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The Binding of the Anthelmintic Pyrvinium Cation to Deoxyribonucleic Acid *In Vitro*

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SUMMARY

Pyrvinium pamoate, an anthelmintic drug with mutagenic activity, binds to duplex DNA *in vitro*. Binding markedly enhances the intrinsic fluorescence of the drug, permitting a characterization of the binding reaction to be performed directly through fluorimetric analysis. The monopyrvinium salt, pyrvinium iodide, binds cooperatively to native calf thymus DNA with an intrinsic binding constant of 1.1×10^4 M^{-1} as determined fluorimetrically and 1.4×10^4 M^{-1} as determined by equilibrium dialysis. The respective binding site was estimated to be 2.5 base pairs. A change in the absorption of bound drug as a function of the binding ratio identifies secondary, nonfluorescent binding which is essentially ionic in character. The binding of pyrvinium removes negative supercoils from PM2-ccc-DNA with an experimental efficiency greater than that observed for the intercalating drug chloroquine. Electron microscopy shows that molecules of pBR322 DNA treated with pyrvinium increase in length by as much as 18% over controls, consistent with an increase in DNA base pair separation upon binding. The evidence indicates that the primary interaction of pyrvinium with DNA involves intercalation.

Pyrvinium pamoate (Fig. 1) is widely used in North America as the drug of choice in the treatment of enterobiasis, a pinworm infestation. Its therapeutic use supercedes that of the more toxic monopyrvinium compound, pyrvinium chloride. While two administrations of a suspension of pyrvinium pamoate, once upon diagnosis and again 2 weeks later, is normally effective in treating the infestation, the drug is known to be used in Mexico and certain southern states as part of a continuous prophylactic regimen.

Pyrvinium pamoate is thought to interfere with the respiratory enzyme systems which are essential to helminths but nonexistent in mammalian tissues (1). However, pyrvinium compounds effectively induce a variety of mutations, including base substitutions, frameshift mutations, and chromosomal breakage events, in yeast (2, 3), and bacterial test systems (3–5). Human toxicity is limited by the drug's insolubility in water and its very poor absorption by the gut. Exposure of the gut to concentrated doses of pyrvinium would appear, though, to impart to this tissue the highest possible risk of genotoxic damage caused by the drug. Pursuant to an investigation of the potential for such an effect in mammals, the present study was undertaken to characterize the interaction between the pyrvinium cation and DNA *in vitro*. An interaction at the molecular level was indicated by changes observed in the spectral parameters for pyrvinium pamoate fluorescence upon the addition of duplex DNA to drug solutions.[1] The phenomenon is reminiscent of the behavior of ethidium bromide, which binds to DNA through intercalation (6).

Pyrvinium is a substituted quinoline, positively charged by virtue of a quaternary nitrogen at the N-1 position. The antimalarial agent chloroquine is a related quinoline derivative which is also a frameshift mutagen (7). Chloroquine binds to DNA through the intercalation of the quinoline residue (8). A planar, aromatic ring system is the essential attribute of molecules which intercalate, and a positive charge significantly stabilizes the interaction (9, 10). Intercalators invoke a number of biologically interesting effects beyond their action as frameshift mutagens (11), such as the inhibition of DNA replication and the promotion of recombination (10, 12). Much current interest is being focused on the molecular aspects of intercalating drugs, particularly how they determine mutagenicity and cytotoxic action (10, 12–14). Consequently, it is compelling to know if the binding of pyrvinium to DNA involves this mechanism and, if so, how effective the association is.

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[1] D. F. Gerson, personal communication.

ABBREVIATIONS: CT, calf thymus; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; EDTA, ethylenediaminetetraacetate; 0.01 SHE, low ionic strength buffer containing 2 mM HEPES, 10 μM EDTA, 9.4 mM NaCl, pH 7.0; 0.1 SHE, high ionic strength buffer; PM2-ccc-DNA, covalently closed, circular PM2 phage DNA; pBR322, circular plasmid DNA; DMSO, dimethylsulfoxide; DU, digitizer unit.

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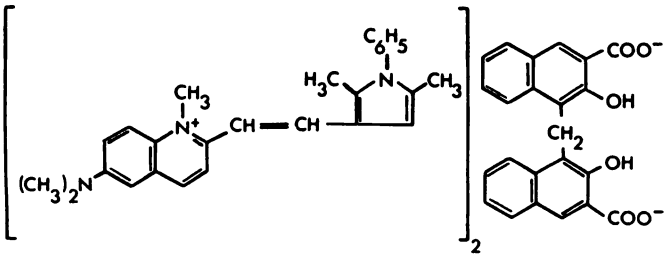

Fig. 1. Chemical structure of pyrvinium pamoate (M, 1151.44). Pyrvinium iodide (M, 509.45) is a simple salt of the pyrvinium cation outlined by the *square brackets*.

The sensitivity of fluorimetry ideally suits the technique for the study of the binding of the sparingly soluble pyrvinium compounds to DNA. It has been possible to define certain binding parameters in terms of the fluorimetric behavior of the drug. The accuracy and interpretation of these parameters are substantiated by the analyses of equilibrium dialysis and absorption spectroscopy. Measurement of the ability of pyrvinium to unwind supercoiled DNA and to linearly extend duplex DNA molecules has led to the conclusion that the primary interaction of pyrvinium compounds with duplex DNA involves intercalation. Intercalation defines a molecular process which may help explain why pyrvinium displays the broad spectrum of biological activity that it does.

Experimental Procedures

Materials

Drugs. Pyrvinium pamoate (medical grade, lot #C434662) was donated by Parke-Davis Canada Ltd. Pyrvinium iodide was custom synthesized by Lancaster Synthesis Ltd. (England). Chloroquine diphosphate and ethidium bromide were purchased from Sigma Chemical Co.

Nucleic acids, enzymes, and buffers. CT DNA (type 1, Sigma Chemical Co.) was dissolved in 2.5 M NaCl to 1 mg/ml and sheared by repeated passage through a 26 gauge syringe needle (Yale) at 0°. Sheared DNA was dialyzed extensively against a low ionic strength buffer of 2 mM HEPES, 10 μM EDTA, 9.4 mM NaCl, pH 7.0 (0.01 SHE). High ionic strength buffer (0.1 SHE) was prepared by the further addition of solid NaCl to 99.4 mM. CT DNA was denatured by heating in 0.01 SHE (15). The isolation of PM2-ccc-DNA has been described elsewhere (16). DNA concentrations, in all cases, were estimated from measurements of absorption at 260 nm, assuming an extinction coefficient of 6600.

