Studies on Interaction of Anthracycline Antibiotics and Deoxyribonucleic Acid: Equilibrium Binding Studies on Interaction of Daunomycin with Deoxyribonucleic Acid†

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ABSTRACT: We have used equilibrium dialysis and fluorescence and absorbance titration to study the interaction of daunomycin with DNA. Our data at 200 mM Na^+ are best fit by the neighbor exclusion model, with $K = 7.0 \times 10^5$ M^{-1} and an exclusion parameter of three to four base pairs. The binding is dependent on ionic strength, with d log K/d log $[Na^+]$ = −0.84, from which we may estimate quantitatively ion release and the binding free energy corrected for the free energy of counterion release. From the temperature dependence of the binding constant, we find the binding to be exothermic, with a van't Hoff enthalpy of −12.8 kcal/mol. Competition dialysis experiments show that G+C base pairs are slightly preferred as binding sites for the drug and suggest that daunomycin binds preferentially to G+C pairs at low $\bar{r}$. Cesium chloride density gradient sedimentation experiments provide an experimental demonstration of this preference. Daunomycin increases the T_m for DNA melting by some 30 °C as binding approaches saturation, with biphasic melting at low drug/base pair ratios. The data from these equilibrium studies are consistent with intercalative binding of daunomycin and provide a solid foundation for further structural and kinetic studies.

Daunomycin is an anthracycline antibiotic widely used in the treatment of various cancers. The drug is believed to act by inhibiting both DNA replication and RNA transcription (Hartmann et al., 1964; Ward et al., 1965; DiMarco et al., 1971) by direct interaction with DNA. The chemistry of daunomycin and its effects on biological systems both in vivo and in vitro have recently been reviewed (Arcamone, 1978; Neidle, 1978).

Numerous studies on the interaction of daunomycin with DNA have been reported (Zunnio et al., 1980, 1972; Gabbay et al., 1976; Plumbridge & Brown, 1977; Barthalemy-Clavey et al., 1973; Huang & Phillips, 1977; Molinier-Jumel et al., 1978; Schütz et al., 1979). However, several important aspects of the daunomycin–DNA interaction have not been treated in these studies. First, binding isotherms reported in these studies as Scatchard plots (Scatchard, 1949) often show pronounced curvature. With the exception of the paper by Schütz et al. (1979), this has been either ignored or interpreted as resulting from two classes of binding sites, "strong" and "weak". This explanation neglects neighbor exclusion effects, and hence formal application of the Scatchard model is probably inappropriate for the case at hand. Second, the effect of ionic strength on daunomycin binding to DNA remains to be clarified. Finally, the specificity of daunomycin binding, with regard to the type of base pair preferred as a binding site, has received little attention.

We report here experiments designed to address these questions in more detail. We have studied the binding of daunomycin to calf thymus DNA as a function of ionic strength and temperature, using equilibrium dialysis and absorbance and fluorescence titration. Competition dialysis experiments (Müller & Crothers, 1975) were used to infer the specificity of daunomycin binding reaction. In addition, the effects of daunomycin on the buoyant density and thermal denaturation of calf thymus DNA were examined. In companion papers, we report on the self-association of daunomycin and the potential effects of self-association on the interaction of the drug with DNA (Chaires et al., 1982) and on the geometry of the daunomycin–DNA complex (Fritzsche et al., 1982).

Materials and Methods

Materials. Calf thymus DNA was obtained from Boehringer-Mannheim (Indianapolis, IN). Daunomycin hydrochloride, *Clostridium perfringens* DNA, and *Micrococcus lysodeikticus* DNA were purchased from Sigma Chemical Co. (St. Louis, MO).

Buffers. The buffers used in this work are designated as follows: TE, 10 mM Tris[1] and 1 mM Na_2EDTA, pH 7.5; BPE, 6 mM Na_2HPO_4, 2 mM NaH_2PO_4, and 1 mM Na_2-EDTA, pH 7.0; BPES, 6 mM Na_2HPO_4, 2 mM NaH_2PO_4, 1 mM Na_2EDTA, and 185 mM NaCl, pH 7.0.

Quality Control. Samples of daunomycin were routinely checked for purity by using thin-layer chromatography on silica gel plates, in a solvent system containing acetic acid–methanol–chloroform (1:1:8). NMR spectra revealed no evidence of degradation products.

DNA Preparation. DNA was dissolved in BPE buffer and dialyzed overnight. Approximately 50 mL (~2 mg/mL) was sonicated at ice bath temperatures for a total of 30 min with a Branson sonifier. Five-minute periods of sonication were used, followed by 5 min of nitrogen bubbling to purge the solutions of oxygen. Following sonication, the Na^+ concentration was raised to 200 mM by addition of NaCl and the solution treated with RNase (Sigma) at 0.5 mg/mL for 30 min at 37 °C. Proteinase K (Boehringer-Mannheim) at 0.5 mg/mL was added, with incubation at 37 °C for 1–2 h. The solution was extracted 3 times with an equal volume of a phenol solution containing 500 g of phenol, 70 mL of *m*-cresol, 1% (w/w) 8-hydroxyquinoline, and H_2O to saturation, adjusted

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[1] Abbreviations: Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetic acid; NMR, nuclear magnetic resonance; $NaDodSO_4$, sodium dodecyl sulfate.

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to pH 8.0. Three extractions with water-saturated ether followed. The DNA was dialyzed overnight against BPES buffer and then fractionated on a column containing Sepharose 4B (Pharmacia). The central region of the elution peak was pooled, concentrated, and dialyzed against BPES buffer. Calf thymus DNA prepared in this way had the following physical properties: s_w^{20} = 6–7 S; M_w 120 000; T_m = 65.3 °C (TE buffer); 34.6% hyperchromism.

DNA concentrations were determined by absorbance measurements at 260 nm with the following extinction coefficients: calf thymus, 12 824 M^{-1} (base pair); *C. perfringens*, 12 450 M^{-1} (base pair); *M. lysodeikticus*, 13 846 M^{-1} (base pair).

Equilibrium Dialysis. DNA solutions (1–2 mL) were dialyzed against buffer containing daunomycin with Spectra/Por2 dialysis tubing (Spectrum Medical Industries, Inc.). Equilibrium was reached within 24 h with this dialysis tubing, with no detectable binding of the drug to the membrane. The concentration of free drug (C_f) in the dialyzate was measured directly from the absorbance at 480 nm or from the fluorescence intensity (λ_{ex} = 480 nm, λ_{em} = 555 nm) with reference to a standard curve prepared from daunomycin solutions of known concentrations. The amount of total drug (C_T) in the retentate was determined directly by absorbance at 540 nm or by dissociating the drug–DNA complex by the addition of $NaDodSO_4$ to a final concentration of 1% and measuring the absorbance at 480 nm. The extinction of the drug at 480 nm was unchanged in the presence of $NaDodSO_4$. The two procedures gave equivalent results. The amount of bound drug (C_B) was determined by difference:

$$C_B = C_T - C_f \quad (1)$$

Phase Partitioning. The technique of phase partitioning has been described in detail, for example, by Waring et al. (1975), Davanloo & Crothers (1976), and Dattagupta et al. (1980). An aqueous buffer containing the drug–DNA complex was shaken with an equal volume of buffer-saturated 1-pentanol. The drug concentrations in the two phases were measured spectrophotometrically. Extinction coefficients of daunomycin at 480 nm were identical (ϵ_{480} = 11 500 mol^{-1} cm^{-1}) in both the phases. The total drug concentration in the aqueous phase was determined after dissociation of the complex with sodium dodecyl sulfate [final concentration 1% (w/v)]. The free drug concentration in the aqueous phase is $C_f = C_{org}/D$, where C_{org} is the concentration of the drug in the organic phase and D is the distribution coefficient. We found D = 13.7 at 25 °C for daunomycin in the BPES–pentanol solvent system. Moreover, C_T in the aqueous phase is equal to $C_f + C_B$. Hence

$$C_B = C_T - C_{org}/D$$

Absorbance Titration Experiments. Titration experiments were performed in a Cary 219 spectrophotometer, maintained at constant temperature by a Neslab RTE 9 refrigerated circulating bath. Plastic cuvettes were used, with continuous stirring throughout the course of the titration. The amounts of free and bound daunomycin were determined as follows. Following each addition of drug, from the absorbance at the isosbestic point, 540 nm, the total drug present was calculated, $C_T = A_{540}/\epsilon_{540}$. This quantity was used to calculate the *expected* absorbance at 480 nm, $A^0 = C_T\epsilon_{480}$. The difference in A^0 and the observed absorbance was used to calculate the amount of bound drug:

$$C_B = A/\Delta\epsilon = (A^0 - A_{obsd})/(\epsilon_f - \epsilon_B) \quad (2)$$

The amount of free drug was determined by difference:

$$C_f = C_T - C_B$$

The extinction coefficient of the bound drug was determined by addition of a known quantity of daunomycin to a large excess of DNA and on the assumption of total binding, $\epsilon_B = A_{480}/C_T$. Alternately, the absorbance of a known quantity of daunomycin was monitored at 480 nm while adding known amounts of DNA until no further change in absorbance was observed.

Fluorescence Titration. Fluorescence titration experiments were performed on a Perkin-Elmer 650-40 spectrofluorometer at ambient temperature. A slit width of 10 nm was typically used, with λ_{ex} = 480 nm and λ_{em} = 555 nm. The ratio of the fluorescence intensity of daunomycin in the absence of DNA (I_0) and in the presence of DNA (I) was used to calculate the amount of bound drug according to

$$C_f = C_T(I/I_0 - P)/(1 - P) \quad (3)$$

where C_T is the known added amount of drug and P is the ratio of the observed quantum yield of fluorescence of the totally bound drug to that of the free drug (Blake & Peacocke, 1968). C_B was then obtained by difference.

The quantity P was determined by adding DNA to a known quantity of daunomycin until no further change in fluorescence emission intensity was observed. The ratio $P = I_\infty/I_0$ was then obtained from the initial value of I and the plateau value of I at high DNA concentration.

Analysis of Binding Data. Plots of r/C_f vs. r, where r is the number of moles of bound daunomycin per mole of DNA base pair, were constructed according to Scatchard (1949). Theoretical curves for the neighbor exclusion model were calculated by using the algorithm of Crothers (1968) or the closed form of McGhee & von Hippel (1974):

$$r/C_f = K_i(1 - nr)[(1 - nr)/[1 - (n - 1)r]]^{n-1} \quad (4)$$

where K_i is the intrinsic binding constant and n is the exclusion parameter in base pairs. The methods gave identical results. The experimental parameters K_i and n were adjusted to produce curves that gave, by inspection, the most satisfactory fits to the experimental data.

CsCl Density Gradient Sedimentation. CsCl density gradient experiments were performed in a Spinco Model E analytical ultracentrifuge, at 20 ± 1 °C, at 40 000 rpm. Equilibrium was reached after 24 h, as judged by comparison of scans at 24 h with those taken at later times. A DNA concentration of 2 μg/mL and a column height of 10 mm were used. The density of CsCl solutions was determined by weight with a calibrated 100-μL micropipet.

Melting Curves. Thermal denaturation experiments were performed in stoppered quartz curvettes in a Cary 219 spectrophotometer. Samples were continuously heated at 0.8 °C/min. By use of the "chart" utility of the temperature readout accessory, the temperature of a dummy cuvette was continuously monitored and used to drive the x axis of the recorder output at 1 °C/cm. Absorbance changes at 260 nm were monitored. DNA samples were dialyzed into BPE/4 buffer for these experiments; typically, the initial DNA concentration was 0.5–0.7 A_{260}.

Results

Optical Properties of Free and Bound Daunomycin. Figure 1 shows the visible absorbance spectra of free and bound daunomycin. Binding of the drug to DNA results in a red shift of the absorbance maximum from 480 to 505 nm. An isosbestic point is observed at 540 nm. We determined the extinction coefficient of the bound drug by adding known

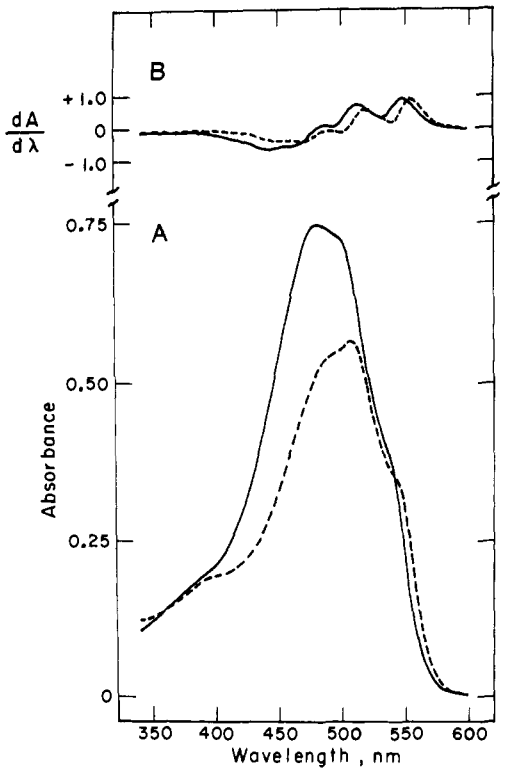

FIGURE 1: Visible absorbance spectra of free and bound daunomycin. (A) Daunomycin concentration of 65 μM in BPES buffer, 20.4 °C: (—) no DNA; (---) 0.25 mM DNA. (B) First derivatives of the spectra shown in (A), used to obtain λ_{max}.

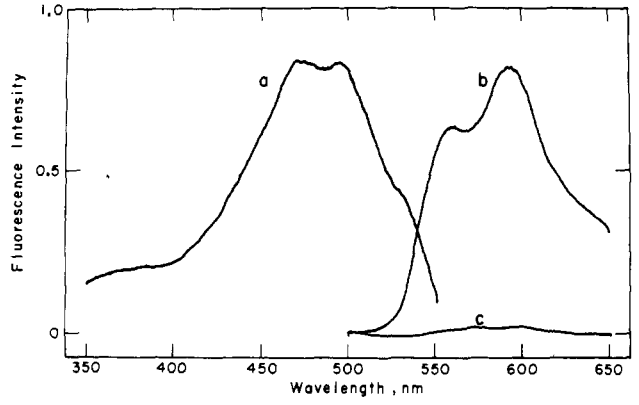

FIGURE 2: Fluorescence emission and excitation spectra of daunomycin. The relative fluorescence intensity of 0.87 μM daunomycin in TE buffer, 20 °C, is shown. The uncorrected emission spectra for free daunomycin (b) and for daunomycin in the presence of 0.17 mM (base pair) calf thymus DNA (c) were recorded with λ_{ex} = 480 nm. The corrected excitation spectra (a) of free daunomycin was recorded with λ_{em} = 592 nm.

amounts of daunomycin to solutions containing a large excess of DNA and assuming that all of the drug was bound. A value of 7000 ± 250 M^{-1} cm^{-1} was obtained. Ionic strength has no apparent effect on the extinction coefficients of the free or bound drug over the range of salt concentrations used in this study.

The fluorescence excitation and emission spectra of daunomycin are illustrated in Figure 2. Emission maxima at 555 and 592 nm are seen; the corrected excitation spectrum is identical with the absorbance spectrum of the free drug seen in Figure 1. In the presence of DNA, the fluorescence emission of daunomycin is quenched (Figure 2, curve c), with no evident shift in the emission maximum. We determined the parameter P, required for titration studies, either by measurement of the

Table I: Optical Properties of Daunomycin in BPES Buffer

	λ_{max} (nm)	ϵ_{480} (M^{-1} cm^{-1})	ϵ_{540} (M^{-1} cm^{-1})	relative fluorescence[a]
free	480	11500	5100	1.0
bound	505	7000 ± 250	5100	0.05 ± 0.02

[a] At λ_{em} = 555 nm with λ_{ex} = 480 nm.

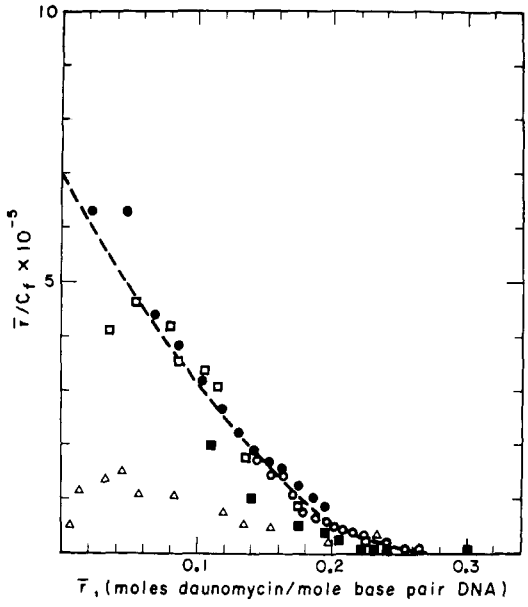

FIGURE 3: Scatchard plot of equilibrium binding isotherm for the daunomycin–DNA interaction. The buffer is BPES, 20 °C. Data are shown from fluoresence titration (●), absorbance titration (○), equilibrium dialysis with absorbance used to quantitate the free and total drug (■), equilibrium dialysis with fluorescence used to detect free drug (□), and solvent partition methods (△). The line (---) was calculated for the neighbor exclusion model, assuming n = 3.5 and $K_i = 7.0 \times 10^5$ M^{-1}.

fluorescence emission of a given quantity of daunomycin in the presence and absence of a large excess of DNA or by addition of DNA to a fixed amount of drug until no further change in fluorescence emission was observed. A value of P = 0.05 ± 0.02 was obtained regardless of the protocol and was used in our calculations.

The optical properties of free and bound daunomycin are collected in Table I. Of crucial importance are the errors associated with ϵ_B and P. The error associated in these quantities propagates directly into the determination of C_B. We thus estimate that we can, using the values indicated, estimate C_B in our titration experiments to at best 5%.

Daunomycin Binds Tightly to DNA and Shows Negative Cooperativity. We began our study of the daunomycin–DNA interaction by studying the influence of DNA concentration on daunomycin binding. DNA solutions were dialyzed against a common daunomycin solution until equilibrium was attained. A free daunomycin concentration of 2.9 μM was reached at equilibrium; the amount of daunomycin bound per base pair of DNA was independent of DNA concentration over the range of 0.1–0.8 mM (base pair) and was found to be 0.22 mol of daunomycin/mol of base-pair DNA. This result indicates that no unusual cooperativity accompanies the drug binding.

The results of equilibrium dialysis experiments and fluorescence titration and absorbance titration studies are presented in the form of a Scatchard plot (Scatchard, 1949) in Figure 3. The agreement between the various methods is quite good. We have fit the experimental data to the neighbor

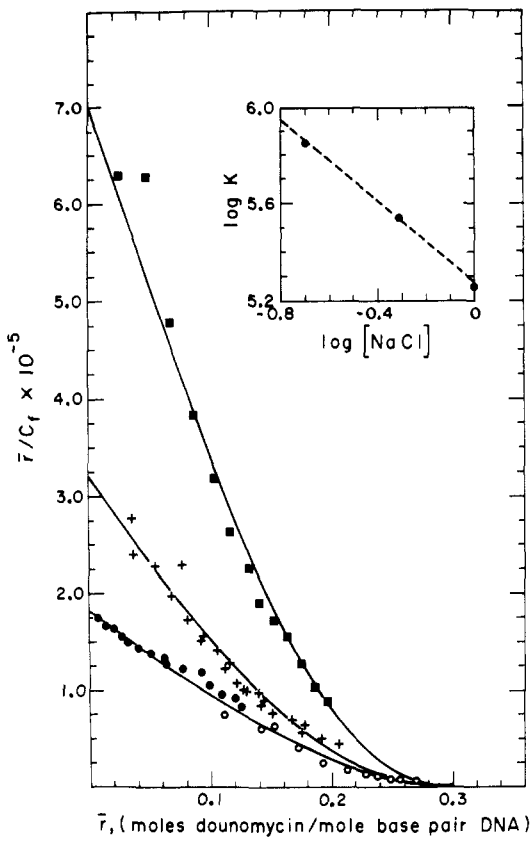

FIGURE 4: Effect of ionic strength on the strength of the daunomycin–DNA interaction. Results are shown from fluorescence titration experiments in BPES buffer (■), BPE buffer plus 0.5 M NaCl (+), and BPE buffer plus 1 M NaCl (●, ○). The data were fit by using the neighbor exclusion model with $n = 3.25$ and the following values for the equilibrium constant: BPES buffer, 7×10^5 M^{-1}; 0.5 M NaCl, 3.2×10^5 M^{-1}; 1 M NaCl, 1.8×10^5 M^{-1}. (Inset) Double log plot illustrating the influence of ionic strength on K_i. The slope is −0.84.

exclusion model (Crothers, 1968; McGhee & von Hippel, 1974), which adequately accounts for the curvature seen in the data of Figure 3. An exclusion parameter, $n = 3.5$ base pairs, and an intrinsic binding constant, $K_i = 7.0 \times 10^5$ M^{-1}, provide the best fit to the experimental data. The uncertainty in these parameters is roughly 10%.

We attempted to use two additional methods to obtain binding data, with poor results. We tried to estimate the free drug concentration by ultrafiltration of solutions containing DNA and drug at equilibrium using Amicon CF 25 Centri flo ultrafiltration membrane cones. We found, however, that virtually all of the daunomycin bound tightly to the filter, rendering the method useless. We also tried to use a phase partition method using 1-pentanol as the organic phase. We obtained results that were not in agreement with other methods used (Figure 3) and that give the illusion of positive cooperativity at low $\bar{r}$ values. We found that dialysis experiments in the presence or absence of 6% pentanol gave identical results, eliminating the possibility that the organic phase somehow perturbs the DNA structure as an explanation of these results. If the DNA–drug complex were soluble to any extent in the organic phase, results giving the illusion of cooperativity might be obtained. We tested for this by labeling a sample of sonicated DNA with ^{32}P and measuring the amount of label found in the organic phase as a function of added daunomycin. In fact, no DNA was found to partition to the organic phase, eliminating this as an explanation and leaving us at a loss to account for the effects seen. Unknown contaminants could be responsible; furthermore, possible

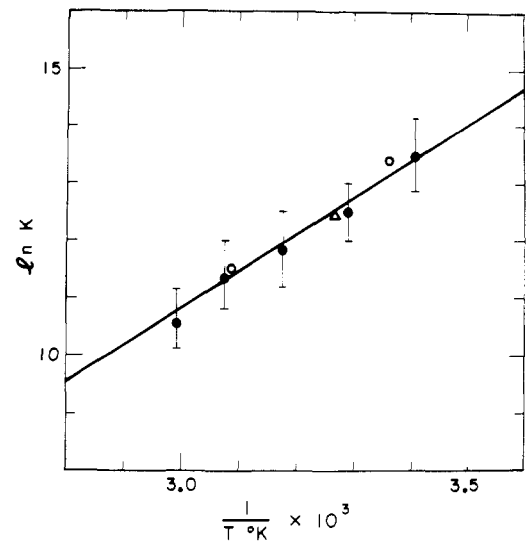

FIGURE 5: van't Hoff plot for the daunomycin–DNA interaction in BPES. Data are shown from complete optical titrations and subsequent fitting to the neighbor exclusion model (○), from variance of T (°C) at a fixed r_{added} (●), or from the equilibrium constant calculated from a temperature-jump relaxation kinetics experiment (△). The slope is 6.4 and yields $\Delta H = -12.8$ kcal/mol.

electrical effects at the phase boundary have so far been ignored in the use of this technique. We have chosen to ignore the phase partition data since there was such a discrepancy between it and the three other methods we have successfully used. These experiments suggest caution in use of the phase partition method to study drug–DNA interaction.

Binding of Daunomycin Is Dependent on Ionic Strength. We have used fluorescence titration to study the effect of ionic strength on the affinity of daunomycin for DNA, with the results shown in Figure 4. The intrinsic binding constant drops from 7.0×10^5 M^{-1} to 1.8×10^5 M^{-1}, as the Na^+ concentration changes from 0.2 to 1.0 M, with the exclusion parameter essentially constant at 3.25 ± 0.25. The inset of Figure 4 shows the logarithmic dependence of the equilibrium constant on Na^+ concentration. A slope of −0.84 is observed, the significance of which will be discussed later.

Daunomycin Binding Is Exothermic. The temperature dependence of the daunomycin binding constant was examined by two procedures. First, complete absorbance titrations, similar to that in Figure 3, were performed at two temperatures, and the data were fit to the neighbor exclusion model. Second, a fixed amount of daunomycin was added to a DNA solution at a constant temperature. The amount of r_b was determined spectrophotometrically, and K_i was calculated from eq 4 by assuming $n = 3.5$. The solution was then equilibrated to a new temperature, and K_i was determined. This was repeated to obtain data at several temperatures. The data are presented in Figure 5 in the form of a van't Hoff plot. The two procedures are in good agreement with one another. An independent estimate of K was also obtained from temperature-jump relaxation kinetic experiments (J. B. Chaires, unpublished results) that also falls on the same line. Linear least-squares fitting of the data yields a slope of 6.4, and $\Delta H^\circ = -12.8$ kcal/mol. Assuming $K_i = 7.0 \times 10^5$ M^{-1} (25 °C), we calculate $\Delta G^\circ_{25°C} = -8.0$ kcal/mol and $\Delta S^\circ_{25°C} = -16.2$ eu, all subject to about 10% uncertainty.

Daunomycin Shows a Slight Preference for G+C Base Pairs as a Binding Site. Competition dialysis (Müller & Crothers, 1975) was used to examine the preferred binding site for daunomycin. DNAs from *C. perfringens* (31% G+C), calf thymus (42% G+C), and *M. lysodeikticus* (72% G+C) at

Table II: Results from Competition Dialysis Experiment[a]

DNA	% C+G	r (daunomycin/mol of base pairs) at C_f (μM) of 1.57	5.04	36.43
M. lysodeikticus	72	0.183	0.282	0.323
calf thymus	42	0.172	0.273	
C. perfringens	31	0.144	0.235	0.287

[a] Samples were dialyzed 4 days at 21 °C in BPES buffer. DNA concentrations were 0.15 mM (base pair).

identical concentrations were dialyzed to equilibrium against various concentrations of daunomycin in BPES buffer. The amount of drug bound per base pair, r_b, was then determined. The results are shown in Table II. Qualitatively, the results are clear: the G+C rich DNAs bind more daunomycin. If there were no base specificity to the binding, the r_b values should be equal. Therefore, daunomycin shows some preference for the G+C base pair. Müller & Crothers (1975) have proposed a more quantitative determination of specificity in terms of the parameter α', defined as the ratio of r values for two DNAs of varying G+C content at the same free drug concentration:

$$\alpha' = r(M.\ lysodeikticus\ \text{DNA})/r(C.\ perfringens\ \text{DNA}) \quad (5)$$

The limiting value of α' as r and C_f approach 0 is α:

$$\alpha = \lim_{r \to 0} \alpha'$$

For two DNAs of varying G+C content, the meaning of α may be specified as

$$\alpha = \sum K_i B_i(\text{DNA I})/\sum K_i B_i(\text{DNA II}) \quad (6)$$

Where K_i is the intrinsic binding constant and B_i is the number of binding sites for each DNA. The quantity α is thus a measure of the relative affinity of the drug for one DNA over another.

From a plot of α' vs. r_b(*C. perfringens*), we obtain from the data of Table II an extrapolated value of $\alpha = 1.45$. For the binding of daunomycin on one side of a G+C base pair at constant K_i, with no binding to A+T pairs, the calculated B_i values lead to prediction of $\alpha = 2.32$ for this pair of DNAs. If binding may occur on either side of the G+C base pair, $\alpha = 1.75$ is predicted. No G+C preference predicts $\alpha = 1.0$, while A+T preference predicts $\alpha < 1.0$. Thus, while we cannot distinguish the two modes of binding to G+C base pairs, we can say that daunomycin shows a moderate preference for G+C base pairs as a binding site but that binding to A+T pairs also occurs.

To interpret the G+C specificity quantitatively, we used a modified version of the algorithm of Crothers (1968), generating an arbitrary DNA sequence containing 42% G+C, and determined the fraction of A+T and G+C sites occupied by drug at various r values. When the ratio of A+T and G+C daunomycin binding constants K_A/K_G was set equal to 1.45, the calculated ratio α was 1.45, in agreement with experiment. This introduction of binding site heterogeneity produced negligible effects on the shape of the calculated binding isotherm.

Preferential Binding of Daunomycin to G+C Rich Regions in CsCl Density Gradients. We attempted to observe the possible effects of G+C preference on CsCl density gradient sedimentation equilibrium profiles, using an artificial mixture of calf thymus and *M. lysodeikticus* DNA. The results are shown in Figure 6. In the absence of drug, the calf thymus

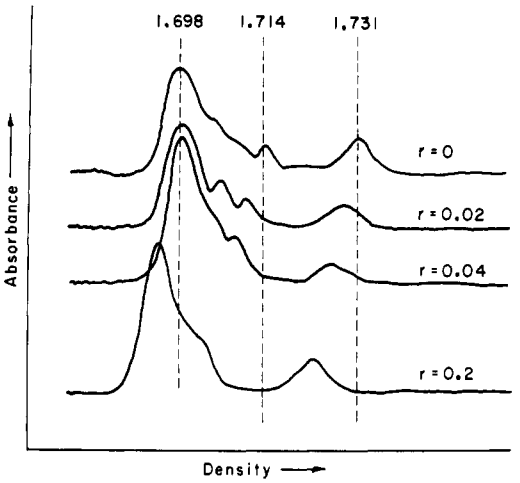

FIGURE 6: Cesium chloride density gradient sedimentation equilibrium of DNA in the presence and absence of daunomycin. A mixture of calf thymus and *M. lysodeikticus* DNA (2 μg/mL) was sedimented in CsCl (initial density 1.716 g/mL) at 40000 rpm, 20 °C, in a Spinco Model E analytical ultracentrifuge for 24–48 h. The photoelectric scanner system was used to obtain the traces shown, with λ = 300 nm. The amount of daunomycin added, r = moles of daunomycin per mole of base-pair DNA, is indicated.

satellite DNAs are well resolved. At low r values, the G+C rich satellite and the *M. lysodeikticus* DNAs are seen to shift to lower density, while the main band calf thymus DNA is stationary. Finally, at high r values, the main band DNA shifts to lower density. It appears that, at low r values, the daunomycin preferentially occupies mostly the G+C rich portions of the DNA mixture and alters their density. The effect is in fact much larger than what is predicted for $\alpha = 1.45$. Clustering of preferred sites in the G+C rich regions may produce an even stronger partitioning to produce the effects seen in Figure 6. Specificity may also be enhanced in high CsCl concentration.

Daunomycin Increases T_m Dramatically. The thermal denaturation of calf thymus DNA in the presence and absence of daunomycin was examined with the results shown in Figure 7A. Under these conditions, calf thymus DNA shows a T_m of 48.5 °C, with a hyperchromicity of 32%. Daunomycin dramatically stabilizes the DNA; at higher r values, the T_m is increased by some 30 °C. At $r = 0.062$, the melting is distinctly biphasic. Figure 7B shows that in the course of this biphasic melting, daunomycin is released only in the second, higher temperature transition.

Discussion

Our results show that daunomycin binds tightly to calf thymus DNA. The neighbor exclusion model provides the best fit to our data, yielding $K_i = 7.0 \times 10^5\ M^{-1}$ and $n = 3.5$ base pairs at 25 °C in BPES buffer. The binding constant decreases with increasing ionic strength, with $d \log K_i / d \log [Na^+] = -0.84$. Binding of the drug is exothermic, with $\Delta H° = -12.8$ kcal/mol (at 25 °C), $\Delta G° = -8.0$ kcal/mol, and $\Delta S° = -16.2$ eu. Daunomycin appears to prefer G+C base pairs as a binding site and prefentially alters the bouyant density of G+C rich DNAs in CsCl density gradients. Finally, daunomycin stabilized DNA toward thermal denaturation.

Comparison of our binding constant with previous work is facilitated by examination of Table III. With a single exception, previous investigators have used the Scatchard model (Scatchard, 1949) to extract a binding constant from their data, from the slopes of linear regions of plots such as those seen in Figure 3, and from the number of binding sites, n, from the x intercept. The Scatchard model formally assumes in-

Table III: Summary of Reported Binding Constants for Interaction of Daunomycin with DNA[a]

no.	K_i (M^{-1})	1/n (mol of DM/ mol of nucleotide)	cation concn (mM)	model	method	source of DNA	curvature	reference
1	1.2×10^7	0.22	10	S	F	CT	yes	Zunnio et al. (1980)
	4.8×10^6	0.16	100					
	1.3×10^6	0.08	1000					
2	3.1×10^6	0.16	100	S	A, D	CT	yes	Zunnio et al. (1972)
3	6.8×10^6	0.17	10	S	A	SS	no	Gabbay et al. (1976)
	9.3×10^6	0.17	10	S	P			
4	1.3×10^6	0.20	50	S	A	CT	?	Plumbridge & Brown (1977)
5	7.3×10^5	0.22	150	S	D	CT	yes	Barthalemy-Clavey et al. (1973)
6	2.1×10^6	0.16	150	S	F, D	CT	no	Huang & Phillips (1977)
7	6.0×10^5	0.13	150	S	polarography	CT	yes	Molinier-Jumel et al. (1978)
8	6.2×10^5	0.33 (DM/bp)	200	NE	A	CT		Schütz et al. (1979)

[a] Abbreviations: S, Scatchard; NE, neighbor exclusion; A, absorbance; D, dialysis; F, fluorescence; P, phase partition; CT, calf thymus; SS, salmon sperm; DM, daunomycin; bp, base pair.

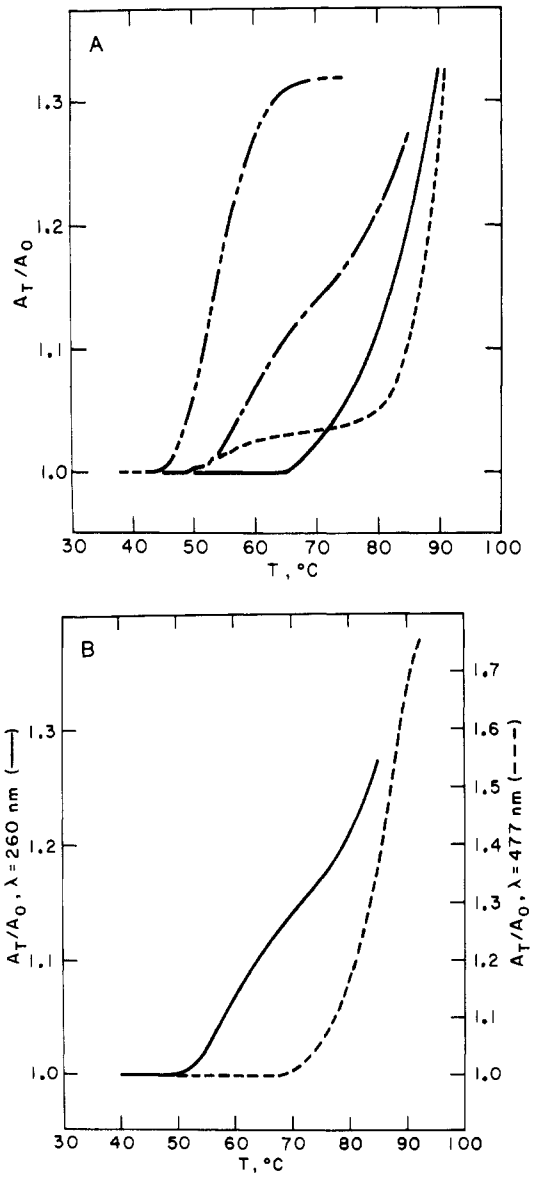

FIGURE 7: Thermal denaturation of calf thymus DNA in 1.5 mM Na_2HPO_4, 0.5 mM NaH_2PO_4, and 0.25 mM Na_2EDTA, pH 7.0. (A) Monitored at 260 nm, with no daunomycin (----), $r = 0.062$ (---), $r = 0.097$ (—), and $r = 0.165$ (---). (B) Monitored at 477 nm (---), showing the release of daunomycin during the melting of the $r = 0.062$ sample.

dependent (noninteracting) binding sites. In several cases, as noted in Table III, curvature is apparent in the reported plots, which has either been ignored or interpreted as representative of two classes of sites with differing affinities for the drug. A more plausible explanation, which accounts fully for the curvature seen in Figure 3, is the neighbor exclusion model (Crothers, 1968; McGhee & von Hippel, 1974), which describes a process in which the binding of a drug molecule to one site influences the binding of subsequent molecules. In particular, subsequent drug molecules are excluded from binding at nearby sites, either by physical blockage or by steric alterations in the DNA. We determine, in Figure 3, an exclusion parameter $n = 3.5$ base pairs. A fractional exclusion parameter has at least two possible meanings: drugs bound three base pairs away may bind but more weakly than those bound four or more base pairs away from a bound drug molecule. Alternatively, the neighbor exclusion range may vary from three to four depending on base sequence. This estimate of the neighbor exclusion distance is in rough accord with the recent crystal structure of a daunomycin–DNA complex (Quigley et al., 1980). The structure shows that daunomycin intercalates between two base pairs but that the sugar residue extends into the minor groove and partially blocks a third base pair. The double helix is also sterically distorted, which may further hamper binding at nearby sites.

Ignoring the effects of neighbor exclusion leads to an artificially high estimate for the drug binding affinity. If one calculates the slope of the linear region of the data in Figure 3 (over the range $r = 0$–0.16) and assigns this value to K_i, the result is 3.3×10^6 M^{-1}, well within the range of the values reported in Table III. The intrinsic binding constant determined with the neighbor exclusion model is lower than this by a factor of 5. Using the neighbor exclusion model, Schütz et al. (1979) determined $n = 3$ base pairs and $K_i = 6.2 \times 10^5$ M^{-1} in 200 mM Na^+, in substantial agreement with the values we obtain.

Failure to account for neighbor exclusion may also lead to erroneous calculation of n, the number of binding sites, and to misinterpretation of the physical significance of the ionic strength dependence of daunomycin binding. For example, Zunnio et al. (1980) have reported that the number of daunomycin binding sites decreases with increasing ionic strength (see Table III) and attributed this to salt-induced alteration in DNA structure. They have, however, used the Scatchard model and obtained n by extrapolating linear regions of their data to obtain the x-axis intercept, which was then equated to n. Examination of the data of Figure 4 shows the danger in this approach; clearly, the linear regions of the curves will extrapolate to different x intercepts. However, all three curves may be fit adequately with the same value for the exclusion parameter. Thus, the number of potential binding sites does not change, only the affinity of the drug for DNA.

Table IV: Calculation of Thermodynamic Equilibrium Constants[a]

$[Na^+]$ (M)	$\ln K_{obsd}$	ΔG_{obsd} (kcal/mol)	f^b	$\ln K_T$	ΔG°_T (kcal/mol)
0.2	13.46	−7.98	1.14	12.32	−7.31
0.5	12.68	−7.52	0.35	12.33	−7.31
1.0	12.10	−7.18	−0.24	12.35	−7.31

[a] At 25 °C. [b] $f = Z\xi^{-1} \ln(\gamma_\pm\delta) - Z\psi \ln [M^+]$ (see the text).

The observed dependence of K_i on ionic strength is a consequence of the linkage between drug and cation binding (Wyman, 1964; Record et al., 1978). The plot shown in the inset of Figure 4 yields

$$d \log K_i / d \log [Na^+] = \Delta r = -Z\psi \quad (7)$$

where Z is the charge on the daunomycin molecule and ψ is the fraction of counterions associated with each DNA phosphate (for double-stranded DNA, $\psi = 0.88$). The quantity Δr is the number of counterions released upon the binding of the ligand with charge Z. We find $Z\psi = 0.84$; thus, $Z = 0.95$ (or 1.0), corresponding to the single charge carried by daunomycin at neutral pH.

Knowledge of the value of $Z\psi$ enables us to assess quantitatively the thermodynamic equilibrium constant for the experimentally observed results. From the results of Record et al. (1978)

$$\ln K_{obsd} = \ln K^\circ_T + Z\xi^{-1} \ln(\gamma_\pm\delta) - Z\psi \ln [M^+] \quad (8)$$

where K_{obsd} is the observed equilibrium constant and K°_T is the thermodynamic equilibrium constant (corrected for the free energy of ion release by using a standard state in which all reactants including ions have unit concentration). $Z\psi$ is defined above, $\gamma_\pm$ is the mean activity coefficient of the Na^+ ion, and the remaining terms are constants for double-stranded DNA: $\xi = 4.2$ and $\delta = 0.56$. Table IV summarizes the results we have obtained from calculations according to eq 8. The large magnitude of K°_T implies extensive nonionic stabilization of the complex.

Our values of $\Delta H^\circ = -12.8$ kcal/mol and $\Delta S^\circ_{25°C} = -16.2$ eu are of the same sign as and similar in magnitude to values found in this laboratory for other intercalators. The binding of proflavin to DNA, for example, was found to have $\Delta H^\circ = -7.8$ kcal/mol and $\Delta S^\circ = -7.7$ eu (Li & Crothers, 1969), while ethidium binding to DNA was characterized by $\Delta H^\circ = -8.4$ kcal/mol and $\Delta S^\circ_{23°C} = -9.2$ eu (Bresloff & Crothers, 1975). The binding of nonintercalating irehdiamine A to DNA, in contrast, was found to have $\Delta H^\circ = +12.0$ kcal/mol, with $\Delta S^\circ_{25°C} = +61.8$ eu (Dattagupta et al., 1978). The binding of daunomycin thus seems to be driven by the large enthalpy term at room temperature. Quadrifollia & Crescenzi (1974) have reported a calorimetric enthalpy for daunomycin binding of −6.5 kcal/mol at 25 °C, in 10 mM phosphate buffer, and Huang & Phillips (1977) have reported a van't Hoff enthalpy of −5.3 kcal/mol in 0.15 M NaCl. We cannot explain the discrepancy between these values and ours, although we note the differences in salt, drug, and DNA concentrations and the use by Huang & Phillips of the formal Scatchard model, which leads to artificially high estimates of the daunomycin binding constants as discussed above.

The data of Table II indicate that daunomycin binding is somewhat specific for G+C base pairs, with a value of $\alpha = 1.45$ intermediate between the values of 1.0 expected for no specificity and 1.75–2.32 expected for total G+C specificity. DuVernay et al. (1979) have reported apparent binding constants for the binding of adriamycin, a compound closely related to daunomycin, to DNAs of varying G+C content. We calculate from their data that the ratio of the apparent binding constant of adriamycin to *M. lysodeikticus* DNA and *C. perfringens* DNA is 1.38, in excellent agreement with the value of α we have determined for daunomycin. As a consequence of this base specificity, G+C base pairs are preferentially filled at low r values. This may be experimentally observed with CsCl density gradient sedimentation (Figure 6.).

Daunomycin increases the T_m of DNA melting by some 30 °C as saturation of potential binding sites is approached. This stabilization of the DNA double helix probably contributes to the inhibition of DNA replication and RNA transcription previously reported (Hartmann et al., 1964; Ward et al., 1965; DiMarco et al., 1971). Both DNA and RNA polymerase require single-stranded templates, the formation of which would be strongly inhibited by the presence of daunomycin. The biphasic thermal denaturation curves seen at low $\bar{r}$ values in Figure 7 are similar to those observed by Patel & Canuel (1978) for poly(dA-dT)–daunomycin complexes, with both proton NMR and UV absorbance spectroscopies. They have interpreted the two phases as resulting from the melting first of regions of DNA containing no drug, followed by melting of regions containing daunomycin. The drug-free regions are presumably generated by the redistribution of drugs on the DNA surface during melting, leading to clustering of drugs on the unmelted DNA surface. Quantitative theoretical models (Crothers, 1971; McGhee, 1976) have shown that biphasic melting curves of ligand–DNA complexes can result from such clustering of the ligands and probably account for the behavior seen in Figure 7.

The equilibrium data here are entirely consistent with the behavior expected for an intercalating drug. The geometry of the daunomycin–DNA complex in solution presented in the following paper (Fritzsche et al., 1982) provides further support for the intercalative binding of daunomycin and compares our results to the recently reported crystal structure of a daunomycin–deoxyoligonucleotide complex (Quigley et al., 1980). The understanding of the equilibrium aspects of the daunomycin–DNA we have obtained here provides a firm basis for kinetic studies of the daunomycin–DNA interactions, as well as for studies on the interaction of the drug with nucleosomes, both of which are under way in our laboratory.

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Studies on Interaction of Anthracycline Antibiotics and Deoxyribonucleic Acid: Geometry of Intercalation of Iremycin and Daunomycin†

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ABSTRACT: The structure of iremycin [10-(α-L-rhodosaminyl)-γ-rhodomycinone] hydrochloride has been confirmed by ^1H and ^{13}C nuclear magnetic resonance (NMR) spectroscopy. We studied the interaction of iremycin and the related compound daunomycin with DNA by transient electric dichroism and by sedimentation analysis of supercoiled closed duplex DNA. The apparent length increase of sonicated calf thymus DNA (150 ± 20 base pairs) in 2.5 mM sodium cacodylate buffer (pH 7) at 12 °C was determined to be 0.40 ± 0.02 nm/bound iremycin, which is significantly higher than the apparent length increase induced by daunomycin (0.31 ± 0.02 nm/bound drug). The Cu(II) complex of iremycin with a metal/drug ratio of 0.7 induces a length increase of DNA of 0.44 ± 0.02 nm/added drug. The alignment of the iremycin chromophore with respect to the DNA helix axis was determined from the electric dichroism of the complex. The tilt (long axis) and twist (short axis) of the chromophore are both 28 ± 4°, whereas for daunomycin the long axis is perpendicular to the helix axis and the short axis is twisted by about 25°. Intercalation of iremycin between DNA base pairs is supported by unwinding of the supercoiled closed duplex form of pBR 322 plasmid DNA from *Escherichia coli*. In 2.5 mM sodium cacodylate buffer at pH 7 and at 25 °C, the unwinding induced by iremycin is 15.0 ± 1.5°/bound drug. Under identical conditions daunomycin shows on unwinding angle of 15.4 ± 1.5°. The superhelical density of pBR 322 DNA ($\bar{\sigma}_0$) was determined to be −0.087 ± 0.002 at standard conditions (0.2 M NaCl, 37 °C).

Recently, the anthracycline antibiotic iremycin (IM)[1] was obtained by selection and characterization of an interspecific recombinant phenotype obtained by hybridization experiments with mutants of various *Streptomyces* species blocked in antibiotic production (Schlegel & Fleck, 1980; Schlegel et al., 1980). The antibiotic IM possesses antimicrobial and cytostatic activity (Schlegel et al., 1979), and IM has been identified by Ihn et al. (1980) as 10-(α-L-rhodosaminyl)-γ-rhodomycinone (I) on the basis of ^1H and ^{13}C NMR, mass spectrometry, infrared and UV–vis spectroscopy, and circular dichroism. The structure of IM is closely related to the antitumor drugs daunomycin (DM, II) and adriamycin (AM,

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[1] Abbreviations: IM, iremycin; DM, daunomycin; NMR, nuclear magnetic resonance; EB, ethidium bromide.

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