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Interactions of the Antitumor Agents Mitoxantrone and Bisantrene with Deoxyribonucleic Acids Studied by Electron Microscopy

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SUMMARY

The interactions of the low cardiotoxic antitumor agents 1,4-dihydroxy-5,8-bis[[2-[(2-hydroxyethyl)amino]ethyl]aminol]-9,10-anthracenedione (mitoxantrone) and 9,10-anthracenedicarboxaldehyde bis[(4,5-dihydro-1*H*-imidazoyl-2-yl)hydrazone] (bisantrene) with pBR322 and PM2 DNA have been examined by electron microscopy. Direct evidence was obtained for intercalative binding of both drugs, with mitoxantrone causing a 13% average length increase in pBR322 corresponding to ~580 drug molecules per circle at saturation and bisantrene causing an 11% increase in length corresponding to ~480 drug molecules bound per circle. Considerations of the known GC preference for non-nearest neighbor binding of the drugs and inspection of the known sequence of pBR322 suggest that the available intercalation sites are occupied and that additional external electrostatic binding of the cationic drugs also occurs. An apparent difference in behavior of mitoxantrone as compared with that of bisantrene in causing no net increase in length of supercoiled pBR322 was shown to be attributable to an offsetting compaction due to extensive supercoiling by mitoxantrone molecules. This conclusion was confirmed by independent experiments with PM2 covalently closed-circular DNA—both native, negatively supercoiled and relaxed—with calf thymus topoisomerase, using ethidium for comparison. Ethidium caused a 21.3 ± 3.6% length increase in nicked, open-circular PM2-DNA, or 2100 molecules bound per 10,300 base pairs. Mitoxantrone caused a 16.6% length increase in nicked PM2-DNA equivalent to ~1700 drug molecules per circle. Electron microscopic measurements on relaxed PM2-DNA with progressively increasing proportions of mitoxantrone (from 1.4:1 to 14:1 drug molecules per base pair) revealed the onset of formation of lacelike networks of DNA circles linked together. This phenomenon, which is not produced by bisantrene, is attributed to inter-DNA links by the charged side arms of mitoxantrone and is in accord with previous reports that mitoxantrone causes severe compaction and distortion of chromatin. Electron microscopic examination of the interaction of six additional mitoxantrone derivatives, two of which produced lacelike DNA networks, revealed strict structural requirements for this phenomenon.

INTRODUCTION

Adriamycin (doxorubicin) is widely used clinically against a variety of human malignancies (1). Its principal clinical limitation, severe risk of irreversible cardiac damage (2–5), has prompted efforts to develop more selective synthetic agents that are less toxic. These studies have been based on the premise that the mode of action of anthracyclines depends, at least in part, on intercalative binding to DNA. They have included derivatization of Adriamycin (6, 7), *de novo* synthesis of chromophore-modified analogues (8), and the synthesis of anthracene derivatives, including 1,4-dihydroxy-5,8-bis[[2-[(2-hydroxyethyl)amino]-ethyl]amino]-9,10-anthracenedione, **1a** (mitoxantrone in Fig. 1) (9, 10), and 9,10-anthracenedicarboxaldehyde bis[(4,5-dihydro-1*H*-imidazoyl-2-yl)hydrazone] (bisantrene in Fig. 1) (11).

Mitoxantrone gives greater than a 500% increase in life-span against P388 murine leukemia, with four of five 60-day survivors, and its efficacy and therapeutic index equal or surpass those of Adriamycin, cyclophosphamide, daunorubicin, methotrexate, and 5-fluorouracil (9). Mitoxantrone is also more effective against L-1210 leuke-

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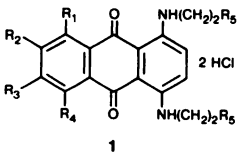

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	R_1	R_2	R_3	R_4	R_5
a	OH	H	H	OH	$NH(CH_2)_2OH$
b	OH	OH	H	H	$N(CH_3)_2$
c	OH	H	H	OH	$CH_2NH(CH_2)_2OH$
d	H	H	H	H	$N(CH_3)_2$
e	OH	H	H	OH	$(CH_2)_2N(CH_3)_2$
f	OH	H	H	OH	$NH(CH_3)$
g	H	H	H	H	$NH(CH_2)_2OH$

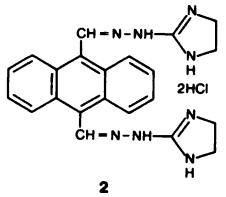

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FIG. 1. *Structures for mitoxantrone* ***1a*** *and derivatives* ***1b–g*** *and bisantrene* ***2***

mia, B-16 melanoma, and colon tumor 26 than Adriamycin and is currently undergoing preclinical toxological evaluation (12, 13). Bisantrene, **2**, similarly shows significant activity against L1210 leukemia, P388 leukemia, B16 melanoma, Ridgeway osteogenic sarcoma, and colon tumor 26 in rodents (11). In cardiotoxicity studies carried out in dogs, both mitoxantrone and bisantrene were significantly less cardiotoxic than Adriamycin (14, 15).

The significant promise of these two agents has prompted several studies on their molecular pharmacology. Mitoxantrone binds to both DNA and RNA in the nuclear chromatin and produces profound changes in its structure (16, 17), prevents progression of cells at the G_2 phase of the cycle, and inhibits Chinese hamster ovary cell colony-forming ability (18). Similar evidence on bisantrene, including the induction of DNA strand breaks as well as associated DNA-protein cross-links (14, 19), strongly implicates nucleic acids as one of the principal cell targets for both compounds.

A full description of the modes of binding of mitoxantrone and bisantrene with DNA has yet to be given, and the nature of the interactions with DNA is still controversial. The evidence to date has indicated no distinction betweeen the actions of these two drugs with DNA. We report an examination by electron microscopy of the interactions of mitoxantrone and bisantrene with pBR322 and PM2 circular DNAs in distinct topological forms which provides direct evidence for intercalative binding of both types of drugs as well as the unique action of mitoxantrone in causing side-by-side association of DNA molecules.

EXPERIMENTAL PROCEDURES

Materials

Drugs, DNAs, and enzymes. Mitosantrone and six additional analogues and bisantrene were provided by Dr. Richard White and his associates of Lederle Laboratories (Pearl River, N. Y.); pBR322 plasmid was a gift from Dr. J. H. Weiner, Department of Biochemistry, University of Alberta. Pst 1 was purchased from Bethesda Research Laboratories Inc. (Gaithersburg, Md.). PM2-CCC[3]-DNA, prepared as described previously (20), and calf thymus topoisomerase, prepared according to the method of Herrick *et al.* (21), were generously provided by A. R. Morgan, Department of Biochemistry, University of Alberta.

Electron Microscopy

Studies with pBR322.

Substrate. pBR322 was partially digested with Pst 1 and stored in 10% formamide/10 mM Tris buffer (pH 7.5)/1 mM EDTA as a mixture of supercoiled, open-circular, and linear molecules. The pBR322 plasmid, which comprises 4362 base pairs, has an average length in the B-configuration of 1470 nm (22). The concentration of the stock solution corresponded to 35 μg of DNA per milliliter.

Incubation procedure. Mitoxantrone, 1 μl of a 0.5 mM solution, was added to 1 μl of the pBR322 (0.05 mM in terms of base pairs, assuming the molecular weight of one base pair of sodium salt is 662). Incubation proceeded at 37° for 60 min. Similarly, bisantrene, 0.5 μl of a 10 mM solution, was added to 1 μl of the pBR322 solution and incubated as above. In both cases the mixtures contained ~10 drug molecules per base pair of the DNA.

Preparation for microscopy. Preparation for electron microscopy was carried out according to the method of Davis *et al.* (23). The DNA:drug complexes were diluted 25-fold into "hyperphase" buffer [100 mM Tris (pH 7.5), 10 mM EDTA, and 40% (v/v) formamide]. The final DNA concentration was 0.7 μg/ml. Cytochrome *c* was added to a concentration of 50 μg/ml, and 50 μl of this solution were spread into 55 ml of the "hypophase," consisting of 10% (v/v) formamide in 10 mM Tris (pH 7.5) and 1 mM EDTA. The DNA was picked up on parlodion-covered grids stained with uranyl acetate in 90% ethanol and rotary-shadowed (8°) with 2 nm of Pt/C.

DNA molecules were measured on photographic prints of electron micrographs with a Hewlett-Packard 9874A digitizer coupled to a Tetronix 4051 graphics computer. Lengths were recorded in terms of "digitizer units."

Studies with supercoiled and open-circular PM2-DNA.

Substrate. The PM2-DNA, which was stored frozen in 10 mM Tris (pH 8.0) and 1 mM EDTA, contained approximately 90% of the molecules in the open-circular form. PM2-DNA comprises ~10,300 base pairs (24) and has a length of ~3.47 μm in the B-configuration.

Incubation procedure. Mitoxantrone, a mitoxantrone analogue, or bisantrene was incubated with the PM2-DNA at 37° for 60 min, following a procedure similar to that described for pBR322.

Preparation for microscopy. PM2-DNA was diluted in 0.5 M ammonium acetate (pH 7.0) to a concentration of 0.5 μg/ml, and mitoxantrone was added to a concentration of 6 μg/ml (approximately 14 drug molecules per base pair). Cytochrome *c* was added to a concentration of 50 μg/ml, and 20 μl of this hyperphase mixture were spread onto a 3-ml hypophase of 5 μg of mitoxantrone per milliliter in 0.1 M ammonium acetate (pH 7.). The DNA was picked up onto parlodion films, stained, shadowed as for studies with pBR322, and photographed in the electron microscope.

Studies with relaxed PM2-DNA.

Substrate. A preparation of PM2-CCC-DNA, which was ~80% CCC as measured by an ethidium bromide fluorescence assay (25), was relaxed by treatment with calf thymus topoisomerase. This sample, when prepared by the aqueous ammonium acetate procedure and examined in the electron microscope, was found to consist exclusively of relaxed molecules. We assume that ~80% of these are relaxed CCC and ~20% are open-circular forms. The length of these molecules (at ×43,000) was 5059 ± 95 digitizer units.

Reaction with ethidium bromide. The relaxed PM2-CCC-DNA was diluted to a concentration of 0.5 μg/ml in 0.5 M ammonium acetate (pH 7), and ethidium bromide was added to a concentration of 4 μg/ml

[3] The abbreviation used is: CCC, covalently closed-circular.

(~13 drug molecules per base pair of DNA). After the addition of cytochrome *c*, the sample was prepared for electron microscopy as described for supercoiled and open-circular PM2-DNA.

Reaction with mitoxantrone. Three different drug concentrations were employed: 50 μg/ml, 0.5 μg/ml, and 0.05 μg/ml for reaction, respectively, with 0.5 μg/ml of relaxed PM2-CCC-DNA. The ratios of the drug molecules per base pair, therefore, were 14:1, 1.4:1, and 0.14:1, respectively. Preparation of the samples for electron microscopy was as described for supercoiled and open-circular PM2-DNA.

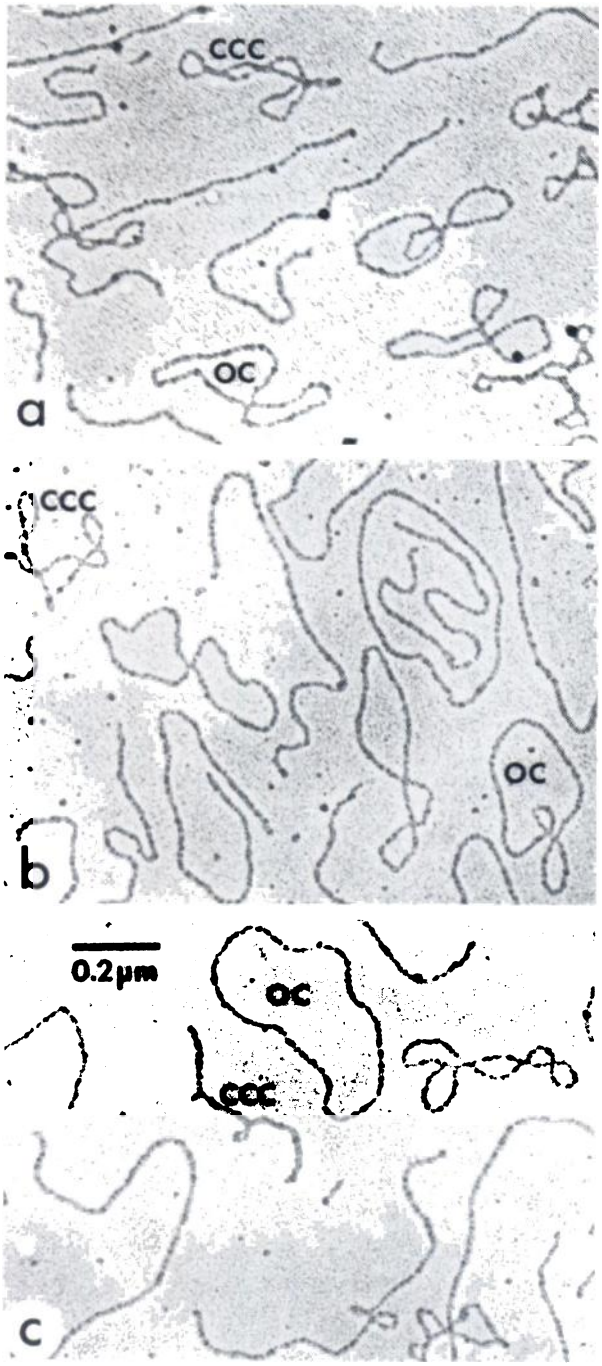

FIG. 2. *Electron micrographs of pBR322 DNA*

a. Control pBR322 DNA in formamide treated with Pst 1 showing supercoiled (*CCC*), open-circular (*OC*), and linear forms. b. pBR322 treated with bisantrene showing length extension of all three forms of the DNA. c. pBR322 treated with mitoxantrone showing length extensions of linear and open-circular forms of the DNA.

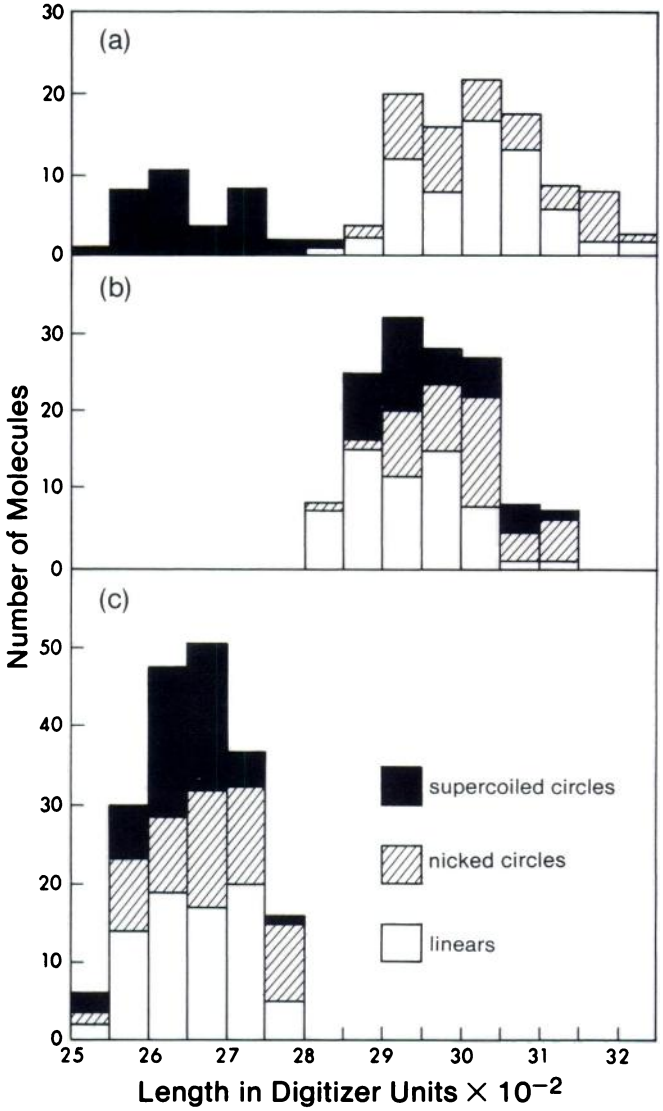

FIG. 3. *Histogram of electron microscopic digitizer measurements of average lengths of three forms of Pst 1-treated pBR322 DNA*

a. pBR322 preparation treated with mitoxantrone at a concentration of 0.5 mM showing increase in length of open circles and linears but no *apparent* increase in over-all length attributable to compensating contraction due to supercoiling. b. pBR322 preparation treated with bisantrene at a concentration of 1.0 mM showing increase in average length of all three forms. c. Control DNA.

RESULTS

Studies with pBR322. Measurement of average lengths of the pBR322 drug complexes by electron microscopy showed (Fig. 2) pBR322 control = 2668 ± 70 digitizer units, pBR322 plus bisantrene = 2966 ± 85 digitizer units, and pBR322 plus mitoxantrone = 3027 ± 89 digitizer units as shown on the histogram (Fig. 3). For pBR322 DNA under these conditions, 100 digitizer units = 163 base pairs = 56 nm. The calculated length increase due to intercalation of bisantrene, therefore, was ~11%, or 480 drug molecules per 4382 base pairs of pBR322, assuming that the DNA essentially retains the B-configuration during binding of the drugs. In the case of supercoiled pBR322 molecules, the addition of mitoxantrone

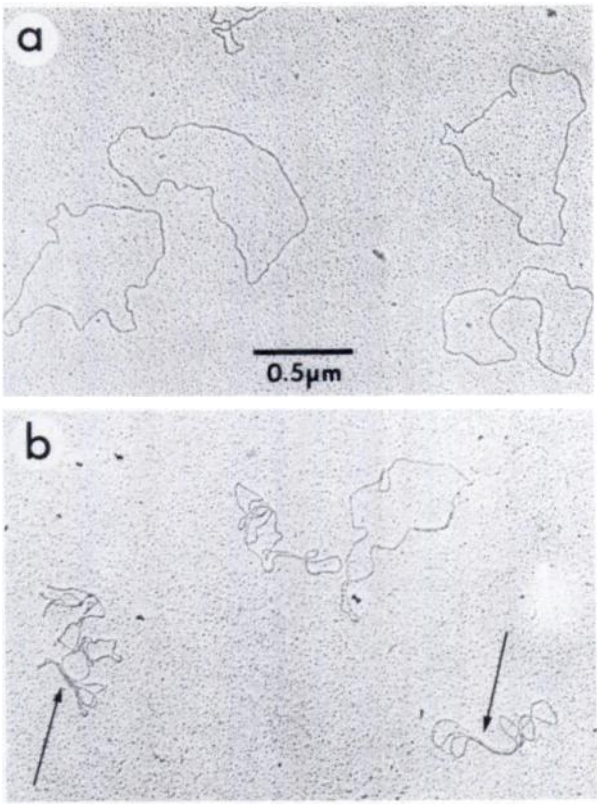

FIG. 4. *Electron micrographs of PM2-DNA*

a. Control PM2-DNA (~80% CCC, ~20% open-circular; relaxed with topoisomerase). b. PM2-DNA treated with mitoxantrone showing the supercoiling and side-by-side aggregation (*arrows*) effects produced by intercalative binding of the drug.

(in contrast to bisantrene) produced no apparent increase in molecular length. This observation could indicate that intercalation of mitoxantrone was hindered by supercoiling, or that intercalation did occur and in turn produced more supercoiling and compaction of the DNA. A close examination of the micrographs suggested that the latter explanation was more likely. Experiments were conducted with PM2-CCC-DNA, both supercoiled and relaxed, in order to investigate this phenomenon further. In this case, it was also decided to eliminate formamide from the preparative procedure and to carry out the mitoxantrone:DNA reaction and preparation for microscopy under aqueous conditions.

Studies with open-circular PM2-DNA. The average length of control nicked (open-circular) PM2-DNA was 5221 ± 60 digitizer units at a magnification of ×43,000 (Fig. 4). Mitoxantrone-treated molecules were 6041 ± 103 digitizer units in length, representing a 16% increase in length, characteristic of intercalation (Fig. 5). In addition, structures were observed in micrographs that resembled intertwined coils. Presumably, the extended, positively charged side chains on mitoxantrone can facilitate close side-by-side packing of DNA duplexes.

Studies with topoisomerase-relaxed PM2-DNA. In the control experiments with this DNA, when ethidium was bound, some 70–80% of the molecules were highly supercoiled (Fig. 6b). Their apparent average length was 4900 ± 72 digitizer units. This measured length is smaller by ~160 digitizer units than the original relaxed PM2-DNA, and reinforces by analogy the earlier argument that mitoxantrone does not bind by intercalation into CCC-pBR322 and that the concomitant increase in supercoiling results in no apparent increase in length of these molecules. The length measured for the portion (~20–30%) of the relaxed PM2-CCC-DNA (presumably nicked open-circular molecules) that remained as relaxed circles after reaction with ethidium bromide was 6136 ± 107 digitizer units (Fig. 5). This represents a length increase of 21.3 ± 3.6% due to drug intercalation, and is equivalent

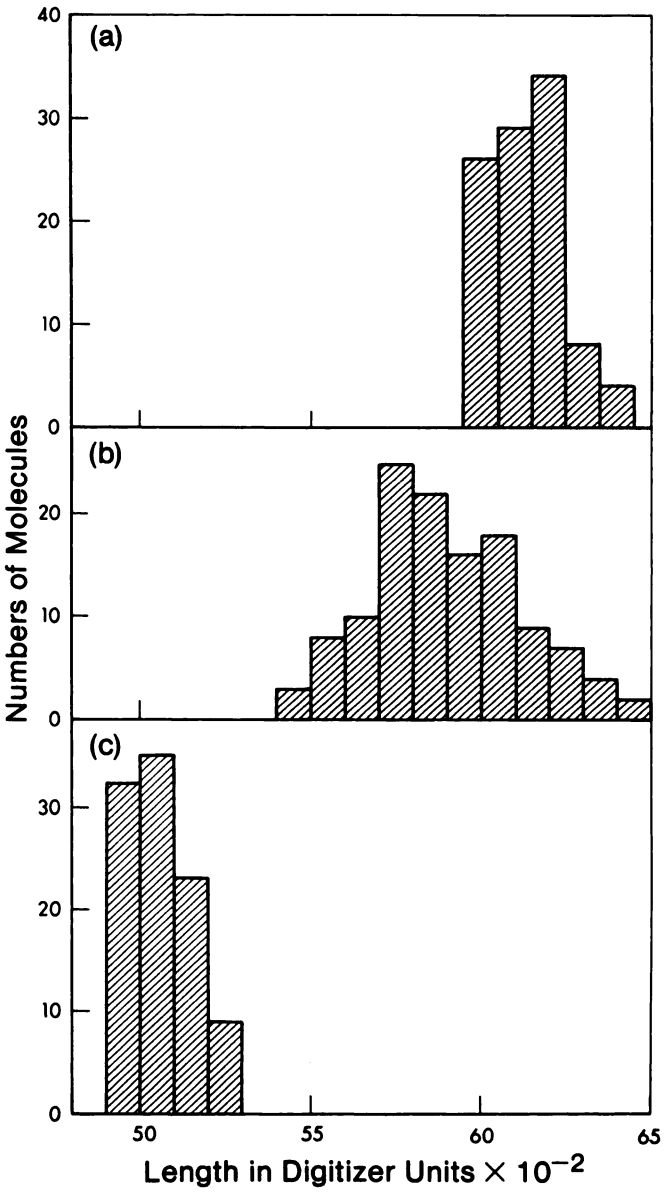

FIG. 5. *Histogram of electron microscopic digitizer measurements of average lengths of topoisomerase-relaxed PM2-DNA*

a. PM2-DNA preparation treated with ethidium bromide at a concentration of 4 μg/ml (~13 drug molecules per base pair) and showing an increase in length of the open-circular form. b. PM2-DNA preparation treated with mitoxantrone at a concentration of 5 μg/ml (~14 drug molecules per base pair) and showing an increase in length of the open-circular form. c. Control PM2-DNA (~80% CCC, ~20% open-circular). Supercoiled molecules were not included in the length measurements.

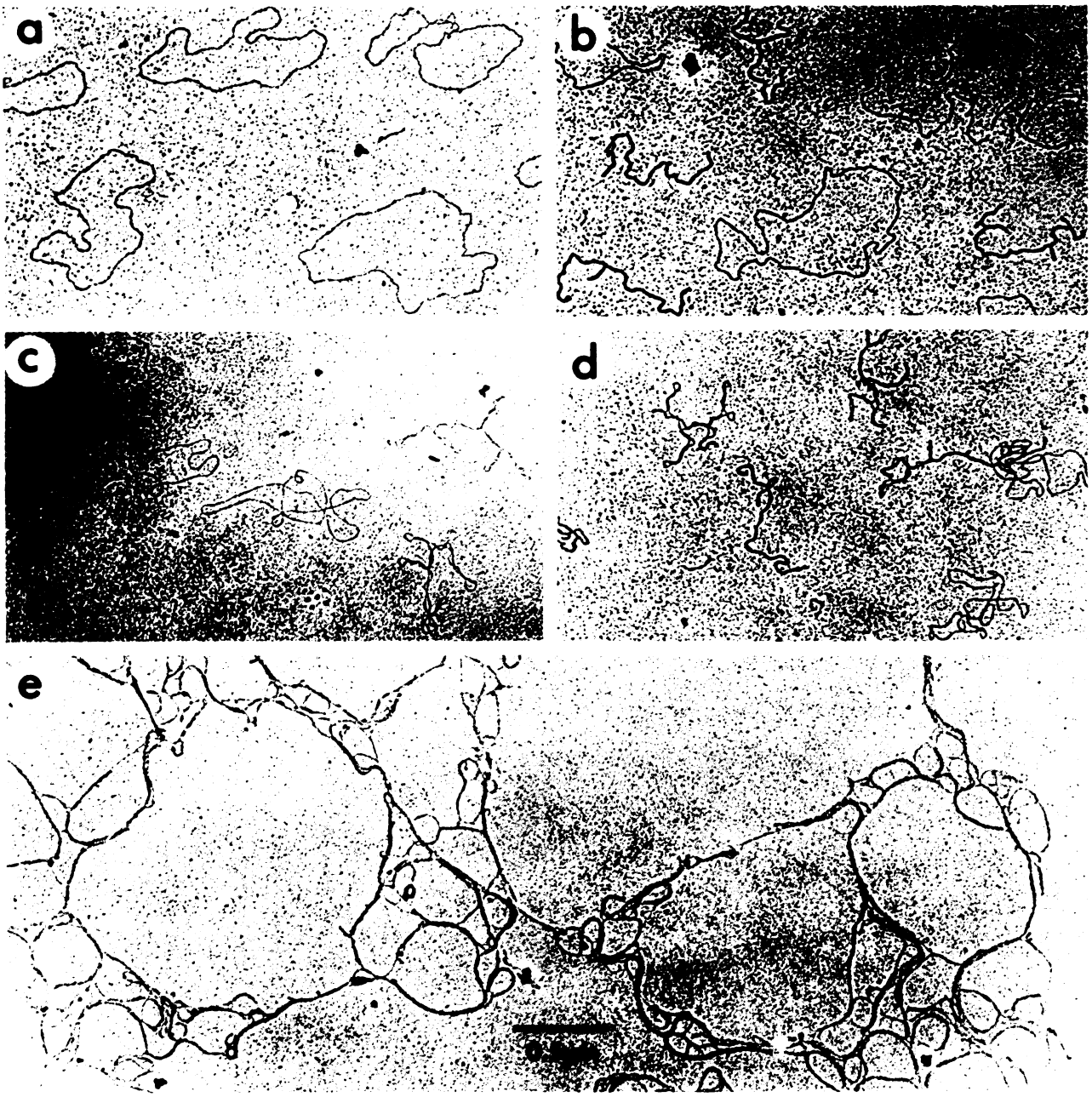

FIG. 6. *Electron micrographs of PM2-DNA*

a. Control PM2-DNA (~80% CCC) relaxed by treatment with calf thymus topoisomerase. b. PM2-DNA as in a, but treated with ethidium bromide, showing length extension of open-circular forms and supercoiling of CCC forms. c. PM2-DNA as in a, but treated with mitoxantrone at a concentration of 0.14 Eq of drug per base pair, showing progressive supercoiling of the DNA. d. PM2-DNA treated with mitoxantrone at a concentration of 1.4 Eq of drug per base pair, showing more extensive supercoiling. e. PM2-DNA treated with mitoxantrone at a concentration of 14 Eq of drug per base pair, demonstrating extensive formation of networks of linked DNA molecules.

to the intercalative binding of ~2100 ethidium molecules into the ~10,300 base pairs of the PM2-DNA.

Similar experiments were performed with mitoxantrone at three different concentrations added to the relaxed PM2-CCC-DNA (Fig. 6b). For the lowest drug concentrations (drug:base pairs = 0.14:1), open circles, twisted circles, and supercoiled circles were observed. The intermediate drug concentration (drug:base pair = 1.4:1) produced mostly twisted-circular and supercoiled DNA molecules. The molecular length of the open circles and twisted circles was 5897 ± 222 digitizer units. This represents a length increase of 16.6 ± 6.3% (see histogram, Fig. 5), and the same average length increase was observed at drug concentrations of both 0.5 μg/ml and 0.05 μg/ml. The length increase of 16.6% represents ~1700 mitoxantrone molecules intercalated into relaxed PM2-DNA. The average apparent length of the supercoiled molecules was only slightly longer than that of control DNA, i.e., 5144 ± 151 digitizer units as compared with 5059 ± 95 digitizer units. This again illustrates the effect of supercoiling on length determination by electron microscopy.

At the highest drug concentration of 14 molecules of mitoxantrone per base pair of relaxed PM2-CCC-DNA, the effect was quite pronounced. Lacelike networks of molecules linked together by the drug were observed. In many areas on the electron microscope grid, no individual molecules remained unincorporated into the network (Fig. 6e). Similar results were obtained with **1a**, **1b**, and **1c** but not with **1d–1g**.

DISCUSSION

Biochemical evidence indicates that nucleic acids represent one of the principal cell targets in the mechanism of action of mitoxantrone and bisantrene (11, 14, 19) and that the binding of these drugs to DNA accounts, at least in part, for the antitumor properties (18, 26). However, the exact nature of the modes of binding has been controversial. Although both drugs bear the requisite planar aromatic chromophore recognized as an essential requirement for intercalation (27), the presence of the two extended side chains on positions 1 and 4 precludes smooth incorporation of all parts of the molecule. This suggestion is supported by the observed increased intercalative binding of the less hindered ethidium molecule (Fig. 5a). Various explanations have been proposed for this phenomenon, including partial intercalation and external binding for mitoxantrone and bisantrene (18, 26).

The electron microscopy experiments employing pBR322 provide clear evidence of substantial intercalation with concomitant increases in average length of the three different topological forms of the DNA for bisantrene. Similarly, pBR322 in the form of nicked circles and linears shows a pronounced increase in length characteristic of intercalative binding when treated with mitoxantrone. An apparent difference in behavior of the two drugs was noted at this stage. When supercoiled pBR322 molecules were measured, it was found that bisantrene produced the same increase in length as it did for linear and relaxed molecules. However, with mitoxantrone there was no *apparent* length increase for CCC molecules. As the experiments outlined below indicate, it is most likely that mitoxantrone does intercalate into CCC-DNA but, in so doing, it increases the amount of supercoiling and compacting of the DNA. In terms of over-all measured length, one effect is evidently offset by the other.

The independent experiments with PM2-DNAs—both nicked, (open-circular) and supercoiled (CCC) treated with topoisomerase—and with the controls treated with ethidium confirm the expected length increase for the different topological forms of the DNA expected for intercalation by the mitoxantrone.

Foye and co-workers (26) have observed a marked preference for GC pairs in the binding of mitoxantrone to DNA (26). The observed binding of ~580 molecules of mitoxantrone at saturation to the 4382 base pairs of the sequence (22) of plasmid pBR322 may be compared with the number of adjacent —GC— and —CG— sites in the DNA:

—GC—	310 sites separated by two or more base
::	pairs and 59 sites separated by zero base
—CG—	pairs
—CG—	283 sites separated by two or more base
::	pairs and 34 sites separated by zero base
—GC—	pairs

The combination of these alternative sites is (a) 418 potential intercalation sites separated by two or more base pairs and (b) 507 binding sites separated by zero or more base pairs. Assuming non-nearest neighbor intercalation, this may represent saturation of the available binding sites and additional external binding of the drug to the DNA, a mechanism which has been suggested previously (18). The latter mode of binding plausibly occurs by electrostatic attraction of the positively charged side chains (**1** and **2** have two principal basic sites each) to the phosphate residues on the outside of the duplex.

The experiments with mitoxantrone and the two DNAs also revealed the unique property of this drug to cause extensive inter-DNA cross-links leading to compaction into networks of linked molecules. These observations provide experimental verification of previously suggested compaction and distortion of chromatin by mitoxantrone (17, 18). Examination of the behavior of seven mitoxantrone analogues (**1a–g**) reveals the following structural requirements for DNA network formation. A necessary but not sufficient requirement is to have two OH groups in ring C of the chromophore. An additional requirement is to have two basic groups in the side arms separated by two carbon atoms.

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