CT topoisomerase was prepared as described (16). It was stored in 50% glycerol at −20° until used. One microliter was used to relax 2.5 μg of PM2-ccc-DNA in 10 min at 37°. The pBR322 plasmid was a gift from Dr. Joel Weiner, Department of Biochemistry, University of Alberta. It was partially digested with *Pst*1 restriction enzyme purchased from Bethesda Research Laboratories Inc. (Gaithersburg, MD). Ribosomal bovine liver RNA was purchased from Sigma Chemical Co.

Spectral Titrations

Absorption spectra derived from the titration of pyrvinium iodide with DNA were performed according to the procedure described by Dougherty (17). Reactions were performed in 1-ml volumes of high and low ionic strength buffers. The initial concentration of DNA was 260 μM. Decreasing concentrations of DNA were achieved by removing a specific volume of the reaction mixture and replacing it with buffer containing the drug only, or without the drug in the case of the blank. Pyrvinium iodide was diluted 1:200 into buffer from DMSO stock solutions to a final and constant concentration of 16 μM. Blanks received an equal volume of DMSO. The DNA and drug were mixed in cuvettes of a 1-cm path length. Spectra were recorded with a Perkin-Elmer 559A Spectrophotometer.

The fluorimetric titration of DNA with pyrvinium was performed in separate test tubes containing various amounts of the drug which had been dissolved in DMSO and diluted 1:200 into assay buffer. Fluorescence was recorded before and after the addition of equal amounts of DNA to the tubes. Blanks received DMSO without the drug. Mixtures were excited at 465 nm and the fluorescence was monitored at 570 nm with a Turner Spectrofluorimeter 430.

Estimations of Binding Affinity

Fluorimetric data were interpreted using the mathematical treatment described by Le Pecq and Paoletti (6) for determining binding equilibria. Scatchard plots were derived from the computed values of $[C]b$, the concentration of bound drug:

$$[C]b = \frac{I_0 - I_1}{(V\text{-}1)K'}$$

where $I_0 - I_1$ is the difference in fluorescence between concentrations of the drug with and without DNA, V is a ratio of the fluorescence of "bound" to "free" drug, and K' is a constant equating the fluorescence of a drug solution to its concentration ($I_1 = [C] \cdot K'$).

Equilibrium dialysis was performed at room temperature in Lucite cells divided into two equal chambers by the insertion of cellulose membranes. Membranes were cut from prewashed dialysis tubing (BRL; exclusion limit 12–14 kDa). Solutions were introduced through individual ports using a Hamilton syringe. Pyrvinium iodide was added to one-half of the cell to a final volume of 100 μl in 0.01 SHE buffer/0.5% DMSO. Equal volumes of CT DNA solution (65 μM in 0.01 SHE) were introduced into opposing chambers. Equilibration was achieved by rotating the cells about a mechanized spit for 24 hr at 20° after which time aliquots were extracted and assayed for the drug. "Free" drug samples (75 μl) were diluted into 2 ml of methanol and the fluorescence was determined. Concentrations were calculated from standard curves prepared by the analogous dilution of known amounts of drug into methanol. Samples of DNA-side drug (75 μl) were assayed for fluorescence after dilution into 2 ml of 50% 0.01 SHE/49% DMSO/1% glacial acetic acid to denature the DNA. Standard curves were prepared in the DMSO/acid solution containing an amount of DNA equal to that employed for the dialysis. Solutions were excited at 465 nm and fluorescence was monitored at 582 nm. The data were analyzed using Scatchard plots as interpreted by McGhee and von Hippel (18).

Unwinding of Superhelical DNA

The "topoisomerase-fluorescent" method (16) for determining unwinding angles was used. The substrate, PM2-ccc-DNA, varied in content, depending on the preparation, from 80% to 97% ccc-DNA. Assay mixtures included 50 μl of buffer containing approximately the A_{260} of DNA, 1 μl of drug (in DMSO), and 1 μl of CT topoisomerase. Calculated unwinding angles were computed on the basis that the superhelical density of the PM2-ccc-DNA was −0.126 (19).

Electron Microscopy

*Pst*1 partial digests of pBR322 plasmid DNA, which contained a mixture of linear and open-circular forms, were incubated with the drug in a 50% (v/v) solution for formamide and buffer (100 mM Tris and 10 mM EDTA, pH 7.5). After 15 min at 37° to ensure equilibration, the hyperphase mixture was prepared for microscopy as described by Davis *et al.* (20). The hypophase consisted of 20% formamide in 10 mM Tris (pH 7.5) and contained, in the case of pyrvinium pamoate, saturating levels of the drug. In the case of pyrvinium iodide, the hypophase contained a ratio of drug to formamide equal to that in the hyperphase. DNA molecules were photographed with a Philips EM300 electron microscope and were measured on photographic prints (overall magnification of × 55,500) with a Hewlett-Packard 9874A digitizer coupled to a Tetronix 4051 graphics computer. Lengths were recorded in terms of "digitizer units." Based on measurements of 4362 base pair, open-

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circular pBR322 molecules that exist in the B-form, 1,964 ± 39 digitizer units were equal to 1 μm.

Results

Pyrvinium fluorescence and the effect of DNA binding. Pyrvinium pamoate and pyrvinium iodide display two excitation maxima and one emission maximum in the visible light region (Table 1). Fluorescence is generally 2.5 times greater at the longer of the two excitation wavelengths. A significant difference between the two drug compounds is reflected in the emission spectra in aqueous solutions. The emission maximum for pyrvinium pamoate is very broad and shifted 30–40 nm to shorter wavelengths. The two drugs respond similarly when dissolved in methanol and when added to DNA in aqueous solutions, suggesting that their disparate behavior in DNA-less buffer may be related to the very poor aqueous solubility of pyrvinium pamoate. With both drugs, the addition of excess DNA brought about a 20-fold increase in the observed fluorescence at the major excitation maximum, which is red-shifted relative to uncomplexed drug. The fluorescence of methanolic pyrvinium solutions is 10 times that observed in aqueous solutions of the same concentration and partially mimics the response to DNA. Methanol is a useful solvent for small amounts of drug.

