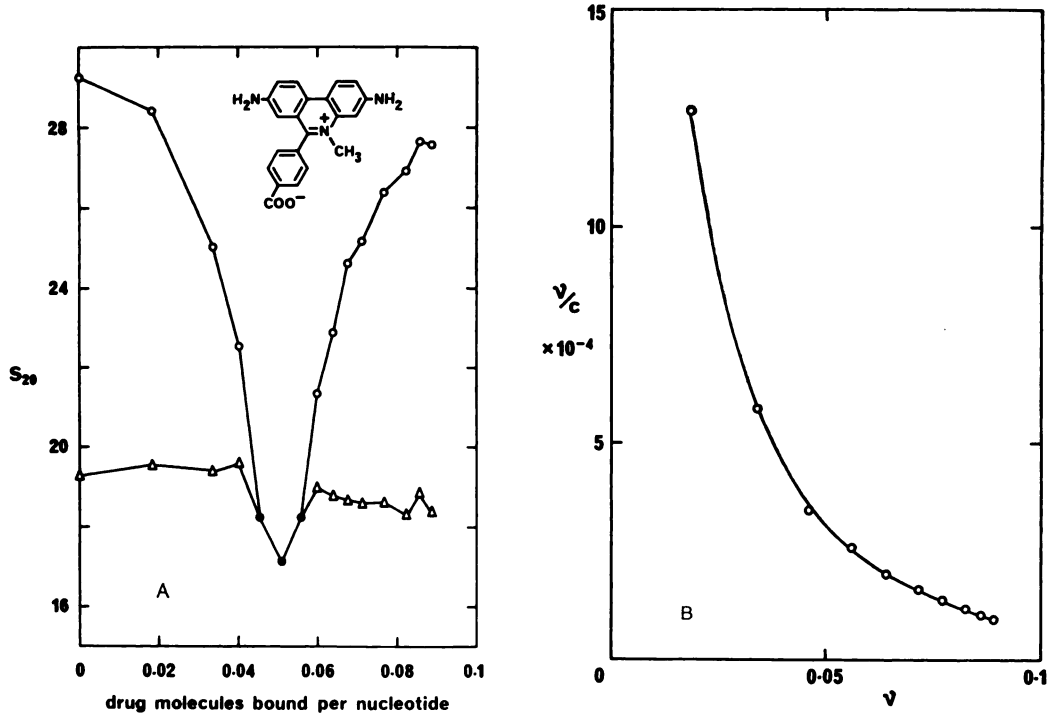


FIG. 3. *Interaction between M&B 3492 and PM2 DNA*

The solvent was HEPES-NaOH buffer, ionic strength 0.02. The DNA preparation contained 80% closed circular molecules. A. Effect on the sedimentation coefficient, with symbols as described in the legend to Fig. 1. The abscissa represents the average level of drug binding to the two DNA components together, taken from spectrophotometric measurements presented in the form of a Scatchard plot (B).

Two such compounds were investigated: phenidium and M&B 3016, which differ in the nature of the quaternising 5-substituent (Fig. 6). The spectra of both drugs showed similar shifts on binding to DNA, each having two isosbestic points in the regions of 370 and 460 nm. The spectrophotometric binding data gave curved Scatchard plots, precluding the determination of exact binding parameters, but it was clear that the strength of interaction was little different, if at all, from that of ethidium and dimidium. Both drugs bear a *p*-amino substituent on the 6-phenyl ring, but in the light of our results on the effect of 6-substituents described above it can be assumed that the presence of this aromatic amino group (uncharged at pH 7.9) is relatively inconsequential. The general shape of the S_{20} plots for both closed and nicked circles (Fig. 6) is exactly as expected for intercalating drugs, and although the calculated unwinding angles are not identical, the estimated error limits overlap (Table 2).

Complete removal of both primary amino groups results in a definitely lower unwinding angle and a 10–20-fold reduction in the binding constant. This compound, des-aminodimidium (M&B 3427), is of special interest because of the opportunity it affords to investigate directly the role of 3,8-diamino substituents in the formation of an intercalated complex. It exhibits a well-defined spectral shift in the presence of DNA, with an isosbestic point at 387 nm (Fig. 7A), enabling spectrophotometric measurement of binding. At low ionic strength (0.02) its binding to PM2 DNA affects the sedimentation coefficient in a manner identical with that caused by binding of ethidium or dimidium, with an equivalence region corresponding to an unwinding angle of 7.7° ± 1.3° (Fig. 8A, filled symbols). The Scatchard plot is linear and yields a binding constant

