Anthramycin Binding to Deoxyribonucleic Acid–Mitomycin C Complexes. Evidence for Drug-Induced Deoxyribonucleic Acid Conformational Change and Cooperativity in Mitomycin C Binding†

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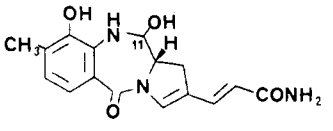
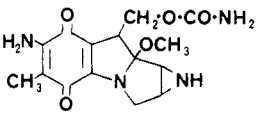
ABSTRACT: Anthramycin and mitomycin C (MC) are two DNA reactive drugs, which bind covalently to GC pairs producing different effects on DNA: anthramycin stiffening and MC distorsion. This paper describes experiments in which we have used anthramycin as a probe to sense quantitatively the effects on DNA of MC binding. Saturation binding experiments show that both anthramycin and MC partially inhibit the binding of the other drug to DNA (maximum inhibition by MC and anthramycin, 22.4% and 19.7%, respectively) but by a mechanism other than direct site exclusion. This suggests that MC binds in the major groove of DNA, since anthramycin is known to bind in the minor groove. An abrupt reduction in the binding of anthramycin to DNA–MC complexes occurs between MC binding ratios of 0.030 and 0.035, which parallels and probably results from sudden intensification of a MC-induced DNA conformational change occurring between these binding ratios. Dialysis measurements indicate that anthramycin is very possibly binding at sites distant from MC sites and suggest a clustering of closely bound MC chromophores resulting from possible cooperative binding. S_1 nuclease digest experiments demonstrate an initial enhancement of nuclease activity in DNA–MC complexes, the magnitude of which correlates well with the reduction of anthramycin binding, relative to the degree of MC binding. The enhanced nuclease activity in these complexes indicates regions of exposed DNA or helix base distortion which is related to or is the result of conformational change.

Anthramycin (I) and mitomycin C (II; MC) are potent



I



II

antitumor antibiotics produced by various *Streptomyces* [I, *S. refuineus* (Tendler & Korman, 1963); II, *S. verticillatus* (Lefermine et al., 1962) and related species (Kirsh, 1967)] which bind covalently to DNA (I, Kohn et al., 1974; Kohn, 1975; Hurley, 1977; Hurley et al., 1979; II, Iyer & Szybalski, 1964) and inhibit nucleic acid synthesis (I, Horwitz & Grollman, 1968; Kohn, 1975; Hurley, 1977; II, Schwartz et al., 1963; Kerstein & Rauen, 1961; Pricer & Weissbach, 1964). While the rate of formation of the covalent adduct with DNA by anthramycin is relatively slow (Kohn & Spears, 1970; Hurley, 1977), mitomycin C's covalent reaction with DNA requires reductive or acidic activation (Iyer & Szybalski, 1964; Lown et al., 1976). Anthramycin shows an absolute specificity for DNA in a double-stranded template (Kohn et al., 1974) and is stable only if the secondary structure of DNA is maintained (Hurley et al., 1979). This is in contrast to MC which will bind to single-stranded DNA and whose covalent interaction with native DNA is stable to denaturing conditions (Tomasz et al., 1974; Lipman et al., 1978). Both anthramycin and mitomycin C demonstrate GC specificity in their binding to DNA (I, Kohn et al., 1974; II, Szybalski & Iyer, 1964b; Tomasz et al., 1974; Lipman et al., 1978). While anthramycin appears to form only one major covalent adduct with DNA (Petrusek et al., 1981; J. M. Ostrander, M. S. Balakrishnan, T. R. Krugh, and L. H. Hurley, unpublished results), mitomycin C binds DNA covalently, both monofunctionally (Szybalski & Iyer, 1964a; Weissbach & Lisio, 1965) and bifunctionally (i.e., cross-linking DNA; Matsumato & Lark, 1963; Iyer & Szybalski, 1963), and also interacts electrostatically with native DNA at binding ratios (drug/base) greater than 0.10 (Lipman et al., 1978). Anthramycin which does not intercalate DNA has been shown to stiffen but not to lengthen the DNA helix (Glaubiger et al., 1974). Recent evidence (J. M. Ostrander, M. S. Balakrishnan, T. R. Krugh, and L. H. Hurley, unpublished results) also demonstrates that anthramycin attaches to the 2-amino group of guanine and lies hidden in the narrow groove of DNA (Hurley & Petrusek, 1979). The point of attachment of mitomycin C to DNA, however, has not yet been identified. Recent studies by D. J. Kaplan and M. Tomasz (unpublished results) demonstrate that MC reduces the DNA radius of gyration through a bending, coiling, or kinking of helical structure, an effect which becomes more apparent, as detected hydrodynamically and through electron microscopy (D. J. Kaplan and M. Tomasz, unpublished results), with increasing molecular weight of DNA in the complex. This effect may well result from an accumulation of conformational changes localized in specific areas of the helix where certain base sequences may be more prevalent (D. J. Kaplan and M. Tomasz, unpublished results).

Since, anthramycin's site of interaction on DNA is well characterized and because anthramycin exhibits specificity, selectivity, and sensitivity to DNA conformation (Kohn et al., 1974; Hurley et al., 1979; Petrusek et al., 1981), we believe anthramycin is potentially a useful probe for monitoring changes in DNA conformation. As previously demonstrated (Hurley et al., 1977), anthramycin has been successfully used in DNA competition drug binding experiments to help discern the number, the number of types, and nature of binding sites in DNA for a series of related drugs. Kohn et al. (1974) have shown, for instance, through similar competition studies, that anthramycin and actinomycin binding to DNA involves similar

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sites of reaction since anthramycin causes the stoichiometric elimination of actinomycin binding sites in DNA. It is therefore evident that anthramycin may serve also as an agent to help identify, more specifically, the sites of interaction in DNA of other drugs such as mitomycin C. Such experiments become even more attractive when one considers that both drugs (anthramycin and MC) bind covalently to GC-rich regions of DNA. Such studies would also prove informative in assessing how two drugs, which impose opposite-like effects in DNA (i.e., I, stiffening, vs. II, bending), effect DNA helical structure, both individually and in combination.

Experimental Procedures

Materials. Native calf thymus DNA from Worthington was dissolved in standard sodium citrate, pH 7.4, precipitated and deproteinized repeatedly with $CHCl_3$ according to the procedure of Marmur (1961), and finally stored in 0.017 M phosphate buffer, pH 7.4. The molecular weight ($\sim 5 \times 10^6$) of this DNA stock was determined previously by viscometry (D. J. Kaplan and M. Tomasz, unpublished results).

Mitomycin C was obtained from Bristol Labs (Syracuse) and was maintained dissolved in CH_3OH–H_2O solution (4.6 mL). An extinction coefficient of 21 840 at 360 nm (Tomasz et al., 1974) was used for concentration determination.

