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The Antitumor Agent Mitoxantrone Binds Cooperatively to DNA: Evidence for Heterogeneity in DNA Conformation†

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ABSTRACT: The equilibrium binding of the antitumor compound DHAQ, or mitoxantrone [1,4-dihydroxy-5,8-bis[[2-[(2-hydroxyethyl)amino]ethyl]amino]-9,10-anthracenedione], to various DNAs has been examined by optical titration and equilibrium dialysis methods. At low r (bound drug/DNA base pair) values, $r < 0.03$, DHAQ binds, in a highly cooperative manner, to calf thymus and *Micrococcus lysodeikticus* DNAs. The binding isotherms for the interaction of DHAQ with *Clostridium perfringens* DNA and poly(dA-dT)·poly(dA-dT) exhibit a small positive slope at low r values, suggestive of cooperative binding. In contrast, the binding of DHAQ to poly(dG-dC)·poly(dG-dC) shows no evidence of cooperative binding even at very low r values. At higher r values ($r > 0.05$), the binding of DHAQ to all the DNAs studied is characterized by a neighbor-exclusion process. A model is proposed to account for the two modes of binding exhibited in the cooperative binding isotherms. The main feature of the proposed model is that local sequence and structural heterogeneity of the DNA give rise to sets of binding sites to which DHAQ binds in a highly cooperative manner, while the majority of the DNA sites bind DHAQ via a neighbor-exclusion process. This two-site model reproduces the observed binding isotherms and leads to the conclusion that DHAQ binds in clusters to selected regions of DNA. It is suggested that clustering may play a role in the physiological activity of drugs.

The anticancer compound DHAQ,[1] mitoxantrone [1,4-dihydroxy-5,8-bis[[2-[(2-hydroxyethyl)amino]ethyl]amino]-9,10-anthracenedione], shown in Figure 1, was first synthesized by Zee-Cheng and Cheng (1978) and independently synthesized by Murdock et al. (1979). The drug has proven pharmacological activity in a number of tumor model systems (Wallace et al., 1979; Von Hoff et al., 1981; Drewinko et al., 1983) and has undergone phase I (Von Hoff et al., 1980) and phase II clinical trials (Anderson et al., 1982). Although the reason for the effectiveness of DHAQ is unknown, physicochemical studies have shown that DHAQ is capable of binding to DNA in vitro and of binding preferentially to DNA and RNA in intact cells, of increasing the stability of DNA to thermal denaturation, of unwinding covalently closed circular DNA, and of causing the cooperative condensation of nucleic acids at high concentrations (Kapuscinski et al., 1981; Johnson et al., 1979). Additional evidence from cell culture studies (Nishio & Uyeki, 1983) shows that DHAQ is an effective

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[1] Abbreviations: DHAQ (mitoxantrone), 1,4-dihydroxy-5,8-bis[[2-[(2-hydroxyethyl)amino]ethyl]amino]-9,10-anthracenedione; TLC, thin-layer chromatography; EDTA, ethylenediaminetetraacetic acid.

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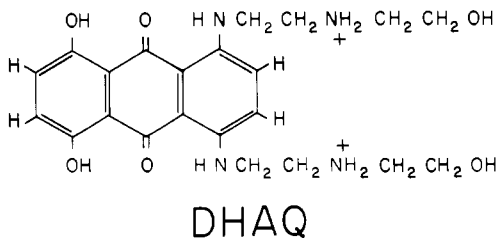

FIGURE 1: Chemical structure of DHAQ (mitoxantrone).

inhibitor of DNA replication and that DHAQ can cause gross structural changes in chromatin (Traganos et al., 1980; Au et al., 1981). This body of information strongly suggests that the binding of DHAQ to DNA is an important factor in its mechanism of action. We have examined the binding of DHAQ to several naturally occurring DNAs, from *Micrococcus lysodeikticus*, *Clostridium perfringens*, and calf thymus, as well as to the synthetic polynucleotides poly(dG-dC)·poly(dG-dC) and poly(dA-dT)·poly(dA-dT). DHAQ binds to *C. perfringens* and calf thymus DNAs in a highly cooperative manner as evidenced by positive slope in the low r value portion of the Scatchard plot binding isotherm.

Cooperative binding of drugs at low levels of bound drug has been observed for many diverse drug–DNA systems: adriamycin + actinomycin D and poly(dA-dT)·poly(dA-dT) (Krugh & Young, 1977); actinomycin D and either calf thymus DNA or poly(dG-dC)·poly(dG-dC) (Winkle & Krugh, 1981); netropsin or distamycin and calf thymus DNA (Hogan et al., 1979; Dattagupta et al., 1980); adriamycin or daunorubicin and calf thymus DNA (Graves & Krugh, 1983a,b); ethidium, actinomycin D, actinomine, and adriamycin with left-handed (Z) forms of poly(dG-dC)·poly(dG-dC) and poly(dG-m^{5}dC)·poly(dG-m^{5}dC) (Walker et al., 1985a,b; Krugh et al., unpublished results). We have asked whether conformational heterogeneity of regions within DNA plays a crucial role in the cooperative binding of specific ligands. A model to account for the binding to the two different types of sites was developed to address this question.

