Equilibrium Studies on the Interaction of Daunomycin with Deoxypolynucleotides†

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ABSTRACT: Fluorescence and absorbance methods were used to study the interaction of daunomycin with four deoxypolynucleotides. The binding may be described by the neighbor-exclusion model for binding ratios greater than 0.05, with intrinsic binding constants decreasing in the order poly[d(A-T)]·poly[d(A-T)] > poly[d(G-C)]·poly[d(G-C)] > poly(dG)·poly(dC) > poly(dA)·poly(dT). The exclusion parameter was found to be approximately 2 for the A-T-containing polynucleotides, 4 for the alternating G-C polymer, and nearly 10 for poly(dG)·poly(dC). Poly(dA)·poly(dT) showed positive cooperativity at low binding ratios. Thermal denaturation studies provided quantitative support for the measured binding parameters; the ΔT_m values measured may be correlated primarily with the differences seen in the exclusion parameter. Sedimentation velocity experiments on daunomycin–deoxypolynucleotide complexes show an unusual nonlinear dependence of S_{app} on the binding ratio for poly[d(A-T)]·poly[d(A-T)], poly[d(G-C)]·poly[d(G-C)], and poly(dA)·poly(dT), indicative of either a nonstandard conformational change accompanying intercalation or cooperative drug binding.

Daunomycin is an anthracycline antibiotic that has found wide clinical use in cancer chemotherapy. The drug inhibits both DNA replication and RNA transcription both in vivo and in vitro (Ward et al., 1965; Hartmann et al., 1964; DiMarco et al., 1971), probably by direct interaction with DNA. Numerous reviews covering the chemistry and pharmacology of daunomycin have appeared (Arcamone, 1978, 1981; Crooke & Reich, 1980; Neidle, 1978).

The interaction of daunomycin with DNA has been extensively studied [see Chaires et al. (1982, 1983) and references cited therein]. These studies support an intercalation model as the probable binding mode to DNA. The crystal structure of a daunomycin–DNA complex has been reported (Quigley et al., 1980), and the geometry of the drug–DNA complex in solution has been probed by using transient electric dichroism (Fritzsche et al., 1982), both of which are in full accord with the intercalation model. The specificity of the daunomycin–DNA interaction remains to be clarified. Are there DNA sequences or conformations that are particularly effective receptors for the drug? In previous publications (Chaires et al., 1982, 1983), this question was partially explored. Using competition dialysis, we found that daunomycin shows a general preference for G-C-rich DNA and as a consequence preferentially alters the buoyant density of the G-C-rich calf thymus satellite DNA at low binding ratios (Chaires et al., 1982). We found that daunomycin binds with considerably higher affinity to free DNA than to DNA structured into nucleosomes, suggesting a specificity for a particular DNA conformation (Chaires et al., 1983).

A logical step toward a more detailed elucidation of the possible sequence specificity in the daunomycin–DNA interaction is to study the binding of the drug to synthetic deoxypolynucleotides of defined sequence. This approach has been used to discern aspects of the specificity of actinomycin (Wells & Larson, 1970), ethidium (Bresloff & Crothers, 1981; Baguley & Falkenhaug, 1978), and echinomycin (Wakelin & Waring, 1976) binding to DNA. Information obtