# Binding of 2,7-Diaminomitosene to DNA: Model for the Precovalent Recognition of DNA by Activated Mitomycin C†

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ABSTRACT: Mitomycin C (MC), mitomycin A, porfiromycin, BMY-25067, and BMY-25287, antitumor antibiotics collectively termed "mitosanes", were found to have no appreciable binding affinity to various natural and synthetic DNAs, as tested by UV spectrophotometry and equilibrium dialysis. Further tests of DNA binding applied to MC including thermal melting measurements, displacement of ethidium fluorescence, and unwinding of closed circular DNA were similarly negative. In contrast, 2,7-diaminomitosene (2,7-DAM), a major end product of the reductive activation of MC, binds to the same series of DNAs by all of these criteria. In the presence of DNA its UV absorbance at the 313 nm maximum decreased and underwent a slight red shift. This effect was used for determining DNA binding constants ($K_b$) by the spectrophotometric titration method. At pH 6.0 the $K_b$s of three natural DNAs with varying GC content, as well as poly(dA-dT)•poly(dA-dT), and poly(dG-dC)•poly(dG-dC), were all in the range of $(1.2-5.3) \times 10^4$ (M nucleotide)$^{-1}$, with no apparent specificity of binding. Poly(dG-m5dC)•poly(dG-m5dC) displayed a slightly higher $K_b$ ($(7.5-8.4) \times 10^4$). Binding of other, closely related mitosenes was tested to calf thymus DNA by equilibrium dialysis. Neither the presence of a 1-OH substituent, removal of the 10-carbamoyl group, nor methylation of the 2-amino group modifies the binding affinity of the mitosenes significantly. The 1-phosphate substituent abolishes binding. The binding of 2,7-DAM to DNA increased with decreasing pH and decreasing ionic strength. It was determined that 2,7-DAM is protonated at the 2-amino group with a $pK_a = 7.55$, and this correlated well with the observed pH dependence of the binding, indicating that the binding affinity has a strong electrostatic component. This was confirmed by the finding that the extrapolated $K_b$ to 1 M $Na^+$ concentration diminishes to only 10% of the value of $K_b$ at 0.01 M $Na^+$ concentration. Viscosity tests showed conclusively that 2,7-DAM intercalates in DNA, in a nonspecific manner. DNA binding by 2,7-DAM is shown to be a close model of the binding of the reduced activated form of MC, previously characterized indirectly [Teng, S. P., Woodson, S. A., and Crothers, D. M. (1989) *Biochemistry 28,* 3901−3907]. The nonspecific precovalent binding of the active form may serve in the cell to concentrate the drug at its critical target, DNA. A CpG-specific minor groove binding mode, previously suggested to explain the observed CpG specificity of the covalent alkylation [Kumar, S., Lipman, R., and Tomasz, M. (1992) *Biochemistry 31,* 1399−1407], is presumably masked by the stronger, bulk nonspecific binding described here.

Molecular recognition is an important factor in covalent reactions of drugs with DNA, with respect both to DNA site selectivity and kinetic efficiency (Warpehoski & Hurley, 1988). It is likely that all DNA-reactive drugs and carcinogens "recognize" DNA, as manifested by binding,[1] in a more or less selective fashion, to DNA structural elements. Given the large variety of drugs which are capable of modifying the same DNA macromolecule, such recognitions must take place by many different mechanisms. Drugs which are natural products display particularly complex precovalent binding interactions, elucidation of which usually requires a battery of different techniques. Examples include calicheamicin (Mah et al., 1994), CC-1065 (Krueger et al., 1985), bleomycin (Chien et al., 1977), and neocarzinostatin chromophore (Dasgupta & Goldberg, 1985).

The mitomycins are a group of antitumor antibiotics whose covalent interaction with DNA includes interstrand and intrastrand cross-linking as well as monofunctional alkylation. It is most likely that this property represents the molecular basis of their biological activity (for review, see Tomasz, 1994). The best-studied member of this group, mitomycin C (MC;[2] **1**), is widely used in clinical anticancer chemotherapy. The design and development of new, therapeutically useful semisynthetic mitomycins is also of great current interest (Verweij & Pinedo, 1990). The major covalent adducts of MC and DNA have been fully characterized. Reductively activated MC alkylates DNA exclusively at the 2-$NH_2$ group of guanines in the minor groove. In the monoalkylation mode the aziridine C1 position is linked to the 2-$NH_2$ group of a guanine, while in bifunctional alkylation the mitomycin is linked to two guanines, at both its C1 and C10 positions, forming a DNA cross-link

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[1] The terms "binding" and "bonding" designate noncovalent and covalent interactions, respectively.

[2] Abbreviations: MC, mitomycin C; 2,7-DAM, 2,7-diaminomitosene; CP-DNA, *Clostridium perfringens* DNA; CT-DNA, calf thymus DNA; ML-DNA, *Micrococcus lysodeikticus* DNA; Etd, ethidium bromide; Col E1-DNA, colicin E1 DNA; SSC, 0.15 M NaCl−0.015 M sodium citrate, pH 7.0; P/D, [polynucleotide]/[drug] ratio; bp, base pair; nt, nucleotide; SDS, sodium dodecyl sulfate; DTT, dithiothreitol.

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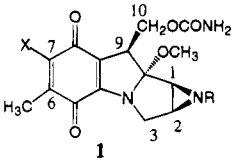
Chart 1



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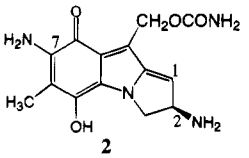

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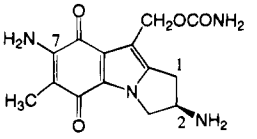
**mitomycin C (MC): X = $NH_2$, R = H** **quinone methide**
**porfiromycin : X = $NH_2$, R = $CH_3$**
**mitomycin A : X = $OCH_3$, R = H**
**BMY-25282: X = N=CHN$(CH_3)_2$, R=H**
**BMY-25067: X = NH-$CH_2CH_2$S-S⟨⟩-$NO_2$**



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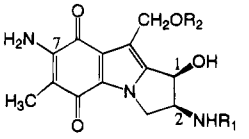
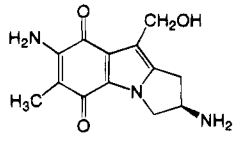
**2, 7 - DAM**



4

**a: $R_1$= H, $R_2$ = $CONH_2$**
**b : $R_1$= H, $R_2$ = H**
**c : $R_1$= $CH_3$, $R_2$= $CONH_2$**



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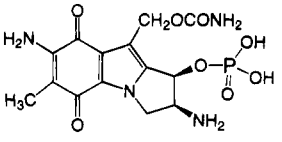

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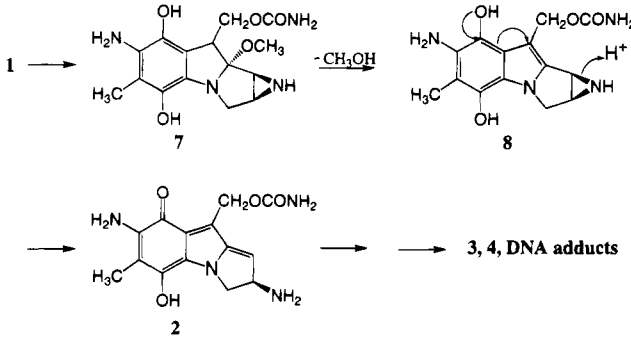
Scheme 1: Reductive Activation of MC



(Tomasz, 1994). The chemical mechanism of MC−DNA adduct formation is well-understood (Tomasz et al., 1988).

In contrast, relatively little is known about precovalent DNA-binding properties of the mitomycins. In a lesser known, but thorough investigation (Rodighiero et al., 1978) no binding of MC to DNA was detectable, using a large variety of techniques. However, the actual DNA-reactive form of MC is known to be generated only upon reductive activation of the original molecule (Iyer & Szybalski, 1964) (see Scheme 1). Its structure is rather well-established as the quinone methide **2** (Chart 1), a short-lived, highly reactive species, characterized only indirectly (Tomasz & Lipman, 1981; Kohn & Zein, 1983; Peterson & Fisher, 1986; Hoey et al., 1988). It is too reactive, therefore, to study its binding to DNA directly. Nevertheless, Crothers and his co-workers (Teng et al., 1989) detected binding of such transient reduced MC species to synthetic oligonucleotides by observing competitive inhibition of the cross-linking of G·C-containing oligonucleotides by oligonucleotides containing only A·T base pairs, i.e., no covalent drug bonding sites. Kinetic analysis of the inhibition fitted a simple model in which the activated mitomycin binds nonspecifically to the oligonucleotides, regardless of base composition and sequence, exhibiting identical dissociation constants for A·T and G·C sites.

These earlier findings, taken together, indicate that MC is not attracted to DNA prior to its reductive activation, but it will bind to the nucleic acid after a conversion to its active form. Such binding is of great importance with respect to the pharmacological efficiency of MC, since it could kinetically facilitate the covalent reactions of the active form **2** with DNA relative to reactions with water and other cellular nucleophiles which detoxify MC (Tomasz & Lipman, 1981).

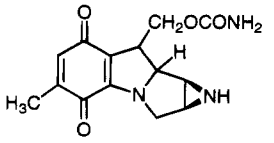
In order to elucidate the structural basis of the precovalent DNA binding of mitomycins, we studied stable "activated form" analogs of MC. These are mitosene[3] quinones (**3**–**6**) which closely resemble the structure of the active form, quinone methide **2**. We also reinvestigated DNA binding by MC itself and included other antitumor mitosanes[3] such as mitomycin A, porfiromycin, BMY-25067, and BMY-25282 (Chart 1). The results led to the interesting conclusion that reductive activation of MC unmasks a DNA-binding activity in addition to the known covalent reactivity. The stability of the "activated form analogs" allowed us to characterize the binding in some detail.

In a preliminary study we reported some of the results of the present work [Tomasz et al., 1990; reviewed by Tomasz (1994)].

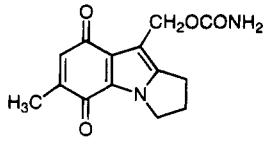
## MATERIALS AND METHODS

Mitomycin C, BMY-25067, BMY-25282, porfiromycin, and mitomycin A were obtained from Bristol-Myers Squibb Co., Wallingford, CT. 2,7- Diaminomitosene (2,7-DAM; **3**) and its decarbamoylated derivative **5** were prepared by catalytic hydrogenation of MC at pH 5.0 (Tomasz & Lipman, 1981) and purified by chromatography on a Sephadex G-25 column followed by preparative HPLC on a Rainin HPLC assembly using a Dynamax C18 column (2.1 × 25 cm). **4a**, **4b**, and **6** were obtained by acid hydrolysis of MC, and **4c** was obtained by acid hydrolysis of porfiromycin, as described (Tomasz & Lipman, 1979). *Clostridium perfringens* DNA (Type XII, 30 mol % GC), calf thymus DNA (Type I, 42 mol % GC), *Micrococcus lysodeikticus* DNA (Type XI, 72 mol % GC), superhelical colicin E1 DNA (53 mol % GC), and ethidium bromide were obtained from Sigma, St. Louis, MO. All natural DNAs except Col E1-DNA were sonicated under ice using a sonicator cell disruptor W185 (Heat Systems: Ultrasonics, Inc., Plainview, Long Island, NY) to molecular weights of the order of (2−2.5) × $10^5$. After sonication the solutions were extensively dialyzed under sterile conditions. Each DNA exhibited the characteristic ultraviolet absorption spectrum with an $A_{260}/A_{280}$ ratio between 1.88 and 1.93 and an $A_{260}/A_{230}$ ratio between 2.12 and 2.22. The thermal melting temperatures ($T_m$) in 0.1x SSC were 83, 71, and 66 °C for ML-, CT-, and CP-DNA, respectively, with hyperchromicity varying from 32% to

[3] The terms "mitosane" and "mitosene" specify the following structures (Webb et al., 1961):



mitosane



mitosene

37%. Poly(dG-dC)·poly(dG-dC), poly(dA-dT)·poly(dA-dT), and poly(dG-m5dC)·poly(dG-m5dC) were products of Pharmacia LKB Biotechnology, Piscataway, NJ. pBR322 DNA (supercoiled) and calf thymus DNA topiosomerase I were purchased from Gibco BRL Life Technologies, Grand Island, NY. The concentration of DNA and polynucleotides in terms of nucleotide was determined spectrophotometrically using the molar extinction coefficient ($\epsilon$) at or near 260 nm reported in the literature (Felsenfeld and Hirschmann, 1965; Wells et al., 1970). For Col E1-DNA $\epsilon_{260} = 6550\ M^{-1}\ cm^{-1}$ was used.

*Preparation of Single Stranded and Heat-Denatured DNA.* Single-stranded DNA was prepared by heating a duplex DNA sample at 98 °C for 10 min in the presence of formaldehyde (0.333 M), followed by rapid cooling as described by Daugherty et al. (1979). Heat-denatured DNA was prepared in a similar fashion, omitting the formaldehyde treatment. The heat-denatured DNA sample thus prepared had ~15% residual hyperchromicity, as shown by absorption measurements at 260 nm at room temperature and at the temperature of complete melting (cf. $T_m$ measurements, described below).

*Citrate−phosphate buffer* at specified pH (Gomori, 1956) contained 5 mM $Na_2HPO_4$. The pH was adjusted in the pH 5−8 range by the addition of citric acid. This buffer provides constant [$Na^+$] (10 mM).

*Determination of Binding Constants ($K_b$) by the Spectrophotometric Titration Method (Peacocke & Skerrett, 1956).* Absorption spectral measurements were performed on a Varian Cary 3 spectrophotometer at 22 ± 1 °C using citrate−phosphate buffer. Usually, a fixed concentration of 2,7-DAM was mixed with varying concentrations of DNA or polynucleotide to give various P/D ratios. The reference cell always contained the same amount of DNA as the sample cell. From the spectrophotometric titration data, the concentration of free and bound 2,7-DAM was estimated for each P/D, and these binding data were cast into Scatchard plots (Scatchard, 1949) of $r/c$ versus $r$, where $r$ is moles of 2,7-DAM bound per mole of DNA nucleotide and $c$ is the concentration of free 2,7-DAM. Two methods were employed to calculate the binding constants ($K_b$):

(1) The Scatchard plots were fitted to a theoretical curve, which was drawn according to the excluded site model (Crothers, 1968) developed by McGhee and von Hippel (1974):

$$r/c = K_b(1 - nr)[(1 - nr)/1 - (n - 1)r]^{n-1} \quad (1)$$

where $n$ is the number of nucleotides occluded after binding of a single ligand molecule. This program generated values of $K_b$ and $n$ for each data set.

(2) $K_b$ was also evaluated from a half-reciprocal plot of the change of the apparent extinction coefficient of ligand versus DNA concentration (Wolfe et al., 1987):

$$[DNA]/\Delta\epsilon_{ap} = [DNA]\Delta\epsilon + 1/(\Delta\epsilon)K_b \quad (2)$$

where $\Delta\epsilon_{ap} = \epsilon_{ap} - \epsilon_F$, $\Delta\epsilon = \epsilon_B - \epsilon_F$, and $\epsilon_{ap}$, $\epsilon_F$, and $\epsilon_B$ are the apparent, free, and bound ligand extinctions, respectively. [DNA] is DNA concentration in base pairs. $\epsilon_B$ is obtained from $\Delta\epsilon$ and the known value of $\epsilon_F$. The slope of such plots is $1/\Delta\epsilon$ and, the $y$ intercept is equal to $1/(\Delta\epsilon)K_b$. (The $K_b$ thus obtained in terms of base pairs was divided by 2 for $K_b$ in nucleotide units.)

Table 1: Extinction Coefficients and p$K_a$s of Mitosanes and Mitosenes

| | $\epsilon$ ($\lambda$; solvent) | p$K_a$ |
|---|---|---|
| MC | 21 840 (367; $H_2O$)[a] | 3.20;[a] 1.5[b] |
| Por | 23 100 (363; $H_2O$)[a] | 0.9[b] |
| MA | 10 400 (320; methanol)[c] | |
| BMY-25282 | 11 200 (396; $H_2O$)[d] | |
| BMY-25067 | 16 800 (373; $H_2O$)[d] | |
| 2,7-DAM | 10 300 (314; $H_2O$)[e] | 7.55[f] |
| 4a | 11 400 (308; methanol)[a] | 7.11[f] |
| 4c | same as above | 7.33[f] |

[a] Stevens et al., 1964. [b] Underberg & Lingeman, 1983. [c] Webb et al., 1962. [d] He, 1994. [e] Tomasz & Lipman, 1981. [f] This report.

*Determination of Binding Constants by the Ethidium Displacement Assay.* This was performed following generally the methods of Lee et al. (1993a,b). The fluorescence measurements were done on an Aminco Bowman spectrofluorimeter. The binding constant ($K_b$) of 2,7-DAM binding to CT-DNA was calculated from the equation

$$K_{Etd}[Etd] = K_b[2,7\text{-DAM}]_{0.5} \quad (3)$$

where $K_{Etd}$ is binding constant of ethidium bromide to CT-DNA ($5.23 \times 10^5\ M^{-1}\ nt^{-1}$, determined by us by the adsorption titration method using citrate−phosphate buffer, pH 6.0), [Etd] = 1.3 $\mu$M, and $[2,7\text{-DAM}]_{0.5}$ is the concentration required to reduce the fluorescence of the Etd−DNA complex to half of its original value (Bauer & Vinograd, 1979).

*Determination of Binding Constants by Equilibrium Dialysis of Drug−DNA Complexes.* These experiments were conducted using a 5-cavity dialysis apparatus with 1-mL volume half-cells (Bel-Art Products, Pequannock, NJ). Dialysis membrane of 6000 MW cutoff (Spectra/Por; Spectrum Medical Instruments, Houston, TX) was used. The membrane was cut to size, washed in 10 mM EDTA, then placed in boiling water for 20 min, and finally secured in the dialysis apparatus. On one side of the membrane 0.5 mL of a CT-DNA solution (3.5 mM) and on the other side 0.5 mL of drug solution at various concentrations were placed. The buffer was variable as specified. Dialysis was performed for 24 h at room temperature under gentle agitation. At the end of this period the samples in both compartments were diluted with the buffer, and solid NaCl was added to a final concentration of 0.5 M in order to dissociate any DNA-bound drug. The absorbance of the drug at its $\lambda_{max}$ in the 310-nm region (see Table 1) was determined, and the original absorbance of the drug in the two dialysis compartments was calculated. All experiments were conducted in triplicate. Binding constants ($K_b$) were calculated from the data obtained under a single condition, based simply on the following binding equilibrium: drug + DNA ⇄ drug−DNA:

$$K_b = \frac{[\text{drug−DNA}]}{[\text{drug}][\text{DNA}]} \quad (4)$$

where [drug], [DNA], and [drug−DNA] are concentrations of free drug, free DNA, and DNA-bound drug, respectively.

$K_b$ can be expressed from the absorbance data as follows:

$$K_b = \frac{(A_1 - A_2 - A_{DNA})/\epsilon_{\lambda max}}{[A_2/\epsilon_{\lambda max} \times (A_{260(DNA)}/\epsilon_{260(DNA)})] - (\text{numerator})} \quad (5)$$

where $A_1$ and $A_2$ are absorbance of drug at its $\lambda_{max}$ in the DNA-containing and DNA-free compartments, respectively; $A_{DNA}$ is absorbance of control DNA at $\lambda_{max}$ of drug; $A_{260(DNA)}$ is absorbance of control DNA at 260 nm; and $\epsilon_{\lambda max}$ and $\epsilon_{260(DNA)}$ are molar extinction coefficients of the drug and DNA, respectively (Table 1). Values of $K_b$ obtained by this method were only used for comparison of binding of a series of closely related drug analogs under identical conditions. They are distinguished from $K_b$s obtained from the absorption titration method by being presented as "relative $K_b$" (Figure 7).

*Thermal Denaturation Temperatures of DNA and Polynucleotides and Their Drug Complexes.* These were determined using a Cary 3 UV-visible spectrophotometer equipped with the "thermal melt" accessory. The change in absorbance of DNA or polynucleotide solution at the absorption maximum was measured using a heating rate of 1 °C/min.

*Viscosity studies* were performed using a Cannon Manning Type 75 semimicro viscometer mounted vertically in a constant temperature bath maintained at 22 ± 0.5 °C. Flow times of DNA alone and DNA−2,7-DAM and DNA−MC mixtures were measured using an electronic stopwatch with an accuracy of 0.01 s. Experiments were carried out at DNA nucleotide concentrations of 0.45 mM. (Col E1-DNA concentration was 0.15 mM.)

*Assay of Supercoiling of Relaxed Circular pBR322 DNA by 2,7-DAM (after Fisher et al., 1985).* pBR322 DNA (2 μg) was incubated with calf thymus DNA topoisomerase I (20 units) at 37 °C for 30 min in 50 μL of "relaxation buffer" containing 50 mM Tris, pH 7.5, 120 mM KCl, 5 mM DTT, 0.5 mM $Na_4EDTA$, and 30 μg/mL bovine serum albumin. Subsequently, 2,7-DAM in the above buffer was added to give 56, 105, and 187 μM concentrations. Incubation continued for an additional 30 min. Reactions were stopped by an addition of 20% SDS (5 μL), and the samples were extracted twice with equal volume of neutralized phenol, followed by extraction with equal volume of chloroform. The samples (20 μL) were mixed with loading dye mix (5 μL) and electrophoresed for 2.5 h on 0.8% agarose, using 40 mM Tris−5 mM magnesium acetate−1mM $Na_4EDTA$, in a field of 3 V/cm, in a horizontal Pharmacia GNA 100 apparatus. Gels were stained by 2 μg/mL Etd in the electrophoresis buffer for 1 h and then photographed under UV light using Polaroid Type 667 (ASA 3000) film and a Polaroid MP4 camera, fitted with a red-orange filter.

## RESULTS

*Lack of Binding of Mitosanes to DNA.* The absorption spectra of MC, mitomycin A, BMY-25067, BMY-25282, and porfiromycin (Chart 1), collectively termed "mitosanes",[3] showed no change in the 300−400 nm range upon titration with calf thymus DNA (Figure 1a). In order to confirm that these substances did not bind appreciably to DNA, equilibrium dialysis was carried out in 0.01 M Tris , pH 7.0, at 80 and 250 μM drug and 3.5 mM DNA concentrations. In all cases, the concentration of the drug after dialysis was found

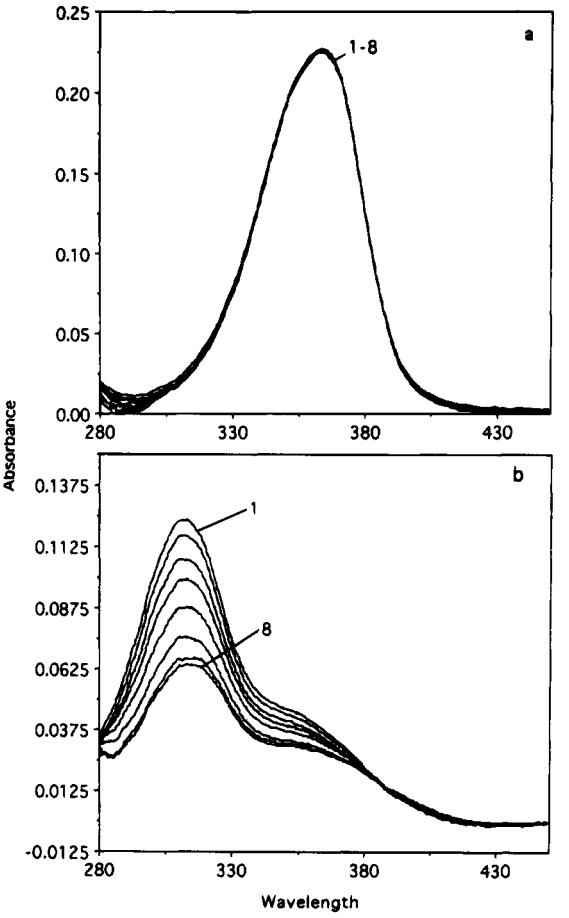



FIGURE 1: Changes in the absorption spectra of mitomycins upon titration with DNA in citrate−phosphate buffer, $[Na^+]$ = 10 mM, pH 6.05 ± 0.05. (a) Spectra of 10.11 μM MC upon titration with 0, 7.3, 30, 60, 120, 240, 479, and 720 μM CT-DNA as represented by curves 1−8, respectively. (b) Spectra of 11.9 μM 2,7-DAM upon titration with 0, 7.1, 30.0, 59.5, 119, 238, 476, and 714 μM CT-DNA as represented by curves 1−8, respectively.

to be identical in the two compartments within experimental variations (data not shown). Lack of binding of MC was also indicated by the lack of increase of $T_m$ of CT-DNA in the presence of MC (Table 3) and the lack of effect of MC on the viscosity of CP-, CT-, and ML-DNA (Figure 8) and of closed circular Col $E_1$ DNA (Figure 9). Furthermore, MC did not displace Etd from DNA at up to 200-fold higher concentration than that of 2,7-DAM which reduced Etd fluorescence by 50% (see Materials and Methods).

*Binding of the Mitosene 2,7-DAM to DNA: Spectrophotometric Titration (Figure 1b).* The absorption spectrum of 2,7-DAM in citrate−phosphate buffer, pH 6.05 shows two major bands centered around 313 and 243 nm. In addition there is a weak broad band in the 560 nm region. In the presence of increasing concentrations of CT-DNA, a strong hypochromic effect is observed in the 313 nm absorption band. This is accompanied by a relatively small bathochromic effect (Figure 1b). A weak isosbestic point around 384 nm characterizes the binding phenomenon. At saturation, a 6 nm red shift of the 313 nm band maximum was observed. Essentially identical changes were observed using all other native natural and synthetic polynucleotides (data not shown). The absorption spectrum of 2,7-DAM in the presence of single-stranded DNA (in 0.33 M formaldehyde solution; see above) was not perturbed, while with heat-denatured DNA effects similar to those with double-stranded DNA were seen.

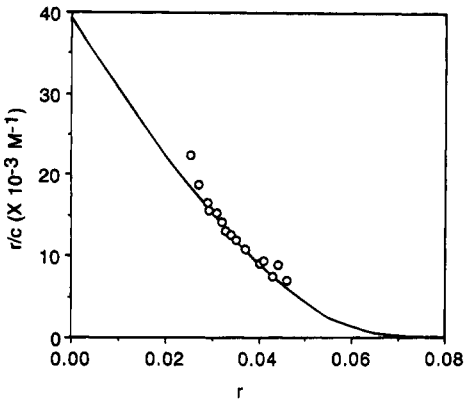



FIGURE 2: Scatchard plot of binding of 2,7-DAM to CT-DNA from data obtained using the spectrophotometric titration method. *r*: Moles of 2,7-DAM bound per mole of DNA mononucleotide; *C*: concentration of free 2,7-DAM. The points represent the measured values, and the curve is the result of fitting them to the McGhee–von Hippel excluded-site model.

In control experiments, it was observed that 0.33 M formaldehyde did not have any effect on the spectra of either native DNA or DNA–2,7-DAM mixtures.

*2,7-DAM Binding Parameters.* The results of the absorption titration were expressed in the form of Scatchard plots. Such plot in the case of CT-DNA is shown in Figure 2. The solid curve represents the best fit of the data to the McGhee–von Hippel equation (1). The binding parameters obtained are listed in Table 2. Another method for evaluation of the same data was also employed as follows. We observe that the number of bound ligands per nucleotide (*r*) is in the range of 0.02–0.05. Since nearest neighbor exclusion effects become significant only at higher *r* values (Bauer & Vinograd, 1970; Bresloff & Crothers, 1975), the data were also analyzed by utilizing simple isotherms which ignore this effect. The double-reciprocal equation of Benesi and Hildebrand (1949), as applied by Schmechel and Crothers (1971), was first converted to half-reciprocal form to give eq (2) (Wolfe et al., 1987). $K_b$s were evaluated from the corresponding plots (Figure 3) and are also listed in Table 2. The two sets of $K_b$s in the table differ slightly in that the excluded-site model gave $K_b$s 1.2 to 2.7 times greater than the half-reciprocal method. The reason for this is not known. In any case, the results can be summarized by saying that 2,7-DAM binds to all duplex DNAs with moderate strength, in the $10^4$ $M^{-1}$ range. The data obtained for the three synthetic polynucleotides indicate no preference for poly(dG-dC)·poly(dG-dC) over poly(dA-dT)·poly(dA-dT); however, approximately 1.5–2.2-fold higher binding is observed to poly(dG-m5dC)·poly(dG-m5dC).

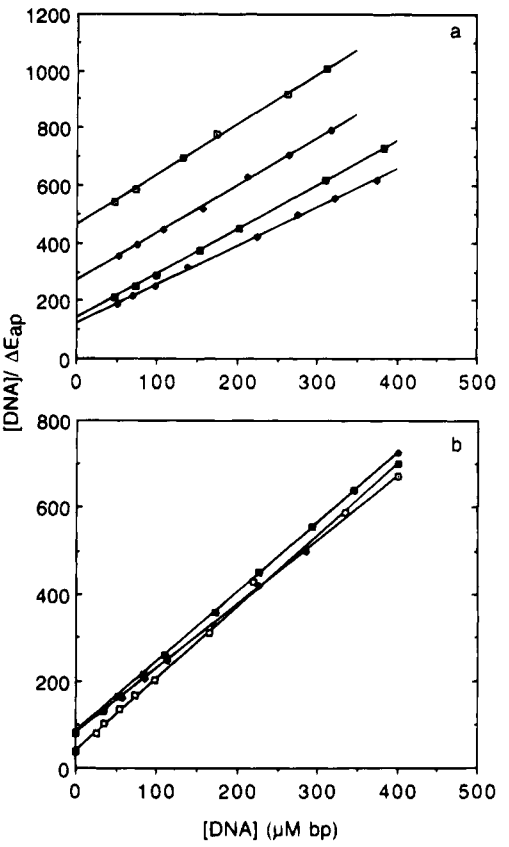



FIGURE 3: Half-reciprocal plots of changes of the apparent extinction coefficients ($\Delta\epsilon_{ap}$) at 313 nm as a function of DNA concentration according to eq (2). (a) (□) CP-DNA; (■) CT-DNA; (♦) CT-DNA (heat denatured); (◊) ML-DNA. (b) (■) poly(dA-dT)·poly(dA-dT); (◊) poly(dG-dC)·poly(dG-dC); (□) poly(dG-m5dC)·poly(dG-m5dC). The values of $K_b$ were calculated from the slopes and *y* intercepts and are listed in Table 2.

*Binding Constants Measured by the Etd Displacement Assay.* Binding of 2,7-DAM to CT-DNA was also determined by this method, giving the value $K_b = (1.25 \pm 0.13) \times 10^4$ $(M\ nt)^{-1}$. This is somewhat lower than the values determined by spectrophotometric titration (Table 2).

*$pK_a$ of Mitosenes.* Spectrophotometric acid/base titration of 2,7-DAM, **4a** and **4c** indicated p$K_a$s of 7.55, 7.11, and 7.33, respectively (Figure 4). Previously, a p$K_a$ of 6.5 was reported for **4a** (Stevens et al., 1964).

*pH Dependence of the Binding.* The binding of 2,7-DAM to CT-DNA was studied in the pH range 5.0–8.0. The

Table 2: Binding Parameters of the Interaction of 2,7-DAM with Various Natural and Synthetic DNAs[a]

| DNA | GCmol % | $K_b/10^4$ $(M\ nt)^{-1}$ from excluded site model[b,c] | *n* | $K_b/10^4$ $(M\ nt)^{-1}$ by half-reciprocal method[b] | $K_b/10^4$ $(M\ nt)^{-1}$ by Etd displacement |
|---|---|---|---|---|---|
| CP-DNA | 30 | 1.4 ± 0.20 | 14.0 | 0.58 ± 0.20 | |
| CT-DNA | 42 | 3.94 ± 0.15 | 12.1 | 2.14 ± 0.17 | 1.25 ± 0.30 |
| ML-DNA | 72 | 4.85 ± 0.14 | 12.7 | 1.80 ± 0.15 | |
| heat-denatured CT-DNA | 42 | 3.27 ± 0.15 | 13.0 | 1.20 ± 0.20 | |
| single-stranded CT-DNA | 42 | no binding | | | |
| poly(dA-dT)·poly(dA-dT) | 0 | 5.32 ± 0.18 | 9.5 | 3.91 ± 0.11 | |
| poly(dG-dC)·poly(dG-dC) | 100 | 4.72 ± 0.15 | 10.0 | 3.75 ± 0.15 | |
| poly(dG-me5dC)·poly(dG-me5dC) | 100 | 7.54 ± 0.15 | 8.5 | 8.42 ± 0.10 | |

[a] Average of three determinations. [b] Determined by the spectrophotometric titration method. Buffer: citrate–phosphate, $[Na^+] = 10$ mM, pH = 6.05. [c] Binding data were limited to D/P ratios corresponding to fraction of bound 2,7-DAM ranging from 0.35 to 0.85.

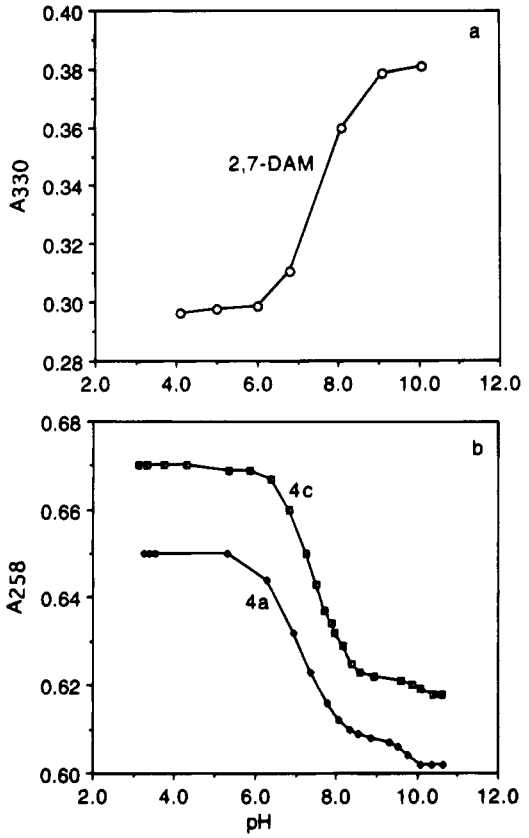



FIGURE 4: Determination of p$K_a$s of the mitosenes 2,7-DAM, **4a**, and **4c** by spectrophtometric acid/base titration. (a) 2,7-DAM: Aliquots of 2,7-DAM in water were added to a series of citrate–phosphate buffers, pH 5–8, and the absorbance at 330 nm was plotted against pH. (b) **4a** and **4c** (50 $\mu$M) in 15 mL of 0.01 M Tris (pH 11)–0.1 M NaCl were titrated with 1 N HCl. The pH and absorbance were determined after each addition of 3 $\mu$L of 1 N HCl and plotted.

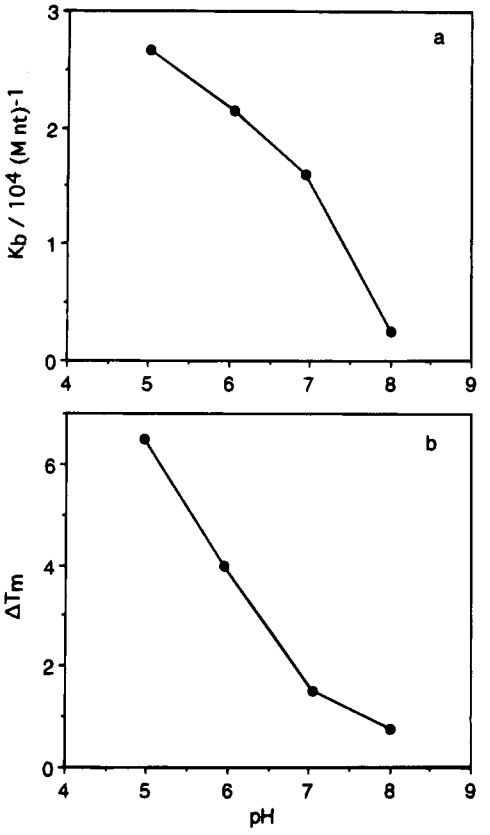



FIGURE 5: (a) pH dependence of $K_b$ of 2,7-DAM/CT-DNA. The spectrophotometric titration method and half-reciprocal analysis of the data were used. Buffer: citrate–phosphate at varying pH. (b) pH dependence of $\Delta T_m$ of CT-DNA in the presence of 2,7-DAM. Conditions: see text.

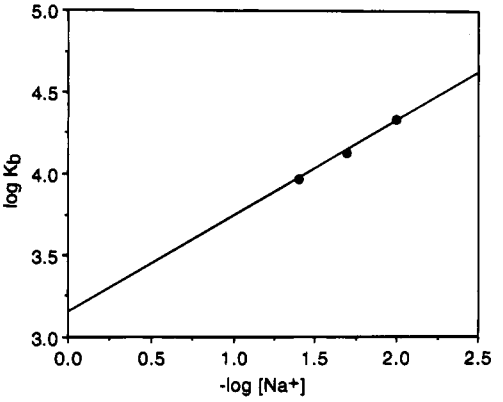



FIGURE 6: $K_b$ as a function of $Na^+$ ion concentration for binding of 2,7-DAM to CT-DNA. The spectrophotometric titration method and half-reciprocal data analysis were used. Conditions: citrate–phosphate buffer, pH 6.05 ± 0.05; the $Na^+$ ion concentrations were 10, 20, and 40 mM.

analysis of the binding by the half-reciprocal method indicates that the binding affinity decreased 10-fold in this range with increasing pH (Figure 5a). The following values of $K_b/10^4$ were obtained: 2.66 (pH 5.00), 2.14 (pH 6.05), 1.59 (pH 6.95), and 0.29 (pH 8.00).

*Ionic Strength Dependence of the Binding.* Binding of 2,7-DAM to CT-DNA was determined at three $Na^+$ concentrations. $K_b/10^4$ were found to be 2.14, 1.35, and 0.933 at [$Na^+$] concentrations of 0.010, 0.020, and 0.040 M, respectively. The plot of log $K$ versus log [$Na^+$] shown in Figure 6 gives a slope of 0.533, indicating the release of 0.533 sodium ion per bound 2,7-DAM (Record & Spolar, 1990).

*DNA Binding by Other Mitosenes: Structure-Activity Relationship (Figure 7).* $K_b$s of a series of mitosenes (**3**–**6**) were measured using the equilibrium dialysis method and were related to the value of $K_b$ of 2,7-DAM, measured under the same conditions. It is seen that the 1-OH-substituted mitosene **4a** has very similar DNA affinity to that of 2,7-DAM. The 10-decarbamoyl derivatives **4b** and **5** showed slightly diminished binding (by 9.4% and 18.4%) compared to **4a** and 2,7-DAM, respectively. In contrast, the negatively charged, 1-phosphate-substituted mitosene **6** exhibited no binding affinity to CT-DNA.

*Indication of Intercalation by 2,7-DAM: Hydrodynamic Properties of DNA–2,7-DAM Complexes.* Viscosity measurements of DNAs from varying sources in the presence of 2,7-DAM indicated (Figure 8) that the viscosity of all three DNAs increased, reaching a saturation value (2.4) of the relative reduced viscosity which was very close to that of the complex of CT-DNA with Etd, a classical intercalator (2.6). Thus, DNA was lengthened upon complexation with 2,7-DAM, a typical effect of intercalators (Lerman, 1961). MC caused essentially no change in viscosity under the same conditions. Another effect of intercalators is unwinding of DNA (Fuller & Waring, 1964). The helix unwinding ability of 2,7-DAM was tested by using the closed circular plasmid Col E1-DNA as probe. Unwinding of such DNA is manifested by release, followed by reversal, of supercoiling

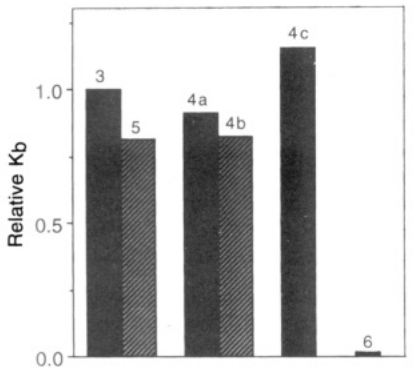



FIGURE 7: Relative binding affinity ("relative $K_b$") of various mitosene derivatives to CT-DNA. The binding constants ($K_b$) were determined by equilibrium dialysis in 10 mM Tris, pH 6 buffer as described in Materials and Methods and were related to the $K_b$ of 2,7-DAM.

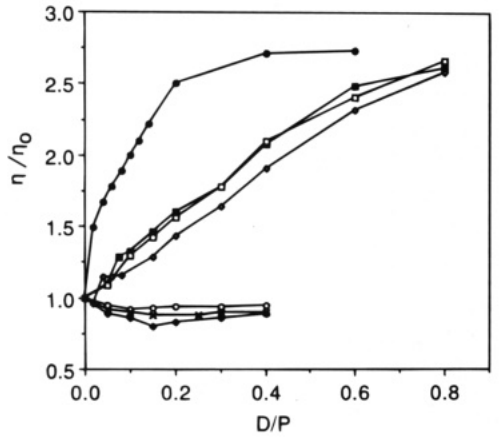



FIGURE 8: Viscometric titration of various DNAs with 2,7-DAM, MC, and Etd. D/P: Ratio of the molar concentration of drug to that of polynucleotide, defined in nucleotide units. $\eta/\eta_0$: Reduced relative viscosity. (●) ML-DNA/Etd; (◇) ML-DNA/2,7-DAM; (□) CT-DNA/2,7-DAM; (■) CP-DNA/2,7-DAM. MC controls: (○) CP-DNA/MC; (×) CT- DNA/MC; (♦) ML-DNA/MC. Buffer: citrate−phosphate, $[Na^+]$ = 10 mM, pH 6.05; temperature: 22 ± 1 °C.

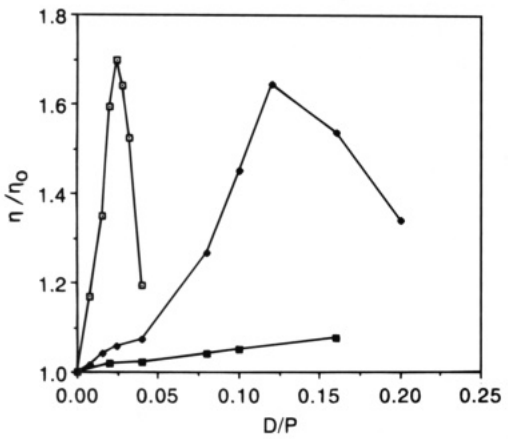



FIGURE 9: Viscometric titrations of closed circular Col E1-DNA with Etd (⊡), 2,7-DAM (♦), and MC (■). Definition of symbols and conditions were the same as in Figure 8, except that the DNA concentration was 0.15 mM.

with increasing drug concentration and can be followed by the rise and subsequent fall of viscosity (Allison & Hahn, 1977; Revet et al., 1971; Cain et al., 1978). As seen in Figure 9, 2,7-DAM induces this characteristic rise and fall of viscosity, indicative of intercalation. The D/P at the maximum is higher than that in the comparative experiment using Etd; this is as expected (Revet et al., 1971) for the 2 orders of magnitude lower binding constants of 2,7-DAM than that of Etd. Significantly, MC did not show any unwinding of supercoiled Col E1-DNA.

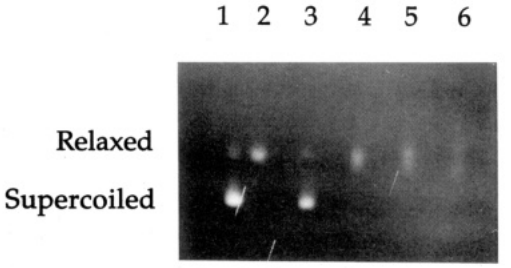



FIGURE 10: Gel electrophoretic assay of supercoiling of relaxed circular pBR322 DNA by 2,7-DAM. For conditions see Materials and Methods section. Lane 1: Control supercoiled pBR322 DNA. Lane 2: Relaxed pBR322 DNA, as a result of topoisomerase I treatment in the absence of 2,7-DAM. Lane 3: Supercoiled pBR322 DNA, formed upon incubation of DNA with topoisomerase I and Etd. Lanes 4−6: Progressive supercoiling of relaxed circular pBR322 DNA, formed upon incubation with topoisomerase I and 56, 105, and 187 μM 2,7-DAM, respectively.

*Supercoiling of relaxed circular pBR322 DNA by 2,7-DAM* was observed upon employing a procedure devised by Fisher et al. (1985) for testing intercalation by bleomycin derivatives. In the photograph of the gel (Figure 10) lanes 1 and 2 show control supercoiled and relaxed pBR322 DNA, respectively. Lane 3 shows the result of incubating relaxed DNA with Etd (10 μM) in the presence of topoisomerase I, indicating a supercoiled product. This effect of Etd is expected of intercalators in general. In lanes 4−6, the reaction products of relaxed DNA with 56, 105, and 187 μM 2,7-DAM, respectively, in the presence of topoisomerase I are shown. Progressive widening (lanes 4 and 5) and appearance of multiple bands (lane 6) indicate that 2,7-DAM induced supercoiling of the relaxed DNA, although at a much lower efficiency than Etd. This is consistent with the much lower binding affinity of 2,7-DAM to DNA than that of Etd expected under the experimental conditions (high ionic strength and pH, see Materials and Methods section). Our attempts to conduct the experiments at lower salt concentration and pH, comparable to the conditions of the viscosity and spectrophotometric studies (pH 6.0), were not successful. Nevertheless, the gel pattern indicates clearly the ability of 2,7-DAM to induce supercoiling of relaxed DNA, as a consequence of intercalation.

*Thermal Stabilization of 2,7-DAM−DNA Complexes.* 2,7-DAM enhanced the $T_m$ of all DNAs and polynucleotides studied. The value of $\Delta T_m$ ($T_m$ of complex − $T_m$ of control DNA) for several DNAs and polynucleotides are given in Table 3. The $\Delta T_m$ for CP- and ML-DNAs is approximately 2.5−3 °C (D/P = 0.8) while for CT-DNA a slightly higher stabilization (4.5 °C) was observed. The A·T polynucleotide displayed higher thermal stabilization than the G·C polynucleotide. $T_m$ measurements of the methylated GC polynucleotide could not be done as it had a $T_m$ >100 °C under our experimental conditions.

## DISCUSSION

The lack of noncovalent interaction of mitomycin C, mitomycin A, and porfiromycin with DNA, observed here, is unusual for DNA-reactive natural products. Nevertheless,

Table 3: $T_m$ of Various DNAs and Polynucleotides in the Presence of 2,7-DAM[a,b]

| polynucleotide | $T_m$ (control) | $T_m$ (+2,7-DAM) | $\Delta T_m$ |
|---|---|---|---|
| CP-DNA | 55.0 | 57.5 | +2.5 |
| CT-DNA | 66.5 | 70.5 (+MC: 66.5) | +4.0 (+MC: 0) |
| ML-DNA | 73.5 | 76.5 | +3.0 |
| poly(dA-dT)·poly(dA-dT) | 34.9 | 40.7 | +5.8 |
| poly(dG-dC)·poly(dG-dC) | 85.1 | 88.5 | +3.4 |

[a] Conditions: 40 $\mu$M DNA or polynucleotide; 32 $\mu$M 2,7-DAM in citrate−phosphate buffer, $[Na^+]$ = 10 mM, pH 6.05. [b] Average of two determinations; difference between the two ≤0.5 °C.

it confirms the earlier findings of Rodighiero et al. (1978) with MC. The semisynthetic analogs BMY-25067 and -25282 show no binding either, ruling out potential hydrophobic interactions of the large 7-substituent of these drugs with DNA. It is apparent from these results that mitosanes[3] in general lack DNA-binding capacity.

In striking contrast, a group of mitosene compounds (**3**−**6**), generated from the parent MC, was shown here to bind to DNA with considerable affinity. The mitosenes **3**, **4a**, and **4b** are major metabolites of MC, both *in vivo* (Cummings et al., 1993) and in cell-free enzymatic reductive activation systems (Tomasz & Lipman, 1981; Pan et al., 1984; Siegel et al., 1990). This investigation focused on 2,7-DAM since it may be regarded as a *stable* structural model of the reduced active form **2**, to which it is related by simple prototropism. Sterically, the only difference between the two molecules is at the C1 carbon (−CH= vs $-CH_2-$). However, as discussed below, the binding does not appear to be sensitive to C1 substituents. Thus we believe that the observed DNA-binding characteristics of 2,7-DAM apply closely to the unsta le activated form of MC, quinone methide **2**. DNA binding by covalently reactive drugs has been frequently characterized by employing stable unreactive metabolites or analogs, for example, in the case of anthramycin (Jones et al., 1990) or calicheamicin (Mah et al., 1994). The following conclusions were drawn from the present results.

*Electrostatic Component of Binding.* The binding of **3** to DNA is greatly increased at acidic pH, parallel to the protonation curve for the 2-amino group (Figures 4 and 5a). For example, a 10-fold increase of the binding constant is observed with CT-DNA as the pH is lowered from 8.0 to 5.0. This, together with the extent of the decrease of $K_b$ with increasing ionic strength (Figure 6), indicates that electrostatic attraction of the protonated mitosene is a substantial component of the binding. Consistent with this is that the magnitude of the "relative $K_b$s" of the three mitosenes 2,7-DAM, **4c**, and **4a** (1.0, 0.9, and 0.8) follow the order of their p$K_a$s: 7.55, 7.33, and 7.10, respectively (Figure 7). Furthermore, the electrically neutral phosphate derivative **6** shows no binding. A nonelectrostatic component with contributing $K_b = 1.87 \times 10^3$ is apparent from the extrapolation of the log $K_b$ *vs* log $[Na^+]$ plot (Figure 6) to 1 M $Na^+$ at which the electrostatic interaction should be negligible. Most of the experiments described here were conducted at pH 6.0 at which 2,7-DAM is 97% protonated.

*The Binding Is Not Specific with Respect to DNA Composition or Sequence.* As is apparent from the data in Table 2, all DNAs and synthetic polynucleotides tested exhibit $K_b$s of the same order of magnitude, i.e., $10^4$ $M^{-1}$, under the standardized conditions. Nevertheless, when the $K_b$s of the series, calculated by the "half-reciprocal method", are compared, a slight *selectivity* in favor of poly(dG-m5dC)·poly(dG-m5dC) is detected. The thermal stabilization of DNA by bound 2,7-DAM, manifested by increased $T_m$, is similarly nonspecific: all DNAs and polynucleotides tested showed a modest increase of $T_m$ without any discernible trend in magnitude within the series. The identical increase of the viscosity of the CP-, CT-, and ML-DNAs in the presence of increasing concentration of 2,7-DAM (Figure 8) also indicates that the binding is not specific to DNA base composition (or other factors different among these DNAs).

*Evidence for DNA Intercalation by 2,7-DAM.* (i) The strong hyperchromism of the 313 nm absorption band of 2,7-DAM upon binding to DNA is analogous to the hyperchromism exhibited by DNA intercalators (Dougherty & Pigram, 1982). (ii) 2,7-DAM causes an increase of viscosity of the three DNAs; this has the same magnitude at saturation as that caused by ethidium bromide, the prototype nonspecific DNA intercalating agent (Figure 6). Viscometric techniques are well-established for investigating helix length extension, associated with intercalation (Cohen & Eisenberg, 1969). In addition, we tested another critical criterion for intercalation: release, followed by reversal, of supercoiling of DNA as the drug concentration is increased. This was assayed by viscosity measurements (Revet et al., 1971). The test showed that 2,7-DAM indeed unwinds and rewinds superhelical Col E1-DNA. Finally, in yet another qualitative test for intercalation, supercoiling of relaxed circular pBR322 DNA upon binding by **3** was assayed by topoisomerase I (Fisher et al., 1985), also with positive results (Figure 10). Taken together, we conclude from these results that the observed binding of 2,7-DAM is, at least in large part, intercalative in nature. The weaker binding to denatured CT-DNA and lack of binding to formaldehyde-treated (single-stranded) CT-DNA (Table 2) is consistent with this conclusion.

*The structure−activity relationship study* of the binding of several mitosenes (Figure 7) suggests that the 10-$CONH_2$ group contributes only weakly, presumably by additional H-bonding, to the overall binding. The presence of a hydroxyl group in the 1-position (in **4a**) or the methyl group on the 2-nitrogen (in **4c**) constitutes neither any steric hindrance nor a significant boost to the binding. On the other hand, electrical neutrality as in **6**, due to the presence of the phosphate group, essentially abolished the binding.

That the absorbance-change method detects most or all of the bound drug molecules is indicated by the fact that the Etd-displacement method gave a similar value of $K_b$ of the 2,7-DAM−CT-DNA complex (Table 2). This method has been shown to detect both intercalation and groove binding modes (Lee et al., 1993a). Therefore, it is concluded that 2,7-DAM and other mitosenes bind to DNA by nonspecific intercalation, dominated by electrostatic attraction between the positively charged drug molecule and polyanionic DNA. This mode of binding explains why the parent mitosane, MC, or the other mitosanes in Chart 1 do not bind to DNA: (i) Their aziridine N is not basic (Iyengar et al., 1986; see also Table 1). (ii) Furthermore, the mitosanes lack the planar indoloquinone system; in fact they are "cup-shaped", with the aziridine bent back sharply, interacting electronically with the quinone ring (Shirahata & Hirayama, 1983). Reductive activation, however, converts MC to mitosenes, possessing a flat indoloquinone system and a basic 2-amino group (p$K_a$

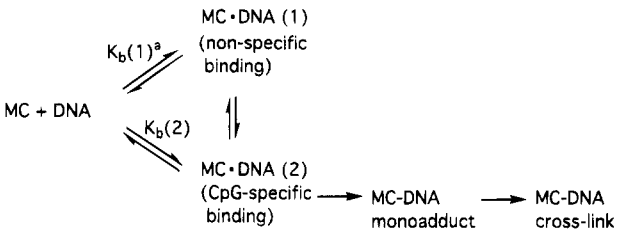
7−7.5), accounting for the characteristics of their affinity to DNA. Electrostatic binding of mitosenes to polyvinyl sulfate and polyphosphate was reported earlier. In this case, drug stacking was likely to provide a hydrophobic component to the binding (Lipman et al., 1978).

As argued above on structural grounds, this mitosene binding model is likely to apply to the binding to DNA of the active form of MC, **2**, which has the same shape and a similar basic p$K_a$ as 2,7-DAM. This assumption is experimentally supported by the observation of Crothers and coworkers (Teng et al., 1989) that the DNA-binding species formed transiently upon reduction of MC in the DNA cross-linking reaction has the same critical characteristic as our binding model: It binds nonspecifically to both A·T and G·C-containing oligonucleotides to the same extent.[4] Striking evidence that **2** has also a similar basic p$K_a$ as 2,7-DAM is provided by the reported pH dependence of the intrinsic covalent alkylating reactivity of reduced MC (Schiltz & Kohn, 1993). From the plot of percent electrophilic product *versus* pH presented by the authors, we could estimate a p$K_a$ = 7.2 of the active form of MC, which is close to our measured p$K_a$ of 2,7-DAM, 7.55 (Table 1). Several other studies confirm that lowering the pH promotes the alkylating or DNA cross-linking activity of reduced MC, giving a similar pH profile (Tomasz & Lipman, 1981; Cera et al., 1989; Siegel et al., 1992; Prakash et al., 1993).[5] Taking all this together, we conclude that the active form of MC, quinone methide **2**, must have essentially the same DNA-binding properties as its stable analog 2,7-DAM, determined in the present work.

*Significance. (i) The results imply that upon intracellular activation of mitomycin C* the positvely charged quinone methide **2** will be drawn to the vicinity of DNA by the relatively long-range electrostatic attraction of the DNA polyanion. Thus the binding mechanism serves to help the activated drug reach its target DNA with greater efficiency. It is likely that the binding kinetically facilitates the subsequent covalent reactions with DNA, at the expense of the competing inactivating metabolic reactions with small cellular nucleophiles or electrophiles in the cellular environment. The finding that the strong nucleophile, GSH competes only weakly with DNA for the activated MC in cell-free model systems supports this scenario (Sharma et al., 1994). These results suggest that designed mitomycins should retain a positively charged basic function for maximum biological efficacy.[6]

*(ii) Relationship to the Base-Sequence Specificity of DNA Alkylation.* The specificity for alkylation by activated MC is known to be determined by covalent reactivity factors, intrinsic to the structure of the guanine nucleoside itself, as demonstrated, for example, by the ready alkylation of dG or d(GpC) while dA, dT, or dC or their dinucleotide combinations give no adducts (Tomasz et al., 1986a,b). Thus, DNA binding plays no role in the specificity for guanine. On the other hand, MC displays high *sequence selectivity* of monoalkylation of guanine at CpG as compared to ApG, TpG, and GpG in duplex DNA (Li & Kohn, 1991; Kumar et al., 1992) and a virtually absolute sequence specificity of cross-linking of the guanines at CG·CG (Teng et al., 1989; Weidner et al., 1989; Borowy-Borowski et al., 1990). In order to explain the CpG selectivity of the alkylation, we postulated (Kumar et al., 1992) a *specific* precovalent binding mode of activated MC at CpG in the minor groove, featuring an H-bond possible only at this sequence. In support of this proposition, the structure of the *covalent* MC monoadduct−DNA complex, determined by an NMR−molecular dynamics study, closely resembles this postulated precovalent binding alignment, showing the presence of the specific H-bond (Sastry et al., 1995). It is clear, however, that the experimentally observed binding described here is different as it is intercalative and nonspecific. It is likely that it represents a stronger, primary binding mode of activated MC which masks the weaker, specific minor groove binding necessary for the covalent reaction to take place. This bulk primary nonspecific binding may serve only to concentrate the drug nonspecifically at the DNA (Warpehoski & Hurley, 1988). These processes are summarized in Scheme 2.

Scheme 2[a]




[a] $K_b(1) \gg K_b(2)$.

*(iii) The nonspecific binding of the MC metabolites 2,7-DAM and* ***4a*** *to DNA,* described here, may account for the weak cytotoxic properties reported for these substances (Iyengar et al., 1989).

## ACKNOWLEDGMENT

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[4] The authors calculated $K_{dissoc}$ = 600 M bp for both oligonucleotides, corresponding to $K_b$ = 1700 (M nt)$^{-1}$ by our calculation, which is lower than our observed $K_b$s. They emphasize, however, that the magnitude of this parameter should be regarded as a very rough estimate only, but (to quote), "...the ability of nonreactive sites (A·T) to bind in a similar manner to the reactive sites (G·C) is a definite conclusion of our analysis".

[5] The mechanism of this effect is not well-understood (Schiltz and Kohn, 1993; Prakash et al., 1993).

[6] Certain $N^{1a}$-acyl mitomycins have been reported to possess cytotoxic and antitumor activity, nevertheless (Fishbein and Kohn, 1987).

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