Tritiated *N*-methyl-MC (a gift of Maria Tomasz) was mixed with MC to give a labeled stock, after radiolabel impurity correction, of 4×10^6 dpm/μmol. The molar ratio of MC to drug in the complex was 20:1.

Anthramycin (as anthramycin-11-methyl ether), both unlabeled and tritiated, was prepared as outlined by (Hurley et al., 1979) and stored in distilled methanol. The antibiotic concentration was determined by absorbance at 333 nm by using an extinction coefficient in CH_3OH of 36 800. The specific activity, after radiolabel impurity correction, of the tritiated methylanthramycin was 8×10^5 dpm/μmol.

S_1 nuclease from *Aspergillus oryzae* type III (Sigma) contained 2×10^5 units of enzymatic activity per mg of protein. The enzyme concentration, which was directly used from the vial, was 0.58 mg of protein/mL.

Preparation of DNA Complexes with MC and Anthramycin. The standard reductive activation procedure of Tomasz et al. (1974) was used to make the DNA–MC complexes. However, $NaBH_4$ was used instead of $Na_2S_2O_4$, and it was added, in equimolar amounts relative to the quantity of MC in mixture, in one application via Hamilton syringe to the DNA–mitomycin C mixture. Reaction time was 35 min, and no significant change in pH occurred upon addition of $NaBH_4$. The nucleic acid–antibiotic complex was separated from unbound drug by a Sephadex G-100 column equilibrated with DSC.[1] This same procedure was used for MC complexes formed from DNA–anthramycin complexes.

DNA–anthramycin complexes were made by adding 0.4 μmol of either unlabeled or tritiated anthramycin (in a volume of 43 μL of distilled CH_3OH) to 1 μmol of calf thymus DNA or calf thymus DNA–MC complex (1 μmol of DNA). The reaction was carried out at room temperature for 3 h and left in a cold room overnight at 4 °C for saturation reactions. Unreacted anthramycin was extracted twice with 3 times the amount of water-saturated butanol with intermittent centrifugation. The aqueous phase containing the complex was then dialyzed in dialysis tubing (12 000–14 000 MS cut off; Spectrum Medical Industries, Inc., Los Angeles, CA) against DSC at 4 °C for 21 h.

For time-dependent (kinetic) reactions, the reaction procedure was carried out with mixtures equilibrated and kept at 24.0 ± 0.5 °C. The reactions were then terminated at 5, 15, 30, and 80 min and dialyzed for 21 h under the conditions indicated above.

Determination of DNA–Drug Binding Ratios. (*a*) *DNA–MC Complexes.* The binding ratios (drug/nucleotide) of DNA–MC complexes were determined by one or both of two methods. Spectrophotometric determinations using the Beckman Model 26 spectrophotometer were completed by measuring the DNA absorbance at 260 nm (molar extinction coefficient of 6412 for calf thymus) and absorbance of MC at 310 nm [extinction coefficient of 11 400 (Tomasz et al., 1974)]. The 260-nm absorbance of DNA was corrected for 260-nm drug absorbance by subtracting 1.5 × 310-nm absorbance. Spectrophotometric-radiolabeled determinations of binding ratio required the measurement of ^3H label in a in a Packard Tricarb C2425 liquid scintillation spectrometer. One-milliliter samples (containing ~ 0.2 μmol of DNA) for radioactivity determination were mixed with 20 mL of Aquassure (LSC High Performance cocktail, New England Nuclear), vials were assayed for 20 min or 10 000 cpm (±2% standard deviation), and the disintegrations per minute were calculated from counts per minute and AES by utilizing percent efficiency values from a ^3H AES efficiency quench curve. Micromoles of drug in complex was calculated from disintegration per minute and measured specific activity of drug. This value was then converted to absorbance units from which the appropriate fraction was subtracted from the DNA absorbance at 260 nm. Binding ratios were then subsequently calculated as in the spectrophotometric procedure.

(*b*) *DNA–Anthramycin Complexes.* Binding ratios of DNA–anthramycin complexes were calculated by one of two or both procedures. The spectrophotometric determination was that of Kohn & Spears (1970) utilizing 260 nm for the DNA absorbance and 343 nm for the drug absorbance. Twenty percent of the 343-nm absorbance was subtracted from the 260-nm absorbance to give the DNA absorbance at this wavelength. The extinction coefficient for bound drug at 343 nm was 28 000. Calculation of binding ratios using radiolabeled anthramycin was essentially the same as that described for DNA–MC complexes.

(*c*) *DNA–MC–Anthramycin Complexes.* Binding ratios for DNA complexes containing both bound MC and anthramycin were determined from two combined procedures: spectrophotometric and radiolabel determinations. The average percentage deviation between these two procedures in the calculation of MC and anthramycin binding ratios was 3.7%. The spectrophotometric assessment of binding ratios was determined from the absorbance measurements at 260, 310, and 343 nm for the complexes. These values were then applied to the equations below which were algebraically derived from the spectra of DNA-bound anthramycin (Kohn & Spears, 1970) and DNA-bound mitomycin C (Tomasz et al., 1974) where the ratios of 310- and 343-nm absorbances for each drug is known:

$$\frac{OD_{343_T} - 0.61(OD_{310_T})}{0.7133} = OD_{343_A}$$

$$0.47(OD_{343_A}) = OD_{310_A}$$

$$OD_{310_T} - OD_{310_A} = OD_{310_{MC}}$$

$$0.61(OD_{310_{MC}}) = OD_{343_{MC}}$$

[1] Abbreviations used: DSC, 0.015 M NaCl and 0.0015 M sodium citrate, pH 7.4; LSC, liquid scintillation counting; AES, automatic external standardization.

Numerical subscripts refer to wavelength, T is total, A is anthramycin, MC is mitomycin C, and OD is absorbance. Calculated absorbances derived from these equations for both drugs were then used to calculate DNA absorbance at 260 nm by subtracting their contribution to the total 260-nm OD. Drug binding ratios were then estimated from these final absorbances. As assessed by comparisons with radiolabel determination of anthramycin binding ratio, this procedure was excellent in deriving the anthramycin binding ratios. This procedure required, however, when MC binding ratios below 0.04 were calculated (in complexes where MC is the second drug bound), the necessity of radiolabel determination of MC binding, since the average percent deviations for MC binding between spectrophotometric and radiolabel methods often became greater than 3.7%. No problem, however, was present in measuring MC binding ratios in complexes made first with MC since its binding ratio was known exactly before anthramycin binding and it was shown (Table I) that MC was not released from these complexes.

The radiolabel method for determining binding ratios in complexes containing both drugs followed this procedure: Conversion of moles of radiolabeled drug to theoretical OD (by extinction coefficient) and the subsequent utilization of this value in the derived equations give the calculated absorption of the unlabeled drug. The subsequent binding ratios for both drugs calculated by this procedure compared well, as indicated, with binding ratios calculated solely from OD data.

Assay for General Electrostatic Interactions. The procedure testing for general electrostatic interaction between drug and DNA was the following: The sample in DSC buffer was made 2 M in NaCl and dialyzed in this same solution overnight at 4 °C. Subsequently, the sample was redialyzed in a large volume of DSC buffer with two changes (5 h each), and then the binding ratios were assessed.

Renaturation Assay. The procedure testing for renaturation entailed heat-denaturing control DNA and complexes in thick-stoppered conical centrifuge tubes for 15 min and then quench cooling in an ice bath. Two milliliters of the various complexes (~1 OD in DNA) was used for these experiments. The buffer was DSC, pH 7.4. The binding ratios were assessed as previously described. Measurement of the 260-nm absorbance of DNA before and after gave hyperchromicity values from which comparisons were made to indicate the extent of relative renaturation in complexes containing MC.

S_1 Nuclease Assay. The method and protocol for the S_1 nuclease digestions was similar to that of Vogt (1973) and Hurley & Petrusek (1979). The procedure required the addition of 0.9 mL of buffer (made from 0.1 M sodium acetate, pH 4.6, 0.5 M NaCl, and 0.05 M $ZnSO_4 \cdot 7H_2O$, respectively, in a volume ratio of 3:0.5:1) to 1.1 mL of DNA sample (3 ODs of DNA in DSC). The substrate mixture was then equilibrated at 37.0 °C for 30 min before the initiation of reaction by the addition of 10 μL of S_1 nuclease. To quench the reaction at various time points, 14 mL of 10% perchloric acid was added, and the reaction mixture was cooled to 0 °C. This mixture was then centrifuged at 10000*g* for 15 min at 4 °C, and then the upper 0.4 mL of supernatant was carefully withdrawn and the absorbance at 260 nm read. The 260-nm acid-soluble absorbance from DNA–MC complexes was corrected, at several binding ratios, for residual drug absorbance contribution to total 260-nm absorbance that appeared in the supernatant of the reactions. Values for correction, as a percent of total 260 nm, were obtained by substracting 1.5 × 310-nm absorbance in supernatant from total 260-nm OD.

Table I: Binding Ratios[a] of Anthramycin and MC in Various DNA Complexes

	binding ratios		
		in double complex	
original complex	in original complex	anthramycin	MC
DNA-anthramycin[b]	0.064[b]	0.061[b]	0.107
DNA-anthramycin	0.043	0.041	0.113[b]
DNA-MC	0.068	0.066[b]	0.064
DNA-MC[b]	0.058[b]	0.050[c]	0.057[b]

[a] Binding ratios were determined as described under Experimental Procedures. [b] Radiolabeled drug. [c] This binding ratio is for a 30-min anthramycin reaction.

Table II: Binding Ratios of Complexes after Incubation in 2 M NaCl

			OD's[b]		
complex	binding ratio[a]	λ (nm)	before incubation	after incubation	OD loss
DNA-anthramycin-MC	Anth = 0.070, MC = 0.050	260	0.777	0.777	0.000
		310	0.155	0.147	0.008
		343	0.240	0.227	0.013
DNA-MC[c]	MC = 0.149	260	1.760	1.760	0.000
		310	0.335	0.274[c]	0.061
$NaBH_4$[e] treated DNA-anthramycin	Anth = 0.070	260	0.698	0.698	0.000
		343	0.198	0.194	0.005

[a] Determined as described under Experimental Procedures. [b] Corrected for dilution due to dialysis. [c] A covalent saturated complex also containing electrostatically bound MC chromophores. [d] Computed binding ratio after incubation = 0.113. [e] Two micromoles (DNA) of a DNA-anthramycin complex (0.07 binding ratio) was incubated in 3 mL of DSC with 4 μmol of $NaBH_4$, under the reductive activation procedure outlined (see Experimental Procedures) for 35 min. The complex was then eluted through a Sephadex G-100 column and then subjected to the conditions of salt treatment.

Ninety percent of total 260-nm OD was taken as acid-soluble nucleic acid for DNA–MC complexes of binding ratios of 0.11 and 0.06. Similarly, 98% was the value used for DNA–MC binding ratios near 0.03.

Results

Stability of Bound Anthramycin and MC in DNA after Reaction with the Other Drug. Table I shows some of the binding ratios of anthramycin and MC in DNA before (in original complex) and after (in double complex) reaction of these respective complexes with the other drug. As shown, both drugs are retained in their respective complexes after reactions with the other drug. The interchange in the use of unlabeled vs. labeled drugs does not make any difference in the measured stabilities, a further support for the validity of the OD and radiolabeled methods in determining binding ratios in complexes containing both drugs. Calculations, furthermore, of antibiotic to base ratios based upon the OD or combined radioactivity–OD measurements produced essentially identical results.

Characteristics of DNA–Anthramycin and –MC Complexes. In Table II is shown the OD loss at 310 and 343 nm, respectively, for bound MC and anthramycin, after incubation of various complexes in 2 M NaCl. As shown, a DNA–MC complex of near 0.15 binding ratio loses a large portion of its drug when incubated in 2 M NaCl. This is expected since significant electrostatic binding of MC to DNA occurs at binding ratios greater than 0.10. The DNA–anthramycin–MC

Table III: Hyperchromicity of DNA Complexes following Thermal Denaturation

complex	binding ratio [a]	λ (nm)	OD's before heating	OD's after heating	hyperchromicity (%)
native DNA		260	1.153	1.468	+27.3
DNA-MC	0.119	260	1.153	1.154	0
		310	0.186	0.205	+10.2
DNA-anthramycin-MC	Anth = 0.067, MC = 0.049	260	0.772	0.818	+6.0
		310	0.156	0.172	+10.3
		343	0.239	0.175	-73.2

[a] Determined as described under Experimental Procedures.

complex reveals a small loss of bound drug as shown by the relatively minor OD losses at 310 and 343 nm. This loss is probably due to a small amount of drugs released during extended dialysis. Likewise, a DNA–anthramycin complex exposed to the reducing agent $NaBH_4$ and subsequently incubated in 2 M NaCl demonstrates no significant loss of anthramycin due either to the conditions of reductive activation used in MC binding or to salt action.

Table III shows the percentage hyperchromicity for various samples subjected to the conditions of thermal denaturation. Native DNA displays a full hyperchromicity of 27.3%, indicating irreversible denaturation. In the presence of MC cross-links, which enables DNA to reversibly denature (Tomasz et al., 1974), DNA–MC complexes display no hyperchromic change. The percentage hyperchromicity in the combined complex of anthramycin and MC is 6%, a value much lower than the 27.3% for the control. Anthramycin does not cross-link DNA and is released from DNA in dilute salt under higher temperatures approaching 100 °C (Kohn & Spears, 1970; Petrusek et al., 1981). As a result, no renaturation occurs. A significant loss of 343-nm absorbance for bound drug is found, which is probably due to heat degradation of bound anthramycin. The 6% hyperchromicity at 260 nm may be partially due to the absorption of the degraded anthramycin in this region. Nevertheless, this 6% value is significantly lower than the 27.3% value displayed by native control DNA and thus, in all probability, indicates that significant MC cross-linking is present and still takes place when MC is bound to a DNA–anthramycin complex.

Saturation Binding of MC to DNA–Anthramycin Complexes. Figure 1 shows the quantity of mitomycin C bound (binding ratio) to DNA–anthramycin complexes of increasing binding ratio. As shown, there is a decrease in MC binding with increasing anthramycin bound in the complex. Linear analysis of the data, based upon several binding experiments for each point, produces a graph showing a decreasing linear relationship between MC binding and the anthramycin binding ratio. The level of maximum inhibition of MC binding (~19.7%) is seen near 0.08 anthramycin binding ratio where it can be estimated that more than three bound anthramycin chromophores block only one MC molecule from binding (e.g., at a 0.060 anthramycin binding ratio, the MC binding ratio is ~0.106, which represents a loss of ~0.019 MC binding ratio unit from the original 0.125; the 0.06/0.019 ratio is greater than 3:1). This stochiometry of inhibition is maintained at all levels of anthramycin binding as expressed by the linearity of the relationship.

Saturation Binding of Anthramycin to DNA–MC Complexes. The results in Figure 2 display the level of saturation binding of anthramycin in DNA–MC complexes of varying binding ratio. With increasing MC binding, there is a gradual decrease in anthramycin binding until a MC binding ratio near 0.035 is reached. Above this, anthramycin binding decreases more sharply. This pronounced drop in anthramycin binding continues until a MC binding ratio of 0.07–0.08 is reached where it again levels off until a final anthramycin binding ratio of 0.062–0.063 (representing a 22.4% inhibition of binding) is obtained. The lower dotted line shows the binding ratios of these same complexes subjected to an additional 21 h dialysis at 4 °C in DSC. As seen, no significant difference in the extent of the ability of anthramycin to dialyze out from control DNA and DNA–MC complexes of various binding ratio during a prolonged period of dialysis is evident. The

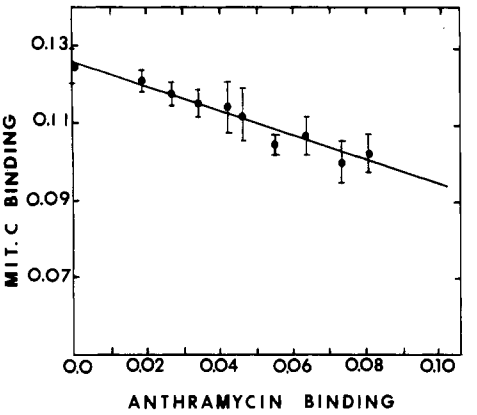

FIGURE 1: Saturation binding ratio of MC in DNA–anthramycin complexes of varying binding ratio. All the MC binding ratios are from a standard reaction procedure where 2 μmol of DNA (DNA–anthramycin) complex is reacted with 4 μmol of MC. Linear analysis was used in deriving the line through the various plotted points. The standard error for each point is also shown. The linear relation possessed a correlation coefficient of ~0.97 with greater than 99.9% degree of certainty. The maximum inhibition of MC binding near 0.08 anthramycin binding ratio is approximately 19.7%.

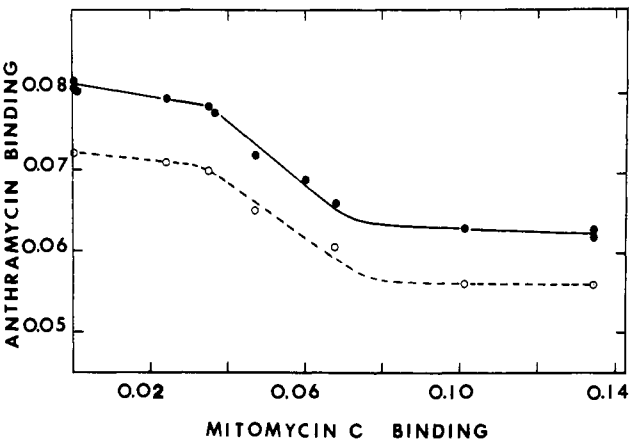

FIGURE 2: Saturation binding ratio of anthramycin in DNA–MC complexes of increasing binding ratio. DNA–MC–anthramycin complexes were prepared and analyzed as described under Experimental Procedures. (●) No additional dialysis; (○) the same complexes subjected to an additional 21-h dialysis in DSC at 4 °C.

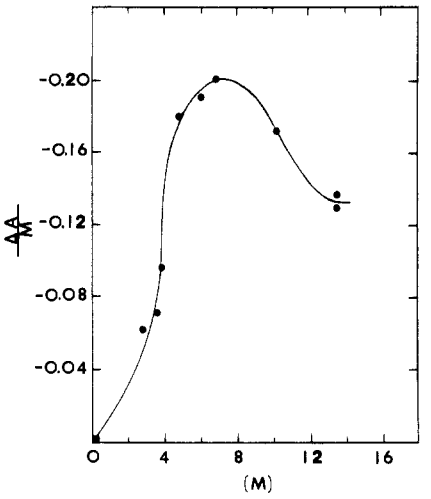

FIGURE 3: Change in the number of anthramycin chromophores bound to DNA per bound MC chromophore. ΔA is the change in the number of bound anthramycin chromophores per 100 nucleotides. M is the number of mitomycin C chromophores bound per 100 nucleotides DNA. $\Delta A/M$ is the change in the number of anthramycin chromophores bound per bound MC chromophore. Data are calculated from Figure 2.

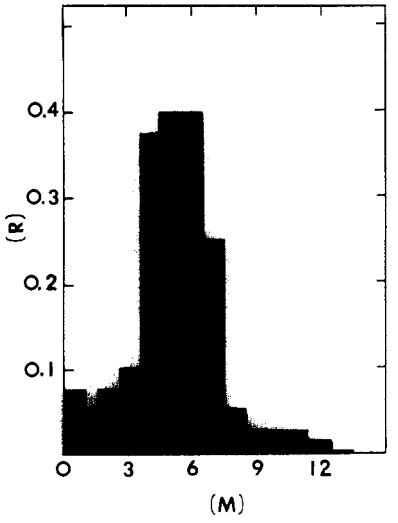

FIGURE 4: Ratio of anthramycin chromophores inhibited from DNA binding per bound MC chromophore [R] at increasing intervals of MC binding per 100 nucleotides [M]. Data are obtained from Figure 2.

stabilizing effect on DNA of the covalently bound MC chromophore (Tomasz et al., 1974) apparently has no significant effect on the ability of anthramycin to slowly dissociate from its DNA complex, suggesting that anthramycin is binding away from MC chromophores.