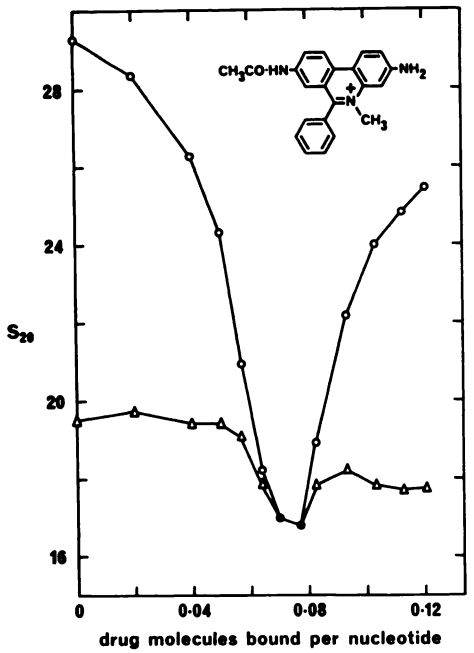


FIG. 4. *Effect of M&B 4594 on sedimentation coefficient of PM2 DNA*

The solvent was HEPES-NaOH buffer, ionic strength 0.02. The DNA preparation contained 85% closed circular molecules. Symbols are described in the legend to Fig. 1. The abscissa represents the average level of drug binding to the two DNA components together, determined by equilibrium dialysis.

10-fold lower than that of ethidium (Table 2). In buffer of ionic strength 0.1 a significant difference becomes apparent, however. When the drug is added, the S_{20} of the closed circles falls to a minimum, reflecting removal of the right-handed supercoils originally present, but fail to rise again (Fig. 8A, open symbols). The binding is evidently too weak to enable intercalation of sufficient drug molecules to generate reversed supercoils. The problem is largely experimental, the range of accessible drug to nucleotide input ratios being limited by the ultraviolet absorption of the free drug in equilibrium with the complex. The Scatchard plot in Fig. 8B shows that in this buffer the binding constant has fallen to 3.32×10^4 M^{-1}, almost exactly 20 times lower than that for ethidium under the same conditions (Table 2). However, it is clear that a considerable change in the helix-unwinding angle must have occurred, and an upper limit of 4.6° per bound drug molecule can be calculated from the binding ratio at the onset of the presumed equivalence region.

Replacement of the amino groups at positions 3 and 8 by bromine atoms yields a derivative (M&B 1765) which is still capable of removing and reversing the supercoiling of PM2 DNA, with the same unwinding angle as ethidium and dimidium (Fig. 9A). In the low ionic strength buffer (0.02) it produces all the usual effects on the sedimentation of closed and nicked circles, with an equivalence point identical with that of ethidium. Spectral analysis reveals clear isosbestic points at 365 and 418 nm (Fig. 7B), and a Scatchard plot derived from the binding measurements is presented in Fig. 9B. It is too obviously curved to allow calculation of binding parameters, but the interaction must be substantially weaker than that of ethidium or dimidium, because only 70% of the added drug was bound to the DNA at the equivalence point. At ionic strength 0.1 the same behaviour noted for the des-amino derivative of dimidium was found: the supercoiling of the closed circles was removed but could not be reversed at accessible drug concentrations (Fig. 9A, open symbols), indicating a lowering of the unwinding angle to some value not greater than 7.2°. The binding constant, derived from the linear Scatchard plot in Fig. 9B (open symbols), was still lower than that for the des-amino compound (Table 2).

The failure of the 3,8-des-amino and dibromo analogues to generate reversed supercoils in closed circular molecules at ionic strength 0.1 is not due to the occurrence of nicking in the presence of these drugs. At all ratios where the sedimenting boundary of closed circles was resolved, the proportion of these intact molecules as a fraction of the total DNA remained constant, reflecting that of the drug-free DNA preparation employed.

Other phenanthridines. A few additional experiments were performed with prothidium, isometamidium (for formulae and descriptions, see ref. 21), and a des-amino derivative of isometamidium. All three drugs bear large, positively charged heterocyclic substituents attached to the 8-amino group