Several explanations have been offered to account for the observed ability of various small molecules to bind to DNA in a cooperative fashion. Cooperative, noncooperative, anticooperative, and neighbor-exclusion effects may be manifested when either adjacent or closely bound ligands interact. For example, McGhee and von Hippel (1974) and Zasedatelev et al. (1971) have derived equations to account for cooperative binding that results from ligand–lattice–ligand interactions. In these models an isolated ligand binds to the lattice with an apparent association constant K. Subsequent ligand binding to adjacent sites is affected because the association constant is changed to ωK, where ω is related to the change in free energy of contiguously bound ligands. For positive cooperativity ($\omega > 1$) a humped Scatchard plot is obtained, in which the degree of curvature is related to the magnitude of ω.

It is also the case that humped Scatchard plots are consistent with an allosteric transition binding model in which the ligand alters the solution structure of the DNA. This alteration is propagated along the helix, converting adjacent sites to the altered form. Crothers and co-workers have shown that the DNA binding of two basic oligopeptides, distamycin and netropsin, can be interpreted in terms of a drug-induced allosteric transition of the solution structure of the DNA (Hogan et al., 1979; Dattagupta et al., 1980). In general, humped Scatchard plots of the form presented in this work may be reproduced by both the allosteric binding model and the two-site binding model. The essential difference between the two models is whether there is an allosteric binding process involving the entire DNA molecule or whether the conformational heterogeneity of the DNA gives rise to a limited number of special sites that preferentially interact with ligands such as DHAQ. We acknowledge, in advance, that the present experiments do not provide definitive support for the concept of local site heterogeneity (i.e., the two-site model), but we offer this alternative for interpreting cooperative binding isotherms to stimulate future experiments.

Materials and Methods

Materials. 1,4-Dihydroxy-5,8-bis[[2-[(2-hydroxyethyl)-amino]ethyl]amino]-9,10-anthracenedione (DHAQ) was obtained from Dr. H. Cox, University of Kansas Medical Center. Additional material was made available through the Chemical Resources Program of the National Cancer Institute and was provided by M. Leaffer, SRI International. All material was checked for purity by TLC according to a protocol kindly provided by M. Leaffer: KC_{18} (Whatman) plates, 4.4 M NH_4CO_2H, pH 4/acetonitrile/H_2O solvent in a ratio of 2:1:1, R_f 0.36; cellulose F (E. Merck) plates, n-BuOH/HOAc/EtOH/H_2O solvent in a ratio of 4/1/2/3, R_f 0.35. Radiolabeled DHAQ was made available by Dr. Robert Engle, Chemical Resources Program, National Cancer Institute, and was provided by M. Leaffer and M. Fong, SRI International. The radiolabeled DHAQ had ^{14}C substituted for each of the 12 carbon atoms in the two aromatic rings for a specific activity of 8.1 mCi/mmol = 18.2 μCi/mg. All drug solutions were prepared fresh on the day of an experiment from dry material stored at −20 °C and were filtered through 0.22-μm Millipore GV filters to remove dust or particulate matter. The DHAQ solid was dissolved in a standard phosphate buffer (0.029 M NaH_2PO_4, 0.023 M Na_2HPO_4, 0.001 M Na_2EDTA, pH 7) with either 0.1 M NaCl, ionic strength μ = 0.2, or 0.2 M NaCl, μ = 0.3, or 0.5 M NaCl, μ = 0.6.

Calf thymus DNA was obtained from Worthington, and *M. lysodeikticus* and *C. perfringens* DNA were obtained from Sigma Chemical Co. These naturally occurring DNAs were prepared according to the procedure of Chaires et al. (1982). Briefly, the DNA was sheared to an average length of approximately 5000 base pairs (bp). The DNA was then treated with T_1 RNase (Sigma) and with proteinase K (Beckman) to remove residual RNA and/or protein contaminants. The DNA was then extracted with phenol/chloroform/isoamyl alcohol (24:24:2) to remove protein and subsequently extracted with diethyl ether and ethanol precipitated. The synthetic polynucleotides poly(dA-dT)·poly(dA-dT) and poly(dG-dC)·poly(dG-dC) were obtained from P-L Biochemicals/Pharmacia and were used without further purification. DNA concentrations were determined absorptiometrically by using ϵ_{260}(calf thymus DNA) = 13 200 Mbp^{-1} cm^{-1}, ϵ_{260}(*M. lysodeikticus*) = 14 000 Mbp^{-1} cm^{-1}, ϵ_{260}(*C. perfringens*) = 12 600 Mbp^{-1} cm^{-1}, ϵ_{254}[poly(dG-dC)·poly(dG-dC)] = 14 400 Mbp^{-1} cm^{-1}, and ϵ_{260}[poly(dA-dT)·poly(dA-dT)] = 13 200 Mbp^{-1} cm^{-1}.

Spectrophotometry. Spectrophotometric analyses were performed on a Cary 219 spectrophotometer. The molar extinction coefficients for DHAQ at 660 nm and at 610 nm were determined by serially titrating a stock solution of drug into buffer. The concentration of the stock drug solution was determined during preparation by weighing dry (72-h vacuum-desiccated at room temperature) DHAQ and dissolving into a given volume. The extinction coefficient at 660 nm of free DHAQ, ϵ_{660}, was determined to be 19 500 M^{-1} cm^{-1}, and the extinction coefficient at 610 nm, ϵ_{610}, was determined to be 16 200 M^{-1} cm^{-1}. The extinction coefficient of the bound DHAQ was determined by two separate methods. In the first

method, a plot of the reciprocal change in absorbance vs. the reciprocal of the DNA phosphate concentration was constructed and extrapolated to infinite DNA concentration to determine ϵ_{bound}. The extinction coefficient at 660 nm for DHAQ bound to the naturally occurring DNAs, calf thymus, *M. lysodeikticus*, and *C. perfringens*, was found equal to 10200 M^{-1} cm^{-1} while for the synthetic polynucleotides, poly(dG-dC)·poly(dG-dC) and poly(dA-dT)·poly(dA-dT), the extinction coefficient at 660 nm for bound DHAQ was found equal to 9800 M^{-1} cm^{-1} and 10000 M^{-1} cm^{-1}, respectively. In the second method, a plot of ϵ_{app} vs. the ratio of $[DHAQ_{total}]/[DNA\ base\ pairs]$ was constructed following the method of Walker et al. (1985a,b). These plots demonstrate a pronounced curvature when cooperative binding is seen. The value of ϵ_{bound} was determined by extrapolating from the minimum value to infinite DNA concentration attained in the curve. The values for ϵ_{bound} determined by the second method agreed with those determined by the first method to within ±5%. This amount of uncertainty in ϵ_{bound} does not change the shape of the binding isotherms, but it does alter the values of r/C_f and r. To illustrate this point, the effect of a ±5% uncertainty in ϵ_{bound} is indicated in the binding isotherm for *C. perfringens* reported in this paper (see Figure 5).

Optical titrations were performed by using a 10-cm quartz cell, wherein a DNA solution of known volume and concentration was titrated with a concentrated solution of DHAQ. The absorbance was read directly from the digital display of the spectrophotometer. The concentration of free DHAQ was always kept below 10^{-6} M as substantial drug aggregation can occur when [DHAQ] > 1 μM. In these experiments it was also important to maintain [free DHAQ] < 1 μM insofar as DHAQ acts as a DNA condensing agent (Kapuscinski et al., 1981). Recently, it has been shown (Kapuscinski & Darzynkiewicz, 1985) that DHAQ can cause the condensation of poly(dA-dT)·poly(dA-dT) at a free ligand concentration of 26.5 μM and of calf thymus DNA at a free ligand concentration of 14.7 μM. All solutions were prepared with free ligand concentrations below that required for DNA condensation. It is of the utmost importance when the optical titration is performed in a 10-cm cell at very low concentration of drug that the solutions be scrupulously free of dust. All buffer and ligand solutins were filtered through 0.22-μm Millipore filters, and DNA solutions were centrifuged prior to use.

Equilibrium Dialysis. For these experiments either a two- or a three-chambered equilibrium dialysis apparatus was used. Each apparatus had a capacity for 12 separate sets of samples. When the three-chambered apparatus was used, the central chamber was loaded with a concentrated solution of radiolabeled DHAQ while the two outer chambers were loaded with identical DNA solutions. When the two-chambered apparatus was used, DNA was placed on one side of the membrane, and radiolabeled DHAQ was placed on the other side. The volume of sample (2 mL) placed in each chamber in any experiment was the same. The ratio of DHAQ to DNA base pairs was varied in each set of 10 samples. Two sets of samples served as controls and were comprised of DHAQ equilibrated against buffer. The entire equilibrium dialysis apparatus was placed on a wrist-action shaker (Burrell) in a constant-temperature room maintained at 22 °C. At various times after the start of the experiment 0.200-mL aliquots were withdrawn from the control samples, placed into scintillation vials containing 10 mL of scintillant, and counted. Equilibrium was assumed to be reached when the concentration of DHAQ was the same within each chamber of the two sets of control samples. A time of 96 h was found to be optimal for equilibration when

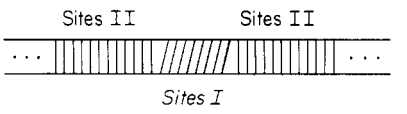

FIGURE 2: A highly schematic drawing illustrating the concept of two different types of DNA binding sites for DHAQ.

Spectrapor No. 6 membrane tubing was used (molecular weight cutoff of 50000). At the end of 96 h the 10 sets of samples were analyzed by withdrawing a 0.200-mL aliquot from each chamber, placing into a vial containing 10 mL of scintillant, and counting. For each set of 12 samples a separate standard curve of counts vs. DHAQ concentration was run to determine the specific activity of the drug at the time of each experiment. In order to minimize loss of radioactive material during transfer, all pipets and pipet tips were siliconized and/or rinsed with cold DHAQ. Drug loss due to binding to the membrane did occur as the membranes were stained blue by the drug; this problem was more severe with dialysis membrane of 10000 molecular weight cutoff. No correction was deemed necessary to account for drug deposition on the membrane other than to ensure equilibration by maintaining a constant equilibration time of 96 h.

Technical Considerations. Since neither free DHAQ nor DHAQ bound to DNA fluoresces or demonstrates a circular dichroism spectrum above 300 nm, we have been unable to exploit these powerful spectroscopic techniques in our study of the interactions of DHAQ with DNAs. A phase-partition technique (Graves & Krugh, 1983a,b) using the ^{14}C-radiolabeled compound was employed with limited success. Numerous organic solvents were examined (straight-chain alcohols of 2–10 carbons, ketones, alkanes, halogenated alkanes), either singularly or in combinations, all of which demonstrated partition coefficients (organic/aqueous) of less than 0.1. In order to increase the partition coefficient, we employed a solute-enhanced phase-partition technique (Krugh et al., 1981) in which varying amounts of sodium tetraphenylborate (NaTPB) were added to the organic phase (1-nonanol). The addition of NaTPB to the system increased the partition coefficient from 0.09, for 1-nonanol–no NaTPB, to as high as 94, 1-nonanol–5.0 mM NaTPB. However, the binding isotherms generated from these experiments were internally inconsistent, such that the intercept on the r/C_f axis was dependent upon NaTPB concentration, suggesting a strong ion-pair formation between DHAQ and TPB in these solutions. In light of these problems the phase-partition experiments were not pursued further.

Two-Site Binding Model Analysis. Naturally occurring DNA represents an inherently complex, heterogeneous lattice of binding sites. The two-site binding model recognizes this inhomogeneity by assuming there to be two independent sets of binding sites (regions) distributed on DNA. These two sets of binding sites are labeled sites I and sites II. This concept is illustrated in Figure 2. Although the equations that describe this model, see below, are written in a general format, in this paper we will refer to the sites that bind DHAQ in a positive cooperative manner as sites I and those sites that bind DHAQ in a noncooperative neighbor-exclusion process as sites II. The size of each set of cooperative binding sites and the distribution of these sites along the helix do not enter into the model except that end effects have been assumed to be negligible. However, the fraction of the total base pairs that bind in a cooperative manner is required as input for the calculations since this determines the relative concentrations of the sites.

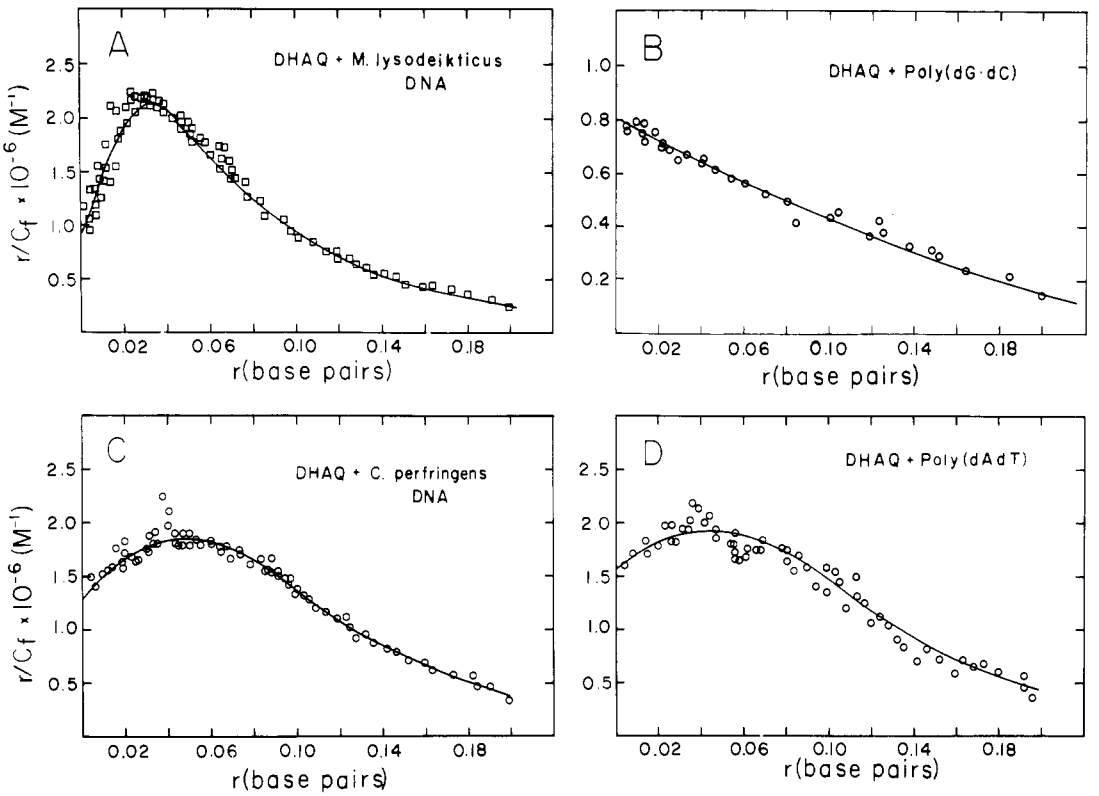

FIGURE 3: Scatchard plots for the equilibrium binding of DHAQ by various DNAs. The binding data were obtained by serially titrating drug into DNA and by monitoring the change in absorbance at 660 nm. The solid line is the best-fit approximation of the two-site binding model: (A) *M. lysodeikticus* DNA, 72% G+C content; (B) poly(dG-dC)·poly(dG-dC), 100% G+C content; (C) *C. perfringens* DNA, 23% G+C content; (D) poly(dA-dT)·poly(dA-dT), 0% G+C content. The solutions are in the standard phosphate buffer with 0.1 M NaCl.

We have used eq 15 of McGhee and von Hippel (1974) to fit the observed binding isotherms for the interaction of DHAQ with DNA. This equation is

$$r/C_f = k(1 - nr) \times \left[\frac{(2\omega - 1)(1 - nr) + r - R}{2(\omega - 1)(1 - nr)}\right]^{n-1}\left[\frac{1 - (n + 1)r + R}{2(1 - nr)}\right]^2 \quad (1)$$

where

$$R = \{[1 - (n + 1)r]^2 + 4\omega r(1 - nr)\}^{1/2} \quad (2)$$

K is the observed binding constant, ω is the cooperativity parameter, C_f is the free drug concentration, and n is the site-exclusion distance. With ω equal to 1, eq 1 reduces to the neighbor-exclusion equation [eq 10 of McGhee & von Hippel (1974)]. For convenience in our computer program we have used eq 1 to fit both the cooperative and noncooperative portions of the binding isotherms. For the noncooperative binding a value of ω equal to 0.99 or 0.995 is used to prevent the denominator from going to zero. The reader may also wish to refer to the supplementary material for additional detail (see paragraph at end of paper regarding supplementary material). The parameters used in this binding model are K_I and K_{II}, the apparent intrinsic association constants, ω_I and ω_{II}, the cooperativity parameters, n_I and n_{II}, the nearest-neighbor exclusion distances for drug intercalation, and P, the percentage of the total base pairs that are cooperative binding sites. We have arbitrarily designated the cooperative sites as type I sites and the noncooperative sites as type II sites.

In order to produce a theoretical fit to an experimental binding isotherm, the concentration of bound drug at each of the two binding sites must be calculated and then summed to provide the total concentration of bound drug. The simultaneous equilibria require that the concentration of free drug seen by type I sites and type II sites be the same. We have written a computer program that requests initial estimates for K_I, ω_I, n_I, K_{II}, ω_{II}, and n_{II}; assuming a starting value of r_I, a value of C_f may be calculated from eq 1 by iteration. A value for r_{II} is subsequently determined by using the calculated value for C_f and the values for K_{II}, ω_{II}, and n_{II}. At any point in the binding isotherm, the observed binding equilibrium is a function of concentration of drug bound to both type I sites and type II sites, as well as the free drug concentration. The calculated values are summed such that

$$r_I + r_{II} = r_{total} \quad (3)$$

and

$$r_I/C_f + r_{II}/C_f = r_{total}/C_f \quad (4)$$

The calculated values, r_{total} and r_{total}/C_f, are then plotted and compared to the observed binding isotherm. This process is repeated until one obtains a good visual match between the experimental and calculated isotherms. No attempt was made to perform a rigorous least-squares regression analysis to optimize the parameters. We want to emphasize that our intention is to illustrate the viability of the two-site model as a plausible explanation for the humped Scatchard plots observed by us and others for a variety of ligand–DNA complexes.

Results and Discussion

In previous papers concerning the interaction of intercalating ligands with various DNAs we have shown that the optical titration method provides reliable data at very low values of bound drug (Winkle et al., 1982; Walker et al., 1985a,b). The binding of DHAQ to *M. lysodeikticus*, calf thymus, and *C. perfringens* DNA and to the two synthetic polynucleotides, poly(dA-dT)·poly(dA-dT) and poly(dG-dC)·poly(dG-dC), has

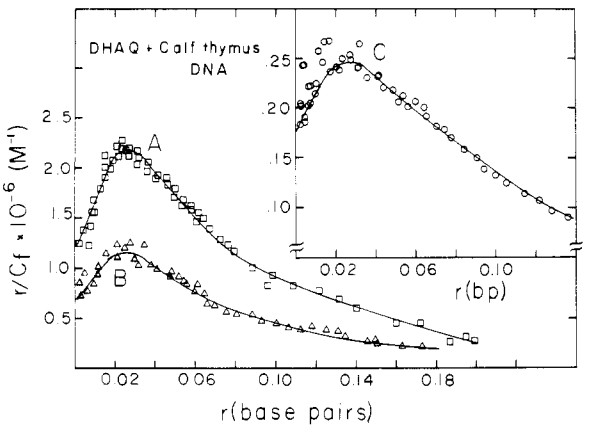

FIGURE 4: Ionic strength dependence of the equilibrium binding of DHAQ by calf thymus DNA. The data shown in these binding isotherms were obtained by monitoring the absorbance at 660 nm. Varying amounts of NaCl were added to the standard phosphate buffer: (A) 0.10 M NaCl; (B) 0.20 M NaCl; (C) 0.50 M NaCl.

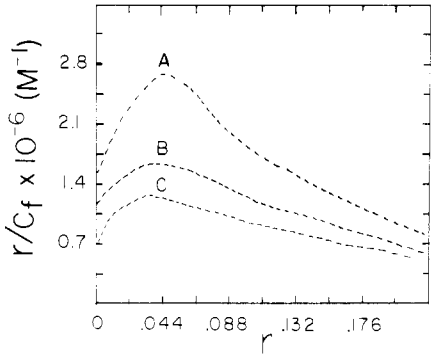

FIGURE 5: Scatchard analyses of the binding of DHAQ to *C. perfringens* DNA. The value for the extinction coefficient at 660 nm of bound drug is varied by ±5% to illustrate the possible effects of experimental uncertainty upon the shape of the binding isotherm: (A) ϵ_{bound} = 10 700 M^{-1} cm^{-1}; (B) ϵ_{bound} = 10 200 M^{-1} cm^{-1}; (C) ϵ_{bound} = 9700 M^{-1} cm^{-1}.

been examined by an optical titration technique. The absorption data were rendered as binding isotherms plotted according to the method of Scatchard (1949), as shown in Figures 3 and 4. The initial increasing slope in the Scatchard plots at low values of r, the ratio of bound drug to total DNA base pairs, is indicative of cooperative binding of DHAQ by DNA. The difference in the shapes of the binding isotherms obtained in the experiments reported here, i.e., concave vs. linear, provides confidence that the humped Scatchard plots are not the result of some systematic artifact.

As noted under Materials and Methods, DHAQ is technically a difficult molecule to study. We have examined the effect that an uncertainty of ±5% in ϵ_{bound} would have upon the shape of the binding isotherms, as shown in Figure 5 for *C. perfringens* DNA. While the magnitudes of the r/C_f and r values are indeed dependent upon the value of ϵ_{bound}, the positive slope in the binding isotherms in Figures 3 and 4 persists for all values of ϵ_{bound} tested.

Equilibrium dialysis was used to provide an independent measure of the binding isotherms, as shown in Figure 6 for calf thymus DNA. While it is reassuring that these data exhibit a positive slope at low r values as do the optical titration data, we note that the Scatchard plots generated by using these two techniques differ in the magnitude of the free drug concentration such that the r/C_f values are substantially smaller for the equilibrium dialysis data (e.g., maximum values of 0.38 × 10^6 M^{-1} vs. 2.3 × 10^6 M^{-1}). Although the uncertainties inherent in these experiments are sufficiently large that the

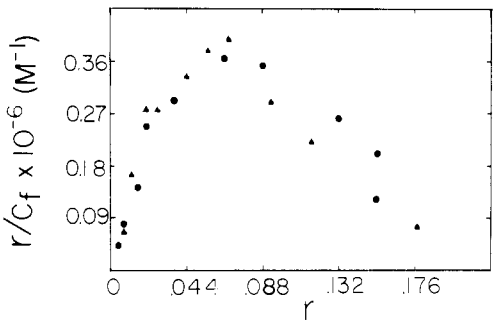

FIGURE 6: Binding isotherm of the interaction of DHAQ with calf thymus DNA in 0.1 M NaCl buffer. The symbols ▲ and ● represent two separate experiments in which the data were obtained using an equilibrium dialysis technique.

dialysis data and the absorptiometric data do agree within experimental error, we suspect that there are limitations in the equilibrium dialysis experiment that affect these data. The equilibrium dialysis experiments were performed many times, and the data are reproducible. However, due to severe adsorption to the membranes and slow equilibration (96 h at 22 °C), equilibrium dialysis was considered less reliable than the optical titrations, although the results of the two experiments could be made to agree by adjusting the value of the bound extinction coefficient. We have chosen to present the data without trying to make the data agree with one another. In this paper we focus on the shape of the binding isotherms at low levels of bound drug as evidence for cooperative binding and do not wish to emphasize the magnitude of the binding constants.

Data Analysis. The parameters obtained from fitting the data shown in Figures 3 and 4 to the two-site binding model are given in Table I (in the supplementary material). The affinity constant K_I is the apparent binding affinity of DHAQ for an isolated cooperative binding site. The results in Table I suggest that the value of K_I is relatively independent of the nature of the DNAs studied and that K_I is slightly greater than K_{II}; for example, K_I = 2.0 × 10^6 M^{-1} and K_{II} = (1.2 − 1.7) × 10^6 M^{-1}. In the present model the cooperativity parameter, ω_I, results from the increase in the free energy of binding of two contiguously bound ligands, which in this case represents two DHAQ molecules bound to DNA and separated by three base pairs. The calculated binding isotherms suggest that the magnitude of ω_I is a function of the G + C content of naturally occurring DNAs and that it tends to fall into two ranges: $\omega \approx 18$ for DNAs with 0–23% G + C content, and $\omega \approx 45$ for DNAs with 40–75% G + C content. However, the synthetic polynucleotide with 100% G + C content, poly(dG-dC)·poly(dG-dC), has $\omega = 1$, so the relationship suggested between the percent G + C content and ω for the three naturally occurring DNAs studied here may be fortuitous.

The two-site binding model computer program calculates the percent occupation of both the cooperative and noncooperative sites for each value of r_{total}. As an example, for *M. lysodeikticus* DNA at an r_{total} of 0.045 the cooperative sites are more than 50% filled while less than 4% of the noncooperative binding sites are occupied. These numbers illustrate a feature of the two-site model, namely, that the drug molecules tend to cluster at low r values into a restricted set of sites. The degree of clustering is a function of the cooperativity parameter, ω; using larger values for the cooperativity parameter results in increased clustering of the ligands (data not shown).

Base Content. The overall shapes of the binding isotherms for the binding of DHAQ by calf thymus (42% G + C) and

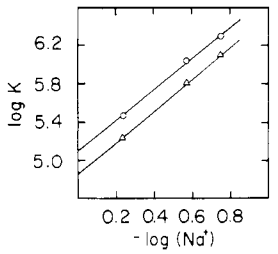

FIGURE 7: A plot of log K vs. the negative log of the sodium ion concentration for the binding of DHAQ to calf thymus DNA. The circles are the K_I values and the triangles are the K_{II} values from Table II (see supplementary material).

M. lysodeikticus (72% G + C) DNAs, Figures 3A and 4A, are qualitatively the same. In contrast, the binding of DHAQ to poly(dG-dC)·poly(dG-dC) showed no evidence of a cooperative interaction, Figure 3B. The apparent association constants for DHAQ binding to the naturally occurring DNAs studied are not very sensitive to the (G + C)/(A + T) ratio of the DNA (Table I in the supplementary material), which is consistent with the data of Richardson and Schulman (1981) and Kapuscinski et al. (1981). Inclusion of poly(dG-dC)·poly(dG-dC) and poly(dA-dT)·poly(dA-dT) leads to the conclusion that DHAQ associates more weakly with (A + T)-containing polymers as noted by Kapuscinski et al. (1981).

The observed binding isotherms for both poly(dA-dT)·poly(dA-dT), Figure 3D, and *C. perfringens* DNA, Figure 3C, are considered to deviate from a neighbor-exclusion isotherm only slightly more than our estimated experimental error, and thus caution must be exercised in the interpretation of the data for these two DNAs. We note that poly(dA-dT)·poly(dA-dT) is an unusual DNA in that it may readily form hairpin loops and may exist in an alternating B-form structure under low-salt conditions (Cohen et al., 1981; Shindo et al., 1980). It is interesting to note that association of nonintercalating ligands with poly(dA-dT)·poly(dA-dT) appears to induce a conformational change such that the dinucleotide repeat nature of the polynucleotide is enhanced, e.g., netropsin (Patel & Canuel, 1977), 3α,5β,17β-dipyrandenium (Patel et al., 1981), and *meso*-tetrakis(4-*N*-methylpyridiniumyl)porphine (Carvlin, unpublished results). Thus, either the apparent small positive slope in the initial portion of the binding isotherm, Figure 3D, results from DHAQ binding to a region of poly(dA-dT)·poly(dA-dT) that is structurally different from the bulk of the polymer or it arises from an allosteric transition in the structure of the polymer as noted for distamycin binding to various DNAs (Hogan et al., 1979) or it may be due to experimental uncertainty in the data.

Effect of Salt. The interaction of the antitumor drug DHAQ with calf thymus DNA has been studied as a function of the sodium ion concentration as shown in Figure 4. The parameters from the two-site binding model used to generate the solid lines in Figure 4 are given in Table II (in the supplementary material). As expected, the binding constants decrease (K_I, from 2.0×10^6 M^{-1} to 3×10^5 M^{-1}; K_{II}, from 1.3×10^6 M^{-1} to 1.7×10^5 M^{-1}) as the sodium ion concentration is increased (from 0.18 to 0.58 M). The cooperativity parameter ω_I does not appear to change markedly with increasing sodium ion concentration within the range of 0.1–0.5 M.

The ion-condensation theory of Record et al. (1976) and Manning (1978) may be used to calculate the number of counterions released. We have plotted in Figure 7 the log of K_I and K_{II} vs. the negative log of the total sodium ion concentration; in both cases a linear relationship exists. Using the data in Table II (in the supplementary material), we find that the calculated number of counterions released when DHAQ binds to the cooperative sites, type I sites, is 1.6; a value of 1.7 was obtained for the number of counterions released when DHAQ binds to the noncooperative, type II, sites. DHAQ exists as a dication at neutral pH with the two positive charges on the aliphatic side chains, presumably available to interact with the phosphate groups of the DNA backbone [see, for example, the extension by Wilson & Lopp (1979) of the ion-condensation theory to consider the effect of intercalation]. The present values are consistent with the intercalation of dicationic DHAQ into DNA.

Possible Origins of the Cooperative Binding of DHAQ. The humped Scatchard plots that have been reported in this and other papers are indicative of a cooperative binding process. That the cooperative binding part of the binding isotherm appears to saturate at low levels of bound drug has led to the proposal that special sites exist which bind the ligands differently than does the majority of the DNA, i.e., the two-site model.

Unfortunately, the equilibrium data in themselves cannot provide the basis for determining the molecular interactions that give rise to the cooperativity. Since DHAQ may intercalate into DNA, as do other anthraquinones, the cooperative binding is likely associated with helix-mediated effects. The ability of DNA to adopt sequence-dependent local structures has been established unequivocally by crystallographic structure determination [e.g., see the reviews by Rich et al. (1984), Dickerson (1983), and Kennard (1984)]. Furthermore, Hogan et al. (1983), among others, have demonstrated that the flexibility of DNA is strongly dependent on base composition. Whether the conformational variations are entirely structural or are intimately associated with modes of breathing or temporary instabilities in the helix (Englander et al., 1980; McGhee & von Hippel, 1977; Englander & Kallenbach, 1984) is not known at the present time. Nevertheless, the cooperative binding of DHAQ is felt to exploit the conformational flexibility of the helix. Other work has shown that two very different conformations may coexist within the same DNA molecule (Peck et al., 1982; Selsing et al., 1979; Quadrifoglio et al., 1982) and that intercalating ligands may cause a highly cooperative interconversion between different conformations, such as the ethidium-induced conversion of Z-form DNA to B-form DNA (Pohl et al., 1972; Walker et al., 1985a,b). However, it is not necessary to suggest that the cooperative binding is associated with regions of such profoundly different structure as Z-form DNA (Pohl et al., 1972; Wang et al., 1979); rather, it is likely that more subtle differences in local conformation are involved.

Interaction between bound DHAQ molecules in the groove of the helix appears to us to be an unlikely explanation for the cooperativity we have observed. However, we note that Kapuscinski and Darzynkiewicz (1985) provide evidence for DHAQ-mediated DNA consensation occurring as the result of the bound drug interactions. Although this effect is cooperative, we believe that the source of the cooperativity is distinctly different in the case of condensation than that which we have have seen for binding at low r values and low drug concentrations. The free drug concentration in our experiments is more than 1 order of magnitude below the critical concentration needed for the onset of DNA condensation observed by Kapuscinski and Darzynkiewicz (1985). Furthermore, we found no evidence for DNA condensation while performing the equilibrium binding experiments, although we were able to induce condensation under the conditions used by Kapuscinski and Darzynkiewicz (1984).

In conclusion, while the present experiments do not prove the validity of the two-site model, we believe the two-site model provides a viable explanation for the humped Scatchard plots observed with selected combinations of ligands and DNAs, although humped Scatchard plots may also be reproduced by an allosteric binding model. The preferential binding of ligands, such as actinomycin D, to selected DNA sequences has been established (Krugh et al., 1979; Van Dyke & Dervan, 1982; Lane et al., 1983); the clustering of drug molecules at certain regions of the DNA results from cooperative binding. These areas deserve an in-depth investigation, since cooperative binding to selected sequences could play an important role in the physiological activity of drugs.

Supplementary Material Available

Details of the calculation of the binding isotherms, parameters obtained from fitting the DNA binding data to the two-site binding model (Table I), and ionic strength dependence of parameters of DHAQ binding to calf thymus DNA (Table II) (2 pages). Ordering information is given on any current masthead page.

Registry No. Poly(dA-dT)·poly(dA-dT), 26966-61-0; poly(dG-dC)·poly(dG-dC), 36786-90-0; mitoxantrone, 65271-80-9.

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