Displayed in Figure 3 is the inhibition seen in the number of anthramycin molecules binding to DNA per bound MC chromophore in the DNA–MC complex. The nonlinear relationship between anthramycin and MC binding is evident in this figure, as there is a rapid and cooperative-like increase in the inhibition of anthramycin binding in the region of low MC binding. There is a maximum inhibition of 0.2 anthramycin per bound MC chromophore near a MC binding ratio of 0.07. Above this binding ratio, this relationship reverses and plateaus near 0.13 MC binding ratio. The reverse is not a contradiction since any point represents the average decrease in anthramycin binding per bound MC chromophore at that level of MC binding. In Figure 4 this relationship is expressed in terms of the number of anthramycin chromophores blocked from binding to DNA per MC chromophore at various intervals of MC binding. Presented here is a pattern demonstrating how each increment of mitomycin C binding is decreasing the actual, rather than the average, anthramycin binding at each point. As shown, the maximum inhibition of anthramycin binding per bound MC (0.4 unit) occurs between 4.5 and 6.5 MC's per 100 nucleotides. At no point in this relationship, therefore, does any one bound MC block one anthramycin from binding. Like Figure 3, Figure 4 indicates a cooperative-like inhibition of anthramycin at certain extents of MC binding. It must be noted, however, that a maximum inhibition of 0.4 anthramycin chromophore per bound MC demonstrates only the absence of a general pattern by which bound MC chromophores directly exclude the binding to anthramycin to specific binding sites, since some MC binding sites could be at anthramycin sites whereas others might not be.

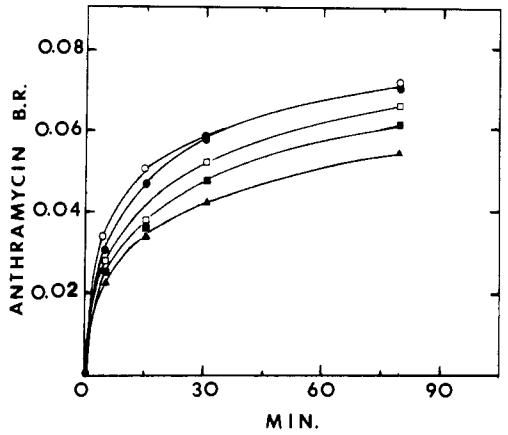

FIGURE 5: Time course of anthramycin binding to DNA–MC complexes of various degrees of binding. DNA–MC complexes were prepared and time course binding of anthramycin followed as described under Experimental Procedures. (○) Control native DNA; (●) DNA–MC, binding ratio = 0.24; (□) DNA–MC, binding ratio = 0.047; (■) DNA–MC, binding ratio = 0.068; (▲) DNA–MC, binding ratio = 0.135.

Kinetics of Anthramycin Binding to DNA–MC Complexes. The relationship of anthramycin binding vs. time for a series of DNA–MC complexes of various binding ratios is displayed in Figure 5. The plateau pattern in the binding ratio per time is evident in all the curves and indicates a saturation of available anthramycin binding sites with time. The findings for control DNA reproduce the results of Hurley et al. (1977). Except for the DNA–MC complex of 0.024 binding ratio where the latter reaction rates coincide with control, it is evident that there is a lower anthramycin reaction rate in DNA–MC complexes of increased binding ratio.

A semilogarithmic plot of the apparent rate constant vs. anthramycin binding ratio for the complexes in Figure 5 is shown in Figure 6. The apparent rate constant (k') (bimolecular constant; Kohn et al., 1974) is a function of the extent of the reaction, r (anthramycin binding ratio); from Kohn et al. (1974), $dr/dt = k'(r)AC(\nu - r)$ where A is the concentration of unreacted anthramycin, C is the total DNA concentration, t is the time of reaction in seconds, and ν (0.21–0.22 unit) is the fraction of nucleotides that are potential binding sites. As shown previously for anthramycin DNA reactions (Kohn et al., 1974) and here for anthramycin reactions with DNA–MC complexes, a plot of $k'(r) = (dr/dt)/[AC(\nu - r)]$ vs. r obeys a consistent function of r for reactions terminated at various times where the apparent rate constant is seen to decrease exponentially with r, a pattern explained by a reduced reaction rate of anthramycin with DNA due to reduced reactivity of the DNA sites after partial

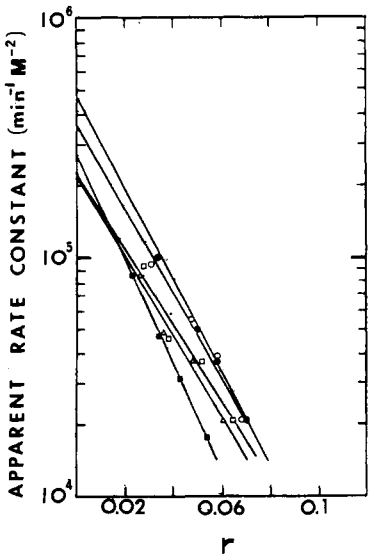

FIGURE 6: Apparent rate constant (k') vs. r (binding ratio) for various DNA–MC complexes. This figure is derived by plotting the anthramycin binding ratio (r) vs. $(r/t)/AC(\nu - r)$. r and t are derived from points plotted in Figure 5. A and C are derived from known DNA anthramycin concentrations in the reaction mixtures of these specific complexes. (●) Control native DNA; (○) DNA–MC, binding ratio = 0.024; (□) DNA–MC, binding ratio = 0.047; (△) DNA–MC, binding ratio = 0.068; (■) DNA–MC, binding ratio ; 0.135. The correlation coefficients for these plots are respectively, in order (as above), –0.991, –0.991, –0.960, –0.989, and 1.000. The average degree of certainty for these plots is approximately 98%.

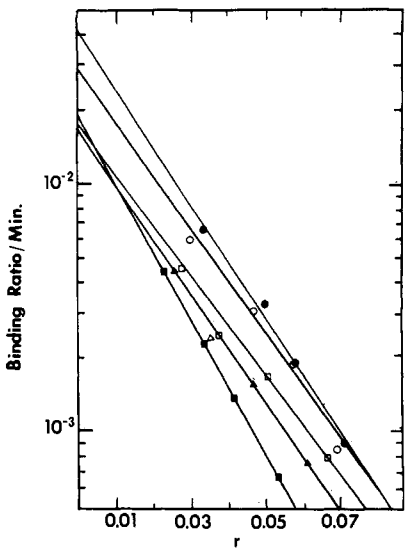

FIGURE 7: Binding ratio/minute vs. binding ratio of anthramycin in DNA–MC complexes. This figure is derived in a similar manner to Figure 6, except only r vs. r/t is plotted. (●) Control native DNA; (○) DNA–MC, binding ratio = 0.024; (□) DNA–MC, binding ratio = 0.047; (△) DNA–MC, binding ratio = 0.068; (■) DNA–MC, binding ratio = 0.135. The correlation coefficients for these plots are respectively, in order (as above), –0.993, –1.000, –0.993, –0.975, and –0.994. The average degree of certainty for these plots is 99%.

reaction with anthramycin (Kohn, 1974). A comparison of the y intercepts in Figure 6, which give the initial apparent rate constants for anthramycin, demonstrates a general decrease (as much as 48%) of this constant in DNAs with greater MC binding. This indicates less available sites for anthramycin binding in DNAs containing bound MC. It must be pointed out, however, that these linear plots are derived from an assumed value of 0.21–0.22 for ν, a value equivalent to the guanine fraction in calf thymus DNA (Sober, 1970). Since anthramycin apparently binds in the minor groove across several bases (Hurley & Petrusek, 1979) it seems most likely, as Kohn (1974) has indicated, that the reaction of anthramycin at one site may interfere with further reactions at nearby sites. The marked slowing down of the anthramycin reaction with time is apparently not due either to the heterogeneity of sites or to preferential reaction at more reactive sites (Kohn et al., 1974). Therefore, it seems appropriate to use the value of 0.21–0.22 for ν in calf thymus DNA, where a final average binding ratio of ~0.08 is indicating a maximum binding condition where bound drugs overlap and block, to a degree, other potential binding sites from further reactions with anthramycin. Quantitatively, however, there is an increment of uncertainty in comparing the plots in Figure 6. This arises since there is some difficulty in estimating a more exact number of available potential binding sites for anthramycin in DNA after it is reacted with MC. Furthermore, estimations of ν are further complicated by considering what effects a conformational change in DNA would have on the total number and spacing of anthramycin binding sites in DNA at various increments of MC binding. In consideration of this problem, plots displaying the relationship of anthramycin binding rate (binding ratio/minute) vs. binding ratio for this same series of complexes (Figure 6) were prepared and are shown in Figure 7. Here, as noted in Figure 6, the ordinate values (binding rate) in these semilogarithmic plots decrease exponentially with r. It is apparent also that the y intercepts, or initial anthramycin reaction rates, drop significantly in the DNA–MC complexes, especially in those of higher MC binding ratio. Furthermore, the y intercepts that may be derived from an extrapolation from defined anthramycin binding ratios (i.e., 0.024, 0.047, 0.065, and 0.135) on the control DNA plot are lower than the actual y intercepts shown for DNA–MC complexes of equivalent MC binding ratio. This further supports a MC inhibition of anthramycin due to a process other than direct site exclusion since a complex containing an amount of anthramycin binding equivalent to a defined MC binding ratio would give, relative to this DNA–MC complex, a much lower y intercept or initial apparent binding rate constant for anthramycin. Support for the validity of these semilog plots, in terms of their linearity and relative spacings of their y intercepts, is derived not only from their high correlation coefficients and high degrees of certainty (see Figure 7) but also from the demonstration that the values for $AC(\nu - r)$ [from $(dr/dt)/[AC(\nu - r)]$] for any series of binding ratios, for a specific complex or control DNA, do not vary significantly relative to the values of dr/dt. This is asserted in view of the fact that the actual linear spacing or y-axis distance in these semilogarithmic plots (Figures 6 and 7) is a logarithm of the $k'(r)$ values, the contribution, therefore, of $AC(\nu - r) = \ln k'(r)$. Since $\Delta \ln (dr/dt)$ is larger than $\Delta \ln [AC(\nu - r)]$, for the extremes of binding, by a factor of approximately 6, as calculated in all complexes and control, it becomes evident that the relative spacing of $\Delta\, dr/dt$ (or y-axis increments) indicates, at the very worst, a relatively good semiquantitative assessment of the relative initial binding rates for anthramycin. This thus provides, in view of the uncertainty of the exact number of anthramycin binding sites in the sample, an alternative and valid method for the analysis of the kinetic results.

A comparison of the final anthramycin binding ratios, the initial anthramycin binding ratio rates, and the relative change in S_1 nuclease sensitivity against the degree of MC binding in DNA–MC complexes is shown in Figure 8. Although some parallelism exists between the final binding and the initial binding rates, a large drop in initial binding rates appears before significant loss of final binding becomes prominent. Suggested here is the presence of kinetically less accessible

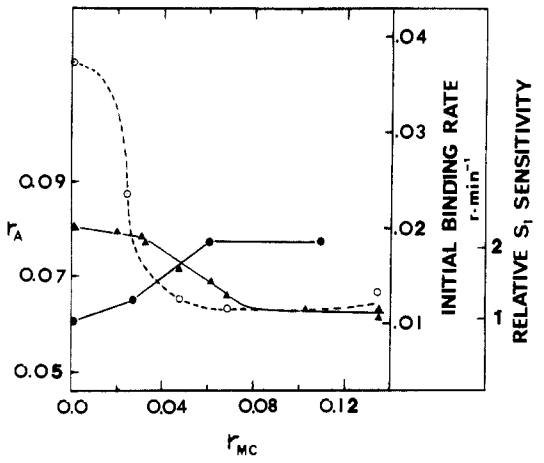

FIGURE 8: Final binding ratio r_A, initial binding rates (binding ratio/minute), and S_1 nuclease susceptibility for anthramycin vs. mitomycin binding ratio (r_{mc}), for a series of DNA–MC complexes. (○) Initial anthramycin binding rate vs. r_{mc}; (▲) final anthramycin binding ratio vs. r_{mc}; (●) increase of acid-soluble OD_{260nm} (relative to control = 1) at 20 min in the S_1 nuclease hydrolysis time course vs. r_{mc}.

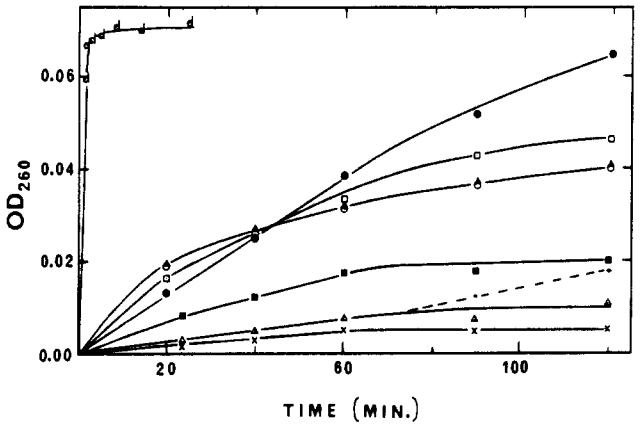

FIGURE 9: Time course digestion of a series of drug-modified DNA samples by S_1 nuclease. Various anthramycin and MC–DNA complexes (single or dual complex) were prepared and subjected to S_1 nuclease digestion as described under Experimental Procedures. (d) Denatured DNA control; (●) control native DNA; (▲) DNA–MC, binding ratio = 0.11; (○) DNA–MC, binding ratio = 0.06; (□) DNA–MC, binding ratio = 0.03; (■) DNA–MC–anthramycin, binding ratio = 0.10 and 0.106 respectively; (△) DNA–anthramycin–MC, binding ratio = 0.08, 0.10; (×) DNA–anthramycin, binding ratio = 0.08. Native unmodified calf thymus DNA control (- - -) was added to an aliquot of incubation at 75 min.

binding sites, at lower MC binding ratios, that bind anthramycin more slowly. Significantly there is an increase in S_1 nuclease sensitivity probably associated with distorsion of DNA due to MC binding (see Discussion).