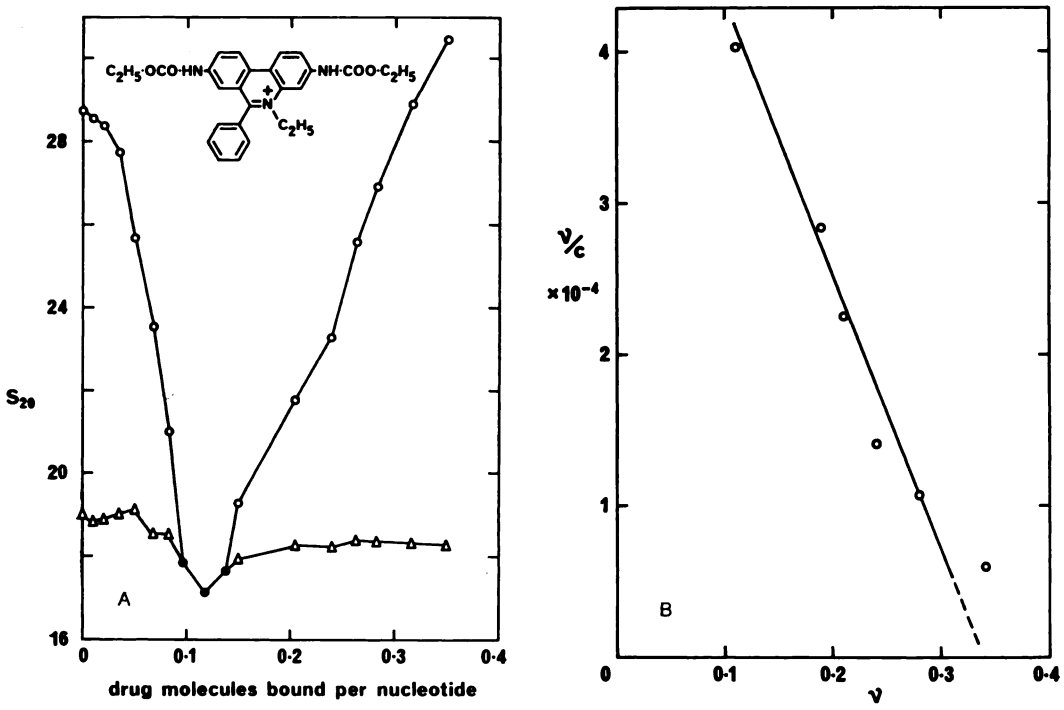


FIG. 5. *Interaction between RD 16101 and PM2 DNA*

The solvent was HEPES-NaOH buffer, ionic strength 0.02. The DNA preparation contained 70% closed circular molecules. A. Effect on the sedimentation coefficient, with symbols as described in the legend to Fig. 1. The abscissa represents the average level of drug binding to the two DNA components together, taken from equilibrium dialysis measurements presented in the form of a Scatchard plot (B).

of the phenanthridine ring system; all were found to remove and reverse the supercoiling of closed circular duplex DNA. Because of difficulties in measuring the binding of these drugs to DNA, the observations were not pursued further, but the fact that their interaction with DNA leads to unwinding of the helix is worthy of note and agrees with our observations on the two amino-substituted drugs reported above (Figs. 4 and 5). An attempt to investigate the effect of an ethidium analogue bearing nitro groups in place of the amino groups at positions 3 and 8 was frustrated by its (reversible) formation of an insoluble pseudobase at pH values above about 3.

DISCUSSION

The most important conclusion to be drawn from the present work is that quite drastic modifications can be made to the structure of ethidium without abolishing its intercalative potential. Of 14 phenanthridines studied, none was found which failed to remove and reverse the supercoiling of PM2 DNA, the only near failures being the des-amino and 3,8-dibromo analogues of dimidium at an unfavourably high ionic strength. Yet even these drugs gave a perfectly normal response at ionic strength 0.02. In fact, the discovery that the dibromo analogue (M&B 1765) can form an intercalation complex having the same geometical characteristics as the ethidium complex may well prove important in future studies of intercalation where a heavy-atom derivative is advantageous.

The next conclusions concern the effect of various substituents on the character and stability of the intercalated complex. The nature of the quaternising group at position 5 appears to be practically immaterial. The role of the 6-phenyl substituent is probably best shown by the precipitation phenomena

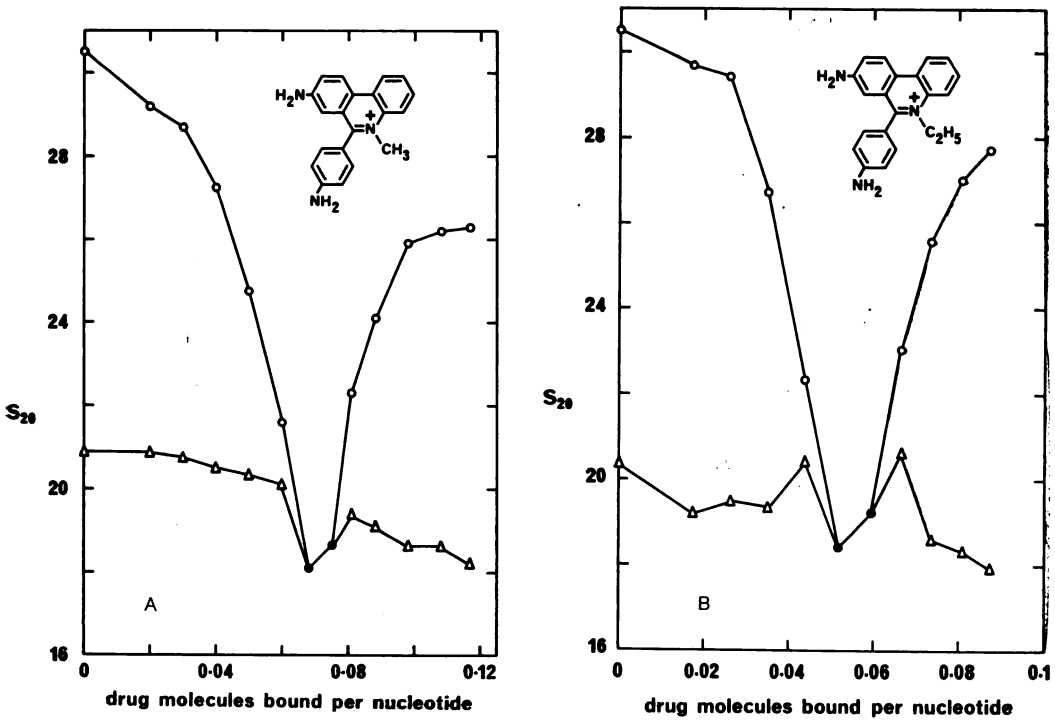


FIG. 6. *Effects of phenidium (A) and M&B 3016 (B) on sedimentation coefficient of PM2 DNA*
The solvent was 0.05 M Tris-HCl buffer, pH 7.9 (ionic strength 0.036). The DNA preparation contained 85% closed circular molecules. Symbols are described in the legend to Fig. 1. The abscissa shows the average level of drug binding to the two DNA components together, determined by the spectrophotometric method.

which occur if it is missing. The precipitates undoubtedly consist of electrically neutral 1:1 complexes in which much of the drug is in the form of stacked aggregates attached to the surface of the DNA helix (see ref. 22). Lacking the out-of-plane phenyl ring, des-phenyldimidium (M&B 2421) is virtually a completely planar molecule and hence can form stacked aggregates much more readily than ethidium or dimidium. Since the formation of secondary (stacked) complexes is known to be highly sensitive to increases in ionic strength (15, 22), the precipitation phenomena are readily explicable, as is the fact that ethidium will only form precipitates quantitatively under practically salt-free conditions (15). Thus the main effect of the 6-phenyl ring seems to be concerned with the distribution of bound drug between an intercalated complex and an externally stacked one.

More information is available concerning the role of the 3,8-diamino substituents. In particular, it is now clear that they are by no means mandatory for the intercalation reaction. One, the 3-amino group, can be removed with no apparent effect other than a small reduction in the unwinding angle (Fig. 6; Table 2). The other, the 8-amino, can be blocked by acetylation (Fig. 4) or substitution with a much larger grouping without major effect on the interaction. Only when both are blocked are the consequences considerable (Table 2), although still not sufficient to prevent removal and reversal of the supercoiling (Fig. 5). The behaviour of this derivative makes interesting comparison with that of a di-*tert*-butyl derivative of proflavine, which has recently been shown to differ markedly from its parent compound, proflavine, not least in that it seems to bind to DNA by a nonintercalative mechanism (16). The carbethoxy substituents of our derivative (RD 16101) are almost as bulky

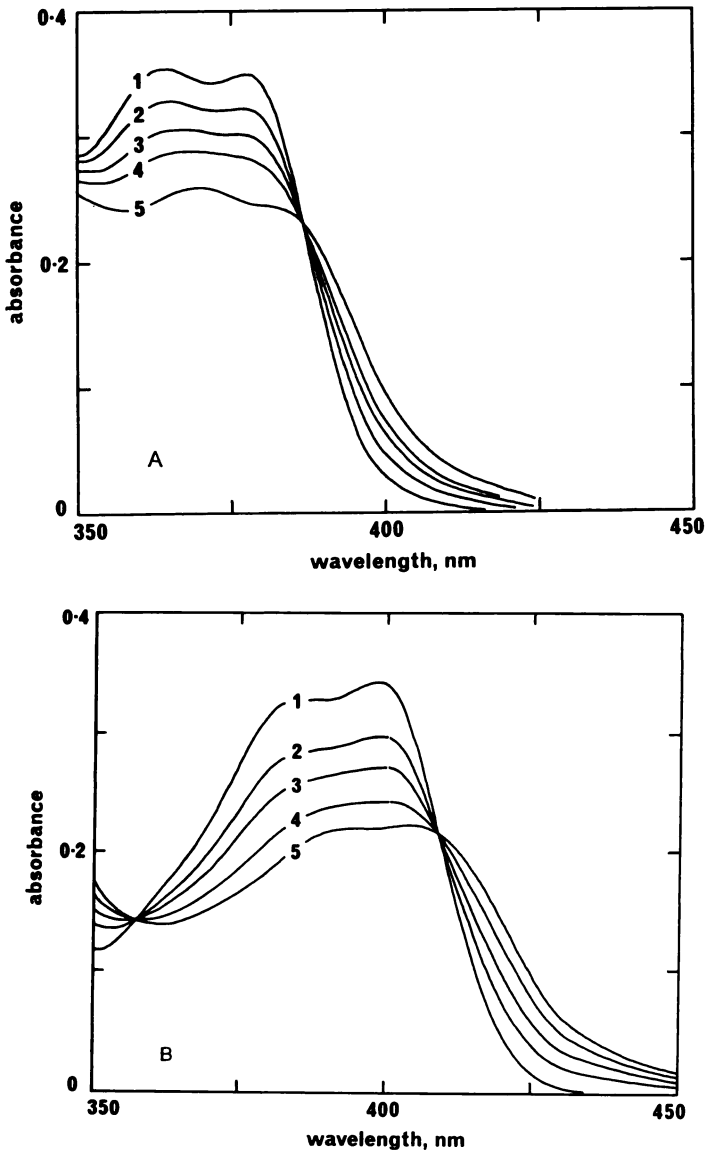


FIG. 7. *Absorption spectra of M&B 3427 (A) and M&B 1765 (B) in the presence of calf thymus DNA*
The solvent was HEPES-NaOH buffer, ionic strength 0.02. The concentration of M&B 3427 was 80.5 μM, with DNA added to the following final concentrations: curve *1*, zero; curve *2*, 44.4 μg/ml; curve *3*, 88.7 μg/ml; curve *4*, 133 μg/ml; curve *5*, 1 mg/ml. M&B 1765 was used at 52.8 μM, in the absence of DNA (curve *1*) or with DNA added to 50 μg/ml (curve *2*), 100 μg/ml (curve *3*), 200 μg/ml (curve *4*), and 1 mg/ml (curve *5*).

as the *tert*-butyl substituents of the proflavine analogue; yet the unwinding effect persists. This observation may point to a fundamental difference between the mechanisms of intercalation by proflavine and ethidium. In any event there are several peculiarities in the DNA-binding behaviour of RD 16101 which suggest that its interaction with DNA may differ substantially from intercalation by ethidium: the helix-unwinding angle is much lower; the shape of the dip in the S_{20} curve for closed circular

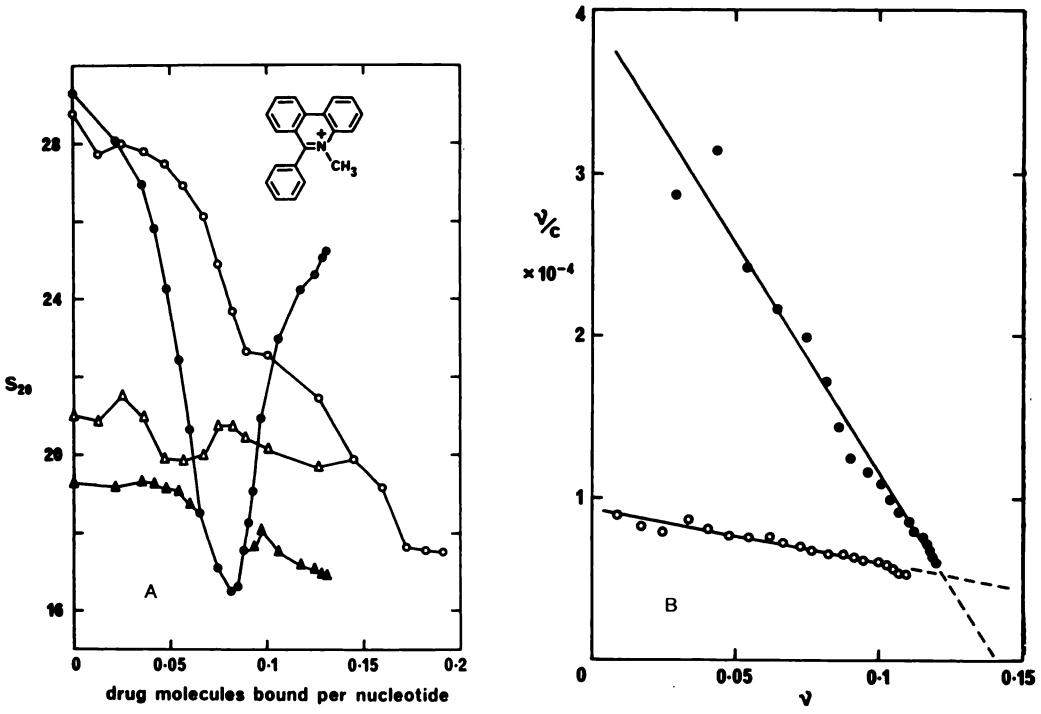


FIG. 8. *Interaction between M&B 3427 (des-amino dimidium) and PM2 DNA*

The DNA preparations contained 90% closed circular molecules. A. Effect on the sedimentation coefficient at ionic strength 0.02 (▲, ●) and 0.10 (△, ○); the S_{20} of closed circular molecules is shown by circles, and that of nicked circular molecules by triangles. No special symbol is used to indicate the S_{20} of unresolved boundaries; they are shown as ● and ○. The abscissa represents the average level of binding to the two DNA components together, taken from spectrophotometric measurements presented in the form of Scatchard plots (B), where ● corresponds to ionic strength 0.02 and ○ corresponds to ionic strength 0.10.

PM2 DNA appears anomalous, especially at high binding ratios (Fig. 5A); the reduction in S_{20} of nicked circles is minimal; the spectral shift in the presence of DNA lacks an isosbestic point; and the number of binding sites (determined by equilibrium dialysis) is surprisingly high. Experiments with molecular models suggest that the bulky nature of the dicarbethoxyamino substituents would indeed prevent intercalation in the normal manner, with maximal overlap of the chromophore and the DNA base pairs, as proposed for proflavine and ethidium (6, 7). Accordingly, the possibility that RD 16101 intercalates "sidewise" (23) or otherwise modifies the intercalation site must be seriously entertained. It would be particularly interesting if the high number of binding sites available to this drug betokened freedom from the neighbour-exclusion principle believed to restrict binding by established intercalating agents (11).

The most direct assessment of the role of 3,8-diamino groups on the phenanthridine chromophore comes from our studies with M&B 3427, which lacks them altogether. As a result the binding is weaker than that of ethidium by a factor of 10 or 20 at ionic strength 0.02 and 0.10, respectively. The only uncertainty lies in the exact magnitude of the binding constants for dimidium, the strict 3,8-diamino analogue of M&B 3427. If the binding constants for ethidium given in Table 2 are applicable to dimidium—and there is every indication that this is so—then the increased free energy of complex formation attributable to the presence of 3,8-diamino groups is, in the extreme case at ionic strength 0.10, only 1.7 kcal/mole. Thus, if hydrogen bonds are formed from

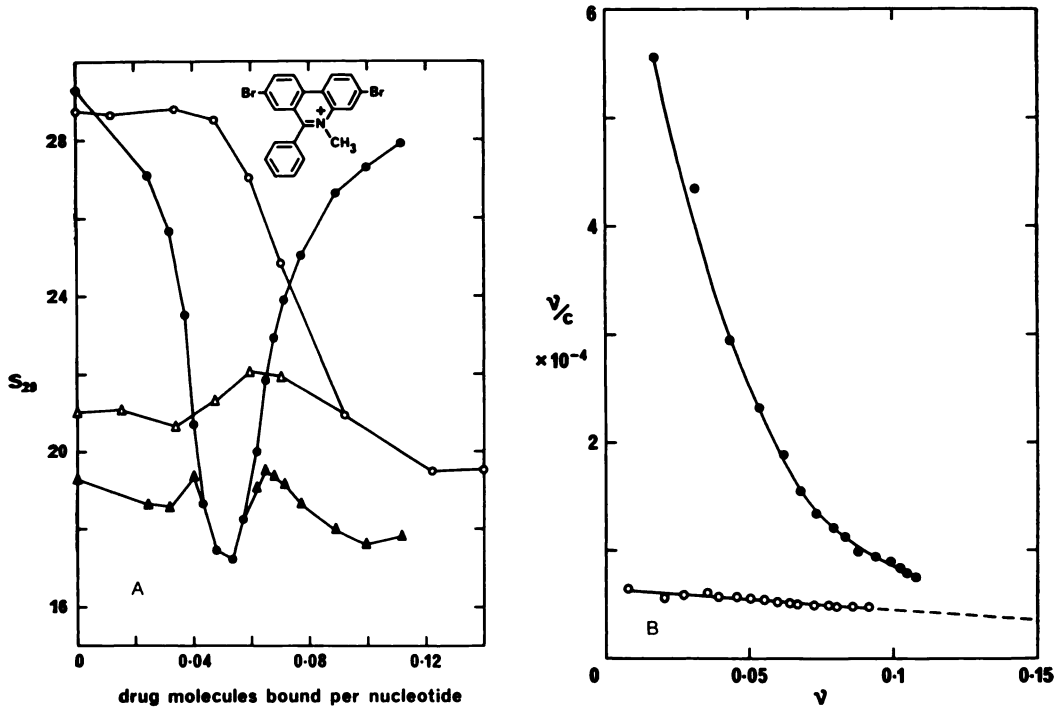


FIG. 9. *Interaction between M&B 1765 and PM2 DNA*

The DNA preparations contained 90% closed circular molecules. A. Effect on the sedimentation coefficient. B. Corresponding Scatchard plots. Symbols used for results at ionic strength 0.02 and 0.10 are described in the legend to Fig. 8. The binding was determined by the spectrophotometric method.

these amino groups, as suggested by Fuller and Waring (7), they are relatively weak, contributing only 0.85 kcal/mole each to the stability of the intercalated complex. They could, however, have an important influence on the orientation of the drug molecule within the intercalation site. Alternatively, the enhanced free energy change may be interpreted in other ways. (a) Chemical evidence indicates that the 3-amino group of ethidium and dimidium is uniquely refractory to attack by electrophilic reagents (24), which is evidence for delocalisation of the positive charge from the 5-nitrogen to the 3-amino nitrogen by conjugation around the phenanthridinium chromophore. This would have the effect of placing the positive charge nearer the negatively charged phosphate oxygens, leading to increased electrostatic interaction and lowering the energy of the system. However, we have shown that removal of the 3-amino group does not noticeably affect the binding. (b) The added stability might be due to enhanced stacking interaction between the chromophore and the DNA base pairs. Partial donation of the lone pairs of the amino nitrogens into the conjugated ring system would increase the π-electron density around the chromophore and increase its electron-donating capacity. The same criticism stated under (a) applies. (c) The presence of the amino groups in ethidium and dimidium facilitates hydrogen bond interaction with water molecules in solution; witness their much higher solubility than the des-amino derivative. On complex formation with DNA, the increase in entropy of the solvent system might consequently be greater for the diamino compounds; hence the larger free energy change. Entropic terms are known to be important in the energetics of the intercalation reaction for ethidium (19). More detailed studies with the des-amino derivative

and other 3,8-substituted phenanthridines, such as the dibromo compound, especially investigation of the thermodynamics of their binding to DNA, would help to distinguish between these various possibilities.

Where unbracketed binding parameters are listed in Table 2 they were derived from Scatchard plots which were linear over most if not all of the range covered by the measurements (Figs. 2B, 5B, 8B, and 9B). The curvature seen in other cases (cf. Figs. 3B and 9B) could result from a variety of causes but is probably due in most instances to the superhelical nature of the PM2 DNA, which affects the shape of binding isotherms because of the changes in superhelix free energy as the level of drug binding rises (10). Curved Scatchard plots were always observed with drugs which bound tightly to the DNA, consistent with this interpretation.

The estimated helix-unwinding angles given in Table 2 were all calculated from Eq. 2 by comparison with ethidium, assuming that each bound ethidium molecule unwinds the helix by 12°. This angle was proposed in 1964 on the basis of molecular model-building studies (7), and as yet no direct experimental proof of its validity has been forthcoming. However, it is widely accepted as a provisional estimate (1–5, 9–13), and such evidence as is available supports the conclusion that it is not seriously in error in sense or magnitude (10, 11, 25). If at some future date this angle is revised, the estimates in Table 2 can be adjusted accordingly. In any event, the conclusions from the present work will not be affected, since they depend only on relative angles for derivatives as compared to that of ethidium.

A more pressing problem concerns explanations for the lower unwinding angles of several derivatives shown in Table 2. In earlier work (1, 3) it was suggested that unwinding angles *per bound drug molecule* (ϕ) lower than 12° might most easily be explained if some fraction of the bound molecules were not in an intercalated state at equilibrium, and that the fraction intercalated might be simply proportional to $\phi/12°$. According to this line of reasoning the presence of a 6-phenyl substituent and unmodified 3,8-diamino groups favours binding in the intercalated state, whereas removal of the phenyl group or interference with the primary amino groups of the chromophore leads to a higher proportion of outside binding, either by facilitating external stacking or by rendering the geometry of the intercalated complex less favourable. This interpretation remains the simplest consistent with the available evidence, but it is by no means the only possible one. Drug-induced structural perturbations of the DNA helix other than mere unwinding, such as movement of base pairs with respect to the helix axis, base pair tilting, or interference with rotational and conformational parameters of the sugar-phosphate backbones, may ultimately have to be considered. Already we are faced with one observation which may demand a novel explanation, i.e., the apparent variation in unwinding angle as a function of ionic strength for the des-amino and dibromo analogues of dimidium (M&B 3427 and 1765; Table 2). This behaviour is quite different from that of ethidium, whose equivalence point for PM2 DNA varies little in the ionic strength range 0.01–0.5 (Fig. 10). At present we are at a loss to account for this variation in ϕ, but if it represents a change in the ratio of drug molecules bound externally as opposed to intercalated, the shift is certainly large.

The values of ν_c for ethidium binding to PM2 DNA determined here (Fig. 10; Table 2) are in satisfactory agreement with values determined by other workers under comparable conditions of salt concentration (26–28), although lower than those reported for 1.5 M CsCl [ν_c = 0.06 (29)] and 2.83 M CsCl [ν_c = 0.08 (30)]. The higher values found in CsCl no doubt reflect the different nature of the salt and the much higher ionic strength. The slight decrease in ν_c with increasing ionic strength implied by our least-squares fitted line in Fig. 10 is not statistically significant, and, since the data over only a limited range of salt concentrations, they do not necessarily contradict the conclusions of earlier workers (30, 31). Smit and Borst (27) and Böttger and Kuhn (26) also found a somewhat complex dependence of ν_c on ionic strength for PM2 DNA.

Finally, it is instructive to compare our

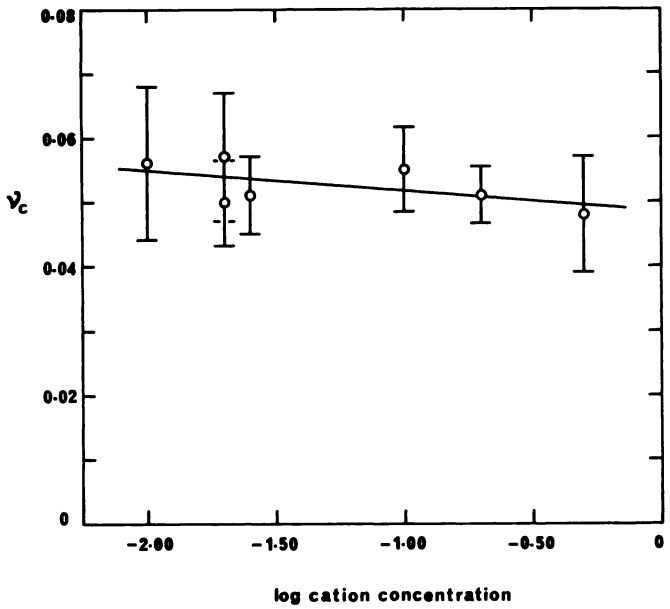


FIG. 10. *Equivalence point for unwinding of supercoiling of PM2 DNA by ethidium as a function of ionic strength*

Error bars indicate the total span of the equivalence region, where closed and nicked circles cosediment as an unresolved boundary, for each determination. The straight line is a least-squares fit to the equation $\nu_c = k(\log_{10} \text{cation concentration}) + \text{const.}$

conclusions with those derived from tests of toxicity and therapeutic efficacy on a large number of phenanthridines nearly 20 years ago (32, 33). In those tests, involving measurements of antibacterial activity as well as toxicity towards trypanosomes, the importance of an aromatic substituent at position 6 as well as free primary amino groups at positions 3 and 8 was noted. While we would hesitate to claim that our studies have elucidated the molecular basis of action of phenanthridines *in vivo*, these drugs are nevertheless believed to inhibit the growth of trypanosomes and bacteria by blocking nucleic acid synthesis as a consequence of their binding to DNA (11, 34, 35). So far as our data on structure-activity relations for the intercalation reaction go, they are in good agreement with those which govern the activity of these drugs *in vivo*.

ACKNOWLEDGMENTS

We thank Mr. N. F. Totty for skilled technical assistance, and many colleagues for helpful advice and criticism. We are especially grateful to Drs. R. Slack, S. S. Berg, and G. Woolfe for generous supplies of the drugs, together with precise analytical data on their chemical constitution.

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