The interaction between pyrvinium pamoate and CT DNA has been titrated fluorimetrically (Fig. 2). The magnitude of the reaction is reflected in the fluorescence increment between samples in the presence and absence of DNA as measured under otherwise identical conditions. Increments are observed to increase dramatically until a mixing ratio of 0.12:0.14 (pyrvinium cation/DNA phosphate) is attained, where the differential is maximal. Fluorescence is lost from the drug-DNA complex at higher binding ratios. It is not clear what contribution the limited solubility of pyrvinium pamoate in aqueous solutions makes to the shape of the titration curve. The nonlinear control curves suggest that the drug is not soluble over the range of concentrations applied. Nonetheless, two observations can be made regarding the specificity of the reaction. There is a requirement for a duplex, polynucleotide structure as indicated by the significantly reduced fluorimetric response elicited by denatured CT DNA and bovine liver ribosomal RNA (Fig. 2A). Also, the significance of ionic interactions in the binding process is apparent from the sensitivity of binding to either high salt or magnesium (Fig. 2B).

A more interpretable analysis of the interaction between the pyrvinium cation and DNA was sought by using a monopyrvinium salt. Pyrvinium iodide was selected for this purpose. There is the added advantage in that pyrvinium iodide is slightly more soluble than pyrvinium pamoate in aqueous buffers. Pyrvinium iodide responds similarly in fluorimetric titrations with DNA (Fig. 3), although the shape of the titration curve is clearly sigmoidal at lower binding ratios. Note that control curves remain linear over the range of concentrations used and there is still the loss of fluorescence from the drug-DNA solutions at high binding ratios. It is therefore likely that this loss of fluorescence from the drug-DNA complex is a consequence of a higher level of binding and is not a solubility-related phenomenon.

TABLE 1

Fluorescence maxima for pyrvinium compounds

Drug concentrations ranged from 2 μM to 4 μM. Aqueous solutions were prepared with a 1:100 dilution from methanol into 0.01 SHE buffer. DNA was added to 300 μM.

Drug	Solvent	Maxima	
		Excitation	Emission
		nm	
Pyrvinium pamoate	Methanol	373,495	565
	Aqueous buffer	373,470	530–550
	Buffer + DNA	373,500	570
Pyrvinium iodide	Methanol	373,483	575
	Aqueous buffer	373,475	580
	Buffer + DNA	373,500	575

Size and affinity of the DNA binding site. From the data in Fig. 3, an estimation of the affinity of the pyrvinium cation for native CT DNA can be calculated using the formula derived by Le Pecq and Paoletti (6). Calculations of *Cb*, the concentration of bound drug, were made having estimated *V* to be 18 from a separate titration of an excess of DNA with pyrvinium iodide under experimental conditions equivalent to those described for Fig. 3. The constant, K', not to be confused with an equilibrium constant, was calculated from the control curve in Fig. 3 to be $10.5 \times 10^6\ M^{-1}$. A Scatchard representation of the computed data is presented in Fig. 4.

Ligands that bind exclusively to one site without cooperative action yield linear Scatchard plots of negative slope. This is not the case with pyrvinium iodide. The positive slope observed at low *r* values in Fig. 4 is indicative of binding influenced by strong positive cooperativity (18). An aberration to the plot is introduced by the unexplained loss of fluorescence at high binding ratios and is seen in the corresponding region of the Scatchard plot as a curvature so profound that the curve doubles back on itself. However, any related error in the determination of the intrinsic affinity coefficient is minimized since this value is obtained directly from the ordinate intercept, i.e., by extrapolation to infinite dilutions. The affinity coefficient as it relates to the form of binding that induces fluorescence augmentation is thus determined from a visual fit curve to be $1.1 \times 10^4\ M^{-1}$.

To establish the validity of this interpretation of the fluorimetric data, binding was also studied by equilibrium dialysis. The results in the form of the binding isotherm are shown in Fig. 5. The corresponding Scatchard plot is presented in Fig. 6. The binding isotherm displays the sigmoidal nature characteristic of ligands that bind cooperatively. In fact, it resembles the fluorescence curves up to and including the break in the curve at the apparent point of saturation. Beyond this point, in contrast to the fluorescence curves, a second phase of binding is indicated by the increasing slope of the isotherm. Scatchard transformations of the equilibrium data display the pronounced curvature indicative of strong positive cooperativity. Following the reasoning of McGhee and von Hippel, (18) the K_a (affinity coefficient) and *n* (site size in base pairs) values are derived from the plot from the ordinate (r/c) intercept and abscissa (r) intercept ($n = 1/r$), respectively. The K_a is $1.4 \times 10^4\ M^{-1}$ and the estimated value of *n* is 2.5 base pairs. The second phase of the binding isotherm signals the onset of binding to secondary, lower affinity sites. A conclusive demonstration of this interaction was not obtained due to the limited solubility of pyrvinium iodide under the imposed experimental conditions.

The existence of multiple binding sites. Dual forms of binding have been described before for positively charged intercalators (21, 22). Intercalation represents the stronger bind-

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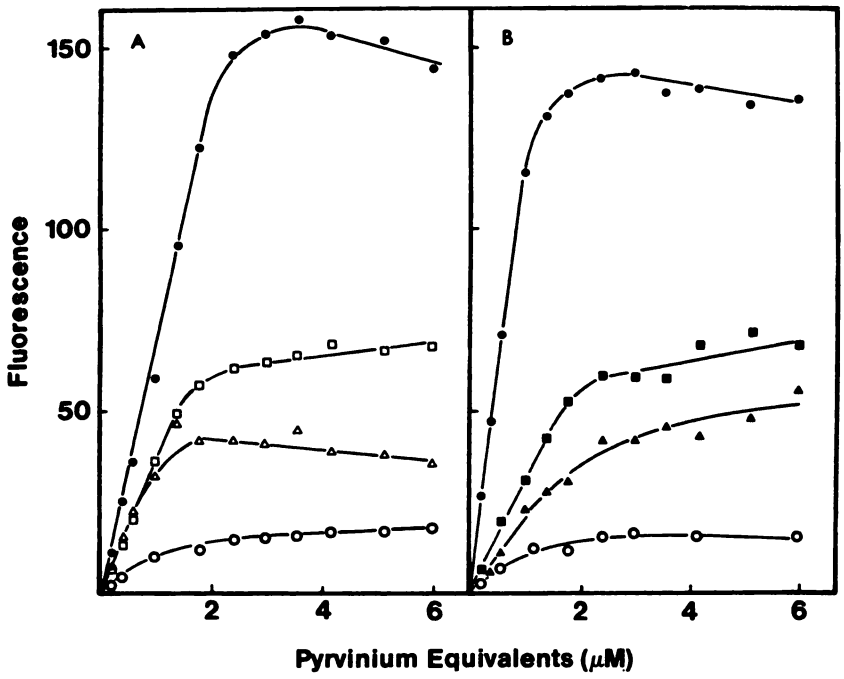

Fig. 2. Fluorimetric titration of pyrvinium pamoate. A, with native CT DNA (●), denatured CT DNA (□), and ribosomal RNA (△). B, with CT DNA under the following conditions: in 0.01 SHE (low salt) buffer (●), in 0.1 SHE (high salt) buffer (■), and in 0.01 SHE plus 0.01 M $MgCl_2$ buffer (△). Controls (○) are in the absence of DNA, in both A and B. Nucleic acids were present at 16 μM. Fluorescence is in arbitrary units.

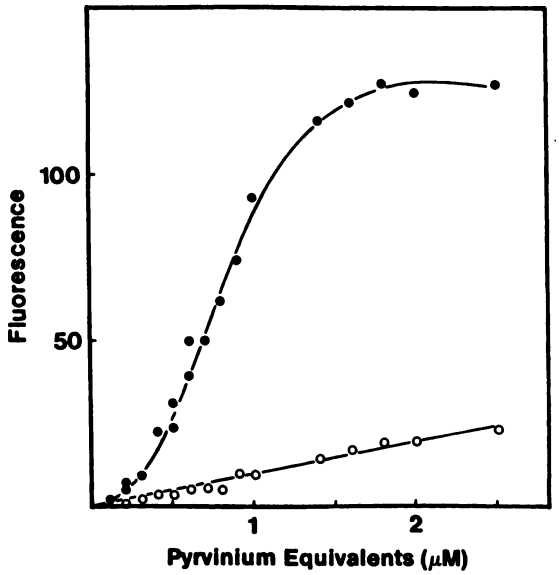

Fig. 3. Fluorimetric titration of pyrvinium iodide without CT DNA (○) and with CT DNA (25 μM) (●) in 0.01 SHE (low salt) buffer. Fluorescence is in arbitrary units.

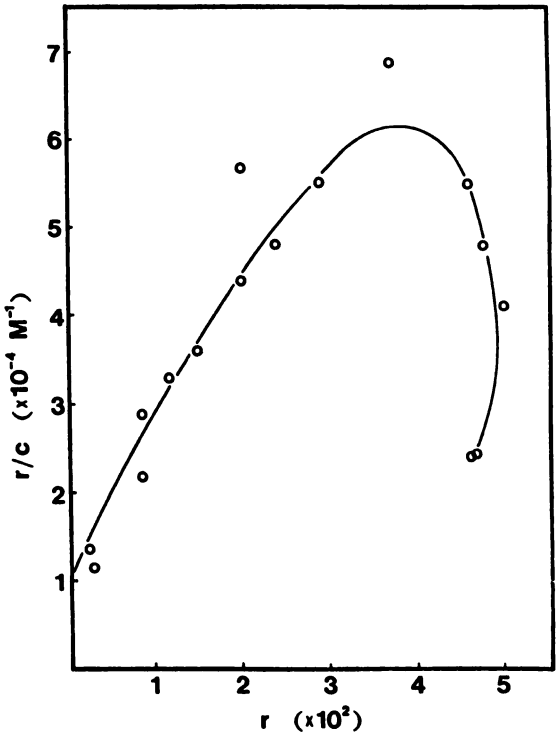

Fig. 4. Scatchard representation of the data in Fig. 3 for determination of the binding parameters from fluorimetric titrations. The number of molecules of pyrvinium iodide bound per base pair is represented by *r*, and the concentration of free drug is represented by *c*.

ing mode. A weaker mode is characterized by the electrostatic interaction of drug with the negatively charged phosphate backbone of the DNA double helix, and is commonly referred to as "external" binding. Changes in the absorption spectra of the phenanthridine derivative, prothidium, when it binds to DNA in low and high salt conditions as a function of the binding ratio, have been interpreted as evidence for these two distinct binding sites (22). Pyrvinium iodide was similarly titrated with DNA in 0.01 and 0.1 SHE buffers. Like other heterocyclic compounds (23), the visible spectrum of pyrvinium changes as it binds to DNA (Figs. 7 and 8). Binding induces a hypochromic and red shift in the absorbance maximum which can be seen by comparing the free-drug spectrum (*curve 13*, Fig. 7) and the bound-drug spectrum (*curve 1*, Fig. 7) in low ionic strength buffer. Superimposed upon the overall shift is an intermediate phase of hyperchromicity with an increased red-directed migration of the maximum (*curves 8–3*, Fig. 7). There is no isosbestic point common to all spectra, and this alone is indicative of multiple forms of binding (23). Results obtained by repeating the experiment under high salt conditions confirm this conclusion (Fig. 8). The intermediate phase described above, which appears as drug is bound at the higher

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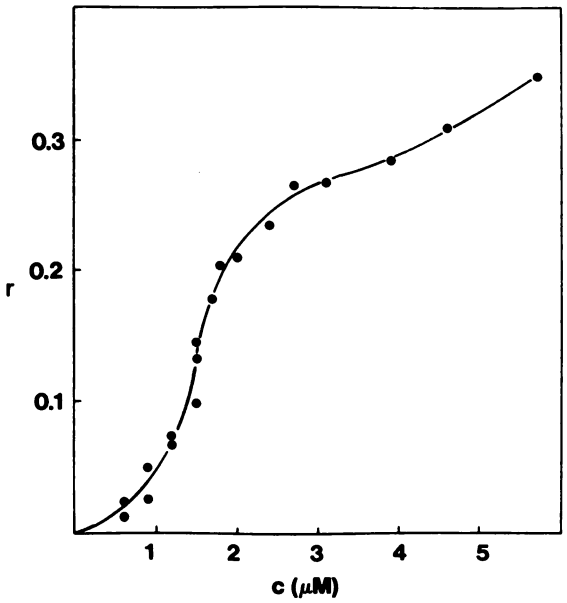

Fig. 5. Binding isotherm of pyrvinium iodide with CT DNA as determined by equilibrium dialysis. The number of molecules of pyrvinium iodide bound per base pair is represented by *r*, and the concentration of free drug is represented by *c*.

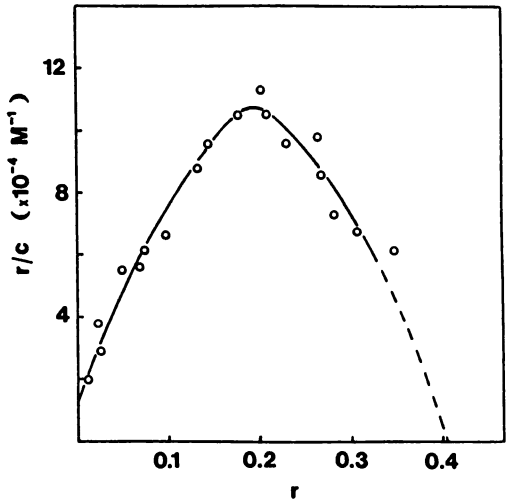

Fig. 6. Scatchard representation of the binding isotherm obtained by equilibrium dialysis as shown in Fig. 5. The number of molecules of pyrvinium iodide bound per base pair is represented by *r*, and the concentration of free drug is represented by *c*.

binding ratios, is essentially absent. What is observed is more or less a direct hypochromic progression from the "free" drug profile (*curve 13*, Fig. 8) to the "bound" profile (*curve 1*, Fig. 8). High salt preferentially inhibits the interaction of pyrvinium with secondary sites which otherwise become occupied at high binding ratios.

There is no analogous transformation of the fluorescence excitation spectrum for pyrvinium going from low to high salt (data not shown). The shapes of excitation spectra are unaffected by changes in the concentration of salt. Either the drug responds identically at both sites spectrofluorimetrically or fluorescence is exclusive to binding at one of the sites. The latter interpretation has been proposed to explain a similar response with ethidium when it binds duplex DNA (24).

Evidence supporting intercalation. Intercalation of a molecule between successive base pairs in a DNA duplex implies that an increase in base pair separation has taken place, ranging from 1.8 to 4.5 Å (25). As well, a local unwinding of the DNA helix should be apparent from the removal of negative supercoils in ccc-DNA. To demonstrate the latter, an unwinding angle was sought for the pyrvinium molecule using supercoiled PM2-ccc-DNA as a substrate; and to discern the former, electron micrographs of treated DNA molecules were examined.

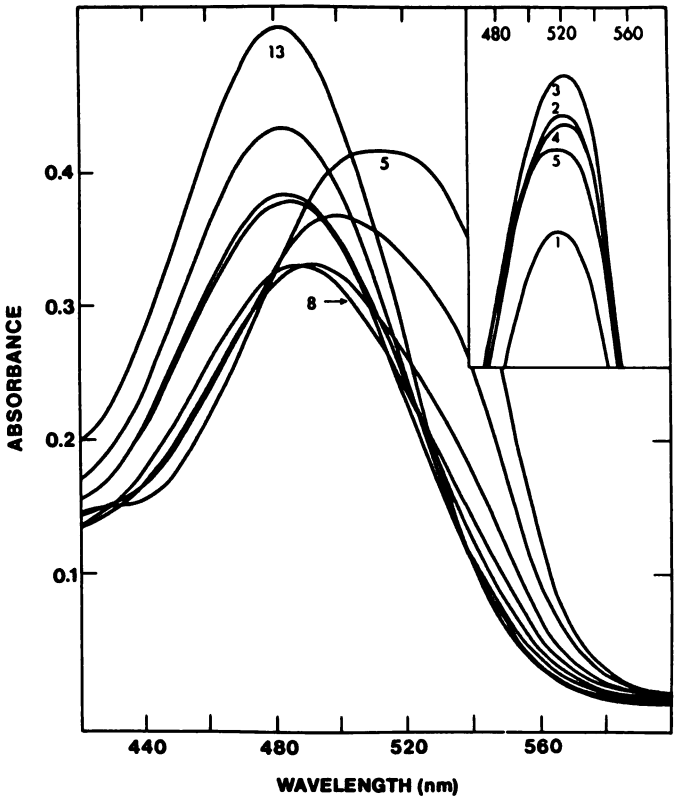

Fig. 7. Absorption spectra of pyrvinium iodide and CT DNA mixtures under low salt conditions (0.01 SHE). The concentration of pyrvinium iodide is 16 μM. The concentrations of CT DNA (in μM) through curves 1–13 (reading curve maxima *right* to *left*, excluding curves *1–5* which are numbered separately in the *inset*) are 260, 156, 94, 56, 34, 20, 12, 8.5, 5.1, 3.1, 1.5, and 0, respectively. Spectrum 12 has been omitted.

The "topoisomerase-fluorescent" assay for measuring unwinding angles is founded upon the observation that the degree of ethidium bromide binding by DNA is a function of the superhelical density of that DNA. Experimentally, supercoiled DNA is incubated with the drug to be tested and the DNA is relaxed with topoisomerase. Removal of the test drug by dilution into buffers containing a large excess of ethidium followed by the direct measurement of the resulting ethidium fluorescence permits a measure of the degree of unwinding (16). Increasingly unwound DNA becomes increasingly more negatively supercoiled after relaxation and displacement of drug. Thus, more ethidium bromide is bound and measured; fluorescence increases.

Increasing concentrations of pyrvinium iodide progressively unwound the PM2 circles in a nearly linear fashion (Fig. 9). This result was observed only under low salt conditions, i.e., at 0.01 M NaCl; no measurable unwinding was seen at the higher salt concentrations (0.1 M NaCl) prescribed by the original assay. The ratio of the number of molecules of pyrvinium iodide to the number of nucleotides at the relaxation point (which defines the point where pyrvinium iodide has completely re-

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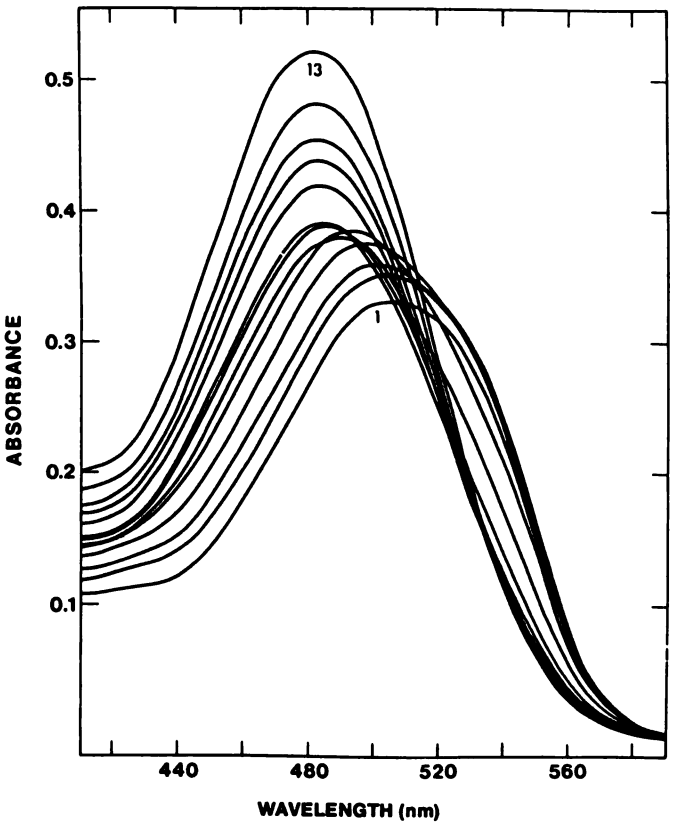

Fig. 8. Absorption spectra of pyrvinium iodide (16 μM) and of CT DNA mixtures under high salt conditions (0.1 SHE). The concentrations of DNA (in μM) through curves *1–13* (reading *right* to *left* as for Fig. 7) are 260, 156, 94, 56, 34, 20, 12, 8.5, 5.1, 3.1, 1.5, 0.76, and 0, respectively.

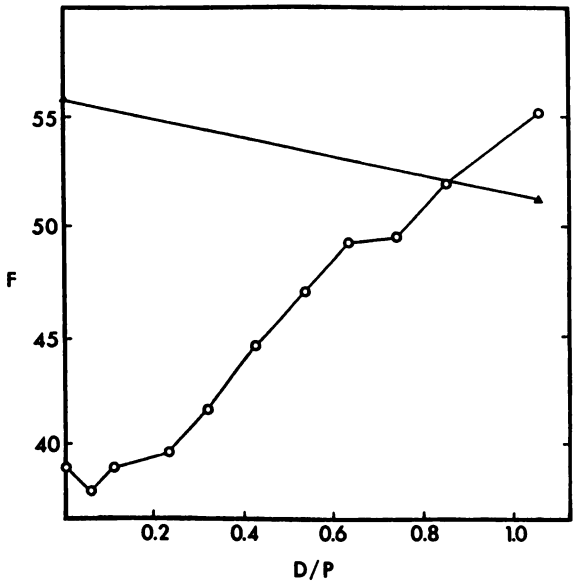

Fig. 9. The unwinding of DNA by pyrvinium iodide as demonstrated by the titration of superhelical turns in PM2 DNA. DNA was incubated in the presence of various concentrations of drug and relaxed with a type I topoisomerase. After a 20-min incubation period an excess of ethidium bromide was added and the fluorescence recorded (○). Increasing fluorescence denotes unwinding by the test drug (see Results). Controls lack topoisomerase (△) and establish a "baseline" response to added drug. Intersection of the two curves indicates the equivalence point where the DNA is completely relaxed by the drug and topoisomerase has no effect.

laxed the DNA and topoisomerase has no effect) is 0.85. To calculate an unwinding angle for the pyrvinium moiety from these data would require an accurate measurement of the amount of the drug which had been intercalated under these conditions. Due to the cooperative nature of intercalative binding and the existence of competitive, secondary binding, this quantitation was not attainable. Therefore, chloroquine was assayed in the same system, within the same concentration range (Table 2), for the purpose of comparison. It is obvious that chloroquine is less capable of relaxing the DNA than is pyrvinium under the assay conditions, even though the two drugs are comparable in their affinity for DNA. Chloroquine, in low ionic strength buffer, has a reported intrinsic binding constant of 2×10^4 M^{-1} (8, 9). By crude extrapolation, an unwinding angle of 6° is estimated for chloroquine. This angle approximates the value found in the early literature of 8.6° for chloroquine measured in 0.07 M NaCl (26). High salt clearly inhibits unwinding by chloroquine.

Electron microscopy has accurately demonstrated the extension of DNA duplexes resulting from the binding of known intercalators (24, 27). At drug/base pair ratios of 30:1, 300:1, and 3000:1, pyrvinium iodide increases the mean length of pBR322 plasmid DNA molecules from 2887 ± 57 DU to 3078 ± 72 DU, 3389 ± 169 DU, and 3114 ± 76 DU, respectively. The mean length of pBR322 treatment with pyrvinium pamoate (at 3000:1, pyrvinium cation/base pair) was 3261 ± 206 DU. The evidence shows that pyrvinium pamoate and pyrvinium iodide extend pBR322 plasmid molecules by as much as 12% and 18%, respectively (Fig. 10). The DNA-ligand complexes photographed in solutions containing a 300-fold, or a 3000-fold (Fig. 11), excess of the drug are distinguished by their propensity to form intra- and intermolecular adhesions (Fig. 11). The number of end-to-end attachments is markedly increased in drug-treated samples. This phenomenon has been demonstrated before with the intercalation of mitoxantrone into PM2 DNA (24).

Discussion

The molecular basis for the anthelmintic efficacy of pyrvinium compounds has long been thought to be related to the sensitivity of respiratory enzymes which are present in pinworms and absent in man (1). In view of the cytogenetic activity

TABLE 2

Unwinding of PM2 superhelical DNA by chloroquine

Low salt buffer contained 0.01 M NaCl and 150 μM DNA. High salt buffers contained 0.1 M NaCl and 139 μM DNA. Otherwise the conditions are as described for the "topoisomerase-fluorescence" assay. Fluorescence is in arbitrary units. Included in parentheses are the equivalent data for pyrvinium iodide taken from Fig. 9.

Buffer condition	Chloroquine concentration	D/P[a]	Enzyme[b]	Ethidium fluorescence	(Pyrvinium)
	μM				
Low salt	0	0	+	34.0	(38.8)
	64	0.43	+	38.4	(44.4)
	128	0.85	+	42.3	(52.5)
	0	0	−	52.5	(56.0)
	128	0.85	−	52.1	(51.6)
High salt	0	0	+	36.7	
	64	0.46	+	38.1	
	128	0.92	+	40.2	
	0	0	−	56.6	
	128	0.92	−	58.7	

[a] D/P, ratio of drug to DNA phosphate.
[b] +, plus topoisomerase; −, less topoisomerase.

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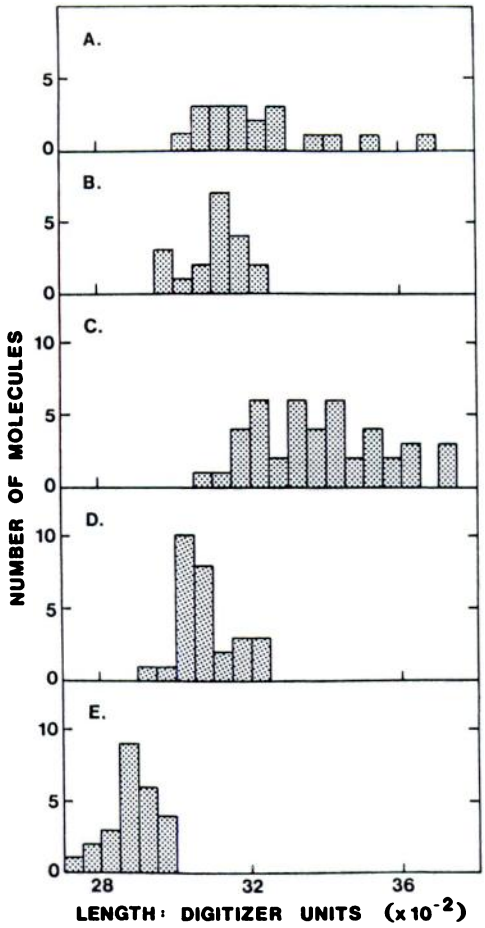

Fig. 10. The effect of pyrvinium iodide and pyrvinium pamoate on the length of pBR322 molecules as determined by electron microscopy. A, pyrvinium pamoate at a ratio of 3000 molecules of pyrvinium moiety/base pair. B–D, pyrvinium iodide at ratios of 3000, 300, and 30 molecules of pyrvinium moiety/base pair, respectively. E, untreated molecules.

of pyrvinium and its ability to bind to DNA *in vitro*, it is very possible that the cytotoxicity of this drug is expressed at the level of the chromosome. The capacity of the pyrvinium cation to intercalate would most certainly make a specific contribution to its broad activity spectrum.

The abilities to unwind and extend the DNA duplex molecule are definitive properties of an intercalating agent (28). The susceptibility of binding to salt and cations such as magnesium, and the mutagenicity profile of pyrvinium, are typical of the behavior associated with intercalators (2, 6, 10). The process of intercalation can induce a range of perturbations in the stereochemistry of the DNA duplex. Actual observations have supported this belief (29), as does a theoretical consideration of the interaction (28). Despite some early controversy over the low unwinding angle reported for chloroquine (26), this drug is now regarded to be an intercalator. It has an unwinding angle of 17°, which is considerably less than the 26° cited for ethidium (13). The "topoisomerase-fluorescent" assay yielded unwinding angles much less than 17° for both chloroquine and pyrvinium. The low values were anticipated because of the high degree of "external" drug bound at nonintercalative sites under the conditions of the assay. Limited by the solubility of pyrvinium iodide and the activity of the topoisomerase, it was not feasible to adjust the NaCl concentration to minimize the amount of secondary-site drug. Consequently, at the observed equivalence point in the assay, considerable drug is bound to the DNA polymer which does not contribute to its unwinding. The result is an underestimation of the unwinding angle. Regardless, it is significant that both drugs unwind PM2 DNA, and that pyrvinium is apparently the more effective of the two. Most likely the two drugs are intrinsically similar in their capacity to unwind DNA, and the experimental difference can be attributed to the cooperativity of pyrvinium binding.

The increase in base pair separation resulting from intercalation is reported with ethidium, which binds to every second base pair (30). A 21% increase with ethidium has been observed previously with the experimental technique used in this paper (27). Pyrvinium iodide, at a ratio of 300 drug molecules/base pair, resulted in an increase of 18%. This high drug/DNA ratio was presumed to meet saturating conditions. Assuming that the intercalation of pyrvinium causes a doubling in the spacing between base pairs, an incremental increase of 18% would correspond to a binding ratio of about one drug molecule for every 6 base pairs—lower than would be predicted for a calculated binding site of 2.5 base pairs. A higher drug ratio resulted in no greater extension of the DNA molecule and may have actually served to diminish the length increments observed at lower ratios. It is conceivable that at high binding ratios there is less intercalative binding. Intercalated drug could be effectively displaced by a larger amount of drug binding to external sites, which was proposed to explain the phenomenon as it appeared with ethidium (30). Proof of a capacity to lengthen DNA distinguishes pyrvinium from a compound like irehdiamine A. Irehdiamine unwinds DNA (unwinding angle 10°), but does so with a negligible effect on the length of the molecule. Although it binds tightly to DNA, it does not intercalate (13).

Binding isotherms generated by equilibrium dialysis and fluorimetric titrations are sufficiently alike to support the conclusion that fluorescence titrations accurately and specifically monitor the primary, intercalative binding process at low binding ratios. A critical limitation to a complete understanding of the analysis is the unexplained loss of fluorescence at high binding ratios. This coincides with binding at secondary sites which is consistent with the displacement of the fluorescence-enhanced intercalated drug as previously discussed. Alternatively, it could be indicative of inner filter fluorescence quenching due to the drug bound at "external" sites. The cooperative nature of intercalative binding by pyrvinium is clearly demonstrated by both methods. Cooperativity may arise through ligand-ligand interactions, perhaps as stacking interactions, between aromatic regions of the pyrvinium molecule in solution or on the surface of a DNA molecule. Chloroquine binding is not cooperative (9). The aliphatic side-chain of chloroquine carries a positive charge which would serve to prohibit self-association. It is interesting to note that the dipyrvinium pamoate compound, which does not dissociate in the buffers used and therefore must interact with DNA in the dimeric form, does not exhibit the profound cooperativity that is characteristic of monopyrvinium iodide, at least not to an extent that is visible from comparisons of the plots in Figs. 2 and 3. Cooperativity, however, may yet arise through drug-induced allosteric transitions in the DNA. Cooperativity of this form can be sensitive to the source of DNA and ionic conditions (31). Neither possibility can be ruled out at this stage.

The pyrvinium cation interaction with DNA is characterized by dual modes of binding. The primary mode, almost certainly

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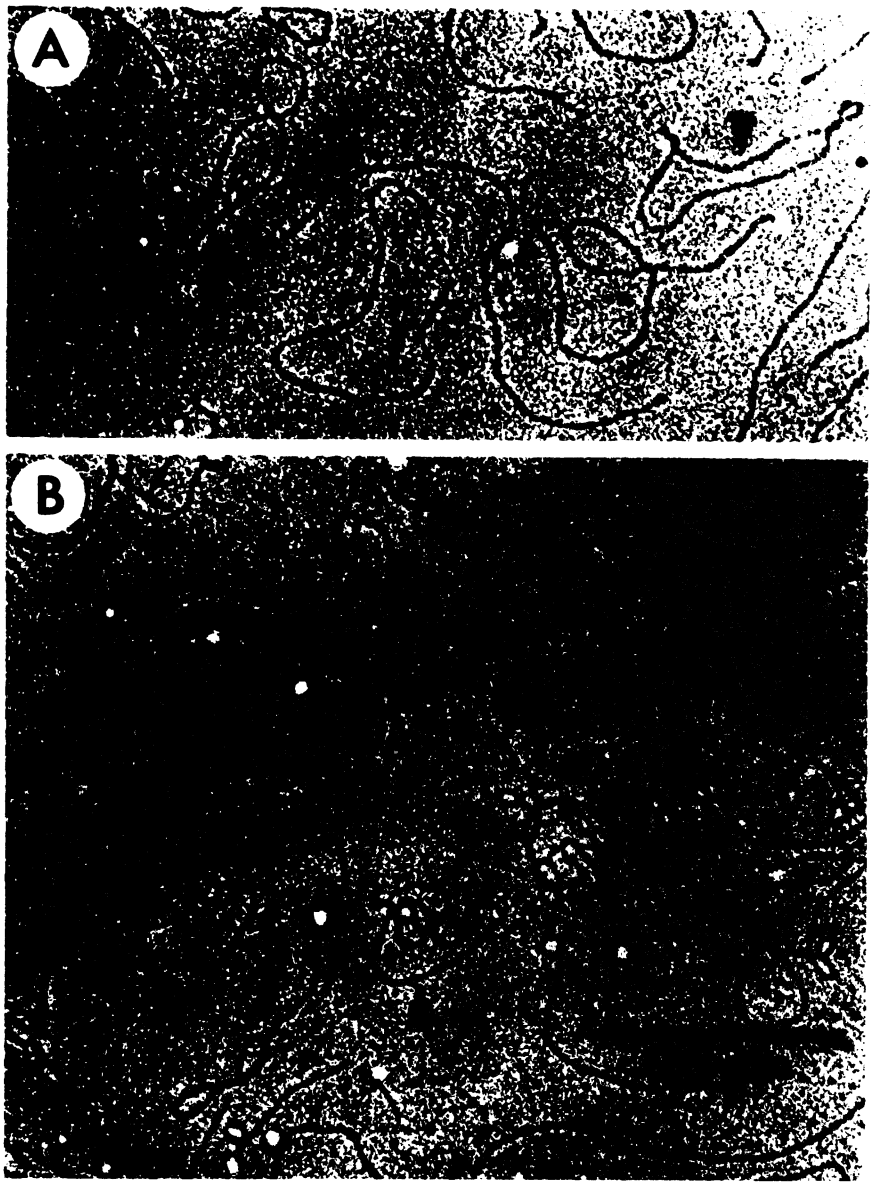

Fig. 11. Electron micrograph of (A) untreated pBR322 plasmid DNA molecules and (B) plasmid DNA molecules treated with pyrvinium iodide at a ratio of 3000 molecules/DNA base pair. The *arrowheads* indicate plasmid circles typical of the molecules included in the measurement data of Fig. 10.

intercalative, displays positive cooperativity. The secondary mode is most likely electrostatic in nature. Evidence for these four attributes, barring the last, is apparent in the fluorimetric data. Further study is necessary to establish a connection between the loss of fluorescence at high *r* values and secondary binding interactions. Nonetheless, it should now be possible to employ fluorimetry as a reliable, preliminary screening technique to compare the interactions of pyrvinium analogues (which display variable mutagenic activities) with selected DNA substrates.

Intercalation of pyrvinium appears to be reflected in the ability of the drug to induce the formation of petite cells in yeast (3), and to cause frameshift mutations and chromosomal breakage events in bacteria and yeast. It is not obvious how intercalation, or the capacity to intercalate, influences the biological specificity of the drug. Recent models attempt to explain the translation of intercalation into biological dysfunction in terms of the poisoning of cellular topoisomerases (10). Topoisomerases I and II, purified from eukaryotic sources, are sensitive to intercalating drugs (14, 32). The sensitivity of topoisomerases is a function of the specific intercalator and, interestingly, the source of the enzyme. Thus, the assignment of genotoxic and cytotoxic specificity to pyrvinium may evolve from considerations of the accessibility of molecular targets either with or without metabolic activation, as well as the susceptibility of the enzymes of DNA metabolism such as cellular topoisomerases.

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