DNA Complexes and S_1 Nuclease Hydrolysis. Shown in Figure 9 is a series of DNA complexes and control DNAs subjected to a time course hydrolysis by S_1 nuclease. The greatest rate of hydrolysis or release of acid-soluble nucleotides, as measured in OD's at 260 nm, occurs for denatured DNA. The hydrolysis of native DNA is considerably less rapid and is presumably due to a process of DNA breathing where transient regions of exposed or single-stranded DNA appear to serve as substrate for the S_1 nuclease enzyme (Vogt, 1973; Beard et al., 1973). In the beginning of the time course hydrolysis, the DNA–MC complexes all display a significant and reproducible increase of hydrolysis over that for control DNA. This increase plateaus near an hours time and becomes stabilized as the hydrolysis rate diminishes below that of control DNA. This pattern of hydrolysis enhancement and then decrease, relative to control DNA, is more prominent in DNA–MC complexes of higher binding ratio (0.06 and 0.11 binding ratios). The coincidence of hydrolysis curves for DNA–MC complexes of higher MC binding suggests that the element in the DNA–MC complex responsible for this enhanced hydrolysis exerts its maximum effect near a MC binding ratio of 0.06. The increased hydrolysis rates seen in these complexes may be attributable to regions of single strandedness, regions resembling mismatched base pairs (Shenk et al., 1975; Dodgson et al., 1977), or more likely areas of major helix base distortion created by bound drug (Fuchs, 1975; Yamasaki et al., 1977; Hurley & Petrusek, 1979; Pulkrabek et al., 1977). The lower plateau attained, relative to control DNA, in hydrolysis rates for the DNA–MC complexes may represent a point where all accessible exposed DNA regions have been enzymatically titrated out leaving hydrolysis-resistant regions of DNA stabilized by bound mitomycin C cross-linking chromophores (Tomasz et al., 1974). The overall stability of the DNA–MC complex to S_1 nuclease activity has been demonstrated previously by Lown et al. (1976) and is presumed to result from the presence of these cross-links.

The hydrolysis rates for the DNA–anthramycin complexes is considerably lower than that of the control DNA, a finding reproducing the results of Hurley & Petrusek (1979). The release of nucleotides is inhibited presumably due to the stabilizing effect this drug has on nucleic acid structure (Hurley & Petrusek, 1979). Through its mode of binding in the minor groove, anthramycin does not protrude outside of the helix or distort the DNA structure, two elements which would contribute to the release of nucleotides (Hurley & Petrusek, 1979). It has been shown, furthermore, that the apparent S_1 nuclease resistance in the complex is not due to nonspecific loss of S_1 nuclease activity (Hurley & Petrusek, 1979).

DNA complexes containing both bound MC and anthramycin demonstrate increased resistance to S_1 nuclease hydrolysis. The DNA–MC–anthramycin complex (made from the reaction of DNA–MC with anthramycin) displays the least resistance to hydrolysis. Bound anthramycin perhaps, by virtue of its stabilizing effect on DNA structure, is making the DNA–MC complex more resistant to the activity of S_1 nuclease. DNA–anthramycin–MC, or "reverse" complex, demonstrates a time course hydrolysis similar to DNA–MC–anthramycin but at a rate intermediate between it and a DNA complex containing only anthramycin. As shown, the addition of fresh DNA at 75 min demonstrates that the activity of the enzyme is not reduced but that the complex is resistant to the activity of the enzyme. The lower rate of hydrolysis displayed in the anthramycin complex, relative to the combined complex, formed first with MC, is probably due to the fact that mitomycin C is binding to a DNA–anthramycin complex which inherently displays and retains, regardless of extensive MC binding, a relatively high degree of resistance toward S_1 nuclease activity. It is also evident that bound anthramycin may be suppressing some of the destabilizing effects bound mitomycin may have on the helix.

Discussion

The initial experiments described in this paper (Tables I, II, and III) demonstrate the validity of the approach and the possible conclusions resulting from this study. For example, the use of anthramycin and MC for competitive binding experiments is valid since neither drug causes the release of the other, once the first drug is bound to DNA. Second, the mode of interaction of either drug in a DNA complex containing both drugs appears to be the same as in its individual complex.

This is especially true for mitomycin C, where Sephadex filtration, incubation in high salt, and hyperchromicity measurements demonstrate that it is binding covalently and is cross-linking DNA in these complexes. Although anthramycin binding to DNA–MC complexes shows kinetic differences, which can be explained partly by a loss of binding sites, the extended dialysis measurements demonstrate no difference in the ability of anthramycin to dialyze from native DNA or DNA–MC complexes. This indicates similarity of anthramycin interaction in DNA–MC complexes as well as in native DNA, a view strengthened by the fact that anthramycin demonstrates specificity and sensitivity in its interaction with DNA (Kohn et al., 1974; Petrusek et al., 1981). Evidence further supporting this premise is revealed by the S_1 nuclease hydrolysis results (Figure 9) which demonstrate, both in control DNA or DNA–MC complex, a stabilizing effect exerted on the DNA helix by bound anthramycin. Presumably a specific mode of anthramycin binding common to both native DNA and DNA bound with MC is needed to facilitate this effect.

The stoichiometry of inhibition of MC binding to DNA–anthramycin complexes does not reveal specifically how the mechanism of inhibition is taking place. The approximate 5:4 (anthramycin/actinomycin) stochiometry of inhibition of actinomycin binding found previously (Kohn et al., 1974) is believed to be due to direct or near site inhibition of actinomycin binding by bound anthramycin chromophores. This is anticipated since actinomycin D, like anthramycin, is guanine–cytosine specific and binds to DNA in the minor groove (Kolchinsky et al., 1975), the groove now proven to be the site of anthramycin's interaction with DNA (J. M. Ostrander, M. S. Balakrishnan, T. R. Krugh, and L. H. Hurley, unpublished results). The stoichiometry of anthramycin's inhibition of MC binding is greater than 3:1 anthramycins/MC. Furthermore, the fact that the bound anthramycin chromophore apparently covers several base pairs (Hurley & Petrusek, 1979) and that its inhibition of MC binding to DNA prevails at all binding ratios makes it less likely that the minor groove is the major site of mitomycin C's interaction with native DNA. Although the binding of MC to phosphate groups in the minor groove, via an esterification process, remains a possibility, it must be considered that the major groove is the more probable site of mitomycin C's interaction with DNA. It must be considered, therefore, that anthramycin's inhibition of MC binding is due to some other effect such as conformational. For instance, anthramycin's stiffening of DNA may serve as a restriction on the ability of MC to bind to DNA if mitomycin's distorting of DNA structure is required for a cooperativity in its binding. If such regions are characterized by base distortion, then the S_1 nuclease experiments support this premise, since a DNA–anthramycin complex bound with MC (Figure 9) relative to a DNA–MC complex alone is less sensitive to S_1 nuclease activity.

The cooperative-like inhibition of anthramycin binding to DNA–MC complexes does not appear to be the result of a direct exclusion of anthramycin binding sites by bound MC chromophores. This is because at no point is there a stoichiometry of inhibition approaching 1:1 (see Figures 3 and 4). The S_1 nuclease experiments indicate that this inhibition pattern may be linked to the MC-induced conformational change. The initial increase in the relative S_1 nuclease hydrolysis rates (Figure 8) for DNA–MC complexes of increasing binding ratio correlates very well with the decrease in final anthramycin binding to these same complexes. It seems evident, therefore, that a direct relationship exists between the quantity of distortion, as assessed by S_1 nuclease activity, and the inhibition of anthramycin binding. This further supports a DNA conformational change as the basis for limiting the degree of anthramycin binding to DNA.

Mercado & Tomasz (1977) have shown that poly[d(G-C)]·poly[d(G-C)] exhibits a profound alteration of its circular dichroism spectrum when modified by mitomycin C. This change is very similar to that occurring when this synthetic polymer is subjected to conditions of high salt concentration, a change which has been reported and interpreted as a conformational change by Pohl & Jovin (1972). ^{31}P NMR studies of this polymer in high salt (Patel et al., 1979) have been a basis for assuming (Wang et al., 1979) that Z-DNA (a left-handed helix; Wang et al., 1979, 1981; Arnott et al., 1980) may be the high salt form of poly[d(G-C)]·poly[d(G-C)]. Through model building, Wang et al. (1979) have shown that the junction between B- and Z-DNAs should have a stacking discontinuity which might result in a kinking of the DNA molecule. The suggestion (Wang et al., 1979) that segments of such Z-DNA can be found in B-DNA when driven by the impetus of guanine alkylation has significance to recent physicochemical studies of DNA–MC complexes (D. J. Kaplan and M. Tomasz, unpublished results) which demonstrate a significant conformational change in DNA induced by MC, a change that is detected as a coiling, bending, or kinking of DNA structure. Since anthramycin requires B-DNA-like structure for binding (Petrusek et al., 1981), one may predict that such distortions or bends in DNA, which may possibly represent areas of the proposed Z-DNA structure, would prevent anthramycin from binding to these areas. It is interesting to note that propagation of Z-DNA at specific sites in DNA by certain agents, such as MC, may lead, as suggested by Wang et al. (1979), to an increased susceptibility of these areas toward further attack by these same agents. This condition would allow for a cooperativity and clustering in the binding of such compounds to the helix. Such interactions with the helix in turn, if associated with increased distortion (as may be the case with MC), can be linked to a cooperativity in the inhibition of anthramycin binding, an inhibition that may stem from the destablization of one region of the DNA helix by an adjacent region [known as telestability (Wells, 1977)]. Such a process of inhibition may be occurring in superhelical SV40 DNA which serves as a poor template for anthramycin binding (Petrusek et al., 1981). The significance of this is related to a theroretical analysis of the transition of B- to Z-DNA structure in torsinally stressed (superhelical) DNA which indicates that this transition is possible in superhelical DNA (Benham, 1980).

The kinetics of anthramycin binding to DNA–MC complexes indicates both a significant lowering of the apparent rate constants and initial binding reaction rates for anthramycin. This cannot be explained by stoichiometric exclusion of anthramycin binding sites by MC chromophores, since a much greater reduction in the constants and rates would have been seen. It is important to note that the decrease in initial reaction rates (Figure 7) for anthramycin is significant in those DNA–MC complexes containing binding ratios greater than 0.024. In this respect, the results of the kinetic studies, in terms of the relative changes seen in anthramycin binding, parallel those of the anthramycin saturation binding studies where the cooperativity in change occurs near 0.030–0.035 MC binding ratio. Figure 8, indicates, however, that a large drop in initial binding rates for anthramycin occurs just before the actual drop in final anthramycin binding to these complexes, a finding that demonstrates that this drug is still binding to these complexes but is binding at a lower rate. The kinetics thus dem-

onstrates the extreme sensitivity of anthramycin to small changes in helical structure that are probably occurring in DNA at low levels of MC binding. In this sense, anthramycin may be demonstrating a sensitivity to a telestability change in DNA occurring just before the large reduction in final anthramycin binding. The initial event that anthramycin senses kinetically may very well be a threshold formation of MC-induced structural transitions (regions of Z-DNA?), at which point after significant cooperative-like MC binding occurs, to facilitate a significant DNA conformational change leading to the large reduction in final anthramycin binding.

For DNA–MC complexes, a number of observations support the premise that anthramycin is binding to helical areas relatively free of MC chromophores. First, the cooperativeness of MC interaction implies a clustering of bound MC chromophores to certain areas of the helix. This is supported by the extended dialysis experiments which indicate that anthramycin is probably binding away from MC chromophores even in complexes containing high MC binding and helix distortion. Second, the binding of anthramycin or MC, respectively, to DNA–MC and DNA–anthramycin complexes makes them less or more susceptible to the action of S_1 nuclease. The larger change in hydrolysis rate is seen when anthramycin is bound to a DNA–MC complex of high binding ratio. Implied here is a condition where extended regions of the helix, relatively free of MC, are available for anthramycin binding so that it can exert its stabilizing (stiffening) effect on DNA structure. Third, the apparent nonsite exclusion inhibition of anthramycin binding by MC is maintained even at MC saturation binding where only a 22.4% maximum inhibition in anthramycin binding is observed (Figure 2).

The results of the experiments described here draws attention to the possible use of anthramycin as a probe to detect small and/or certain changes in DNA helical structure. An interesting situation is also presented which describes how two DNA covalent modifying antitumor agents, which induce diametrical conformational effects (stiffening vs. distortion), affect the helix, both individually and in combination. Insights may also be provided, on the in vitro level, into the possible development of certain antitumor regimes based upon considerations of cooperativity in drug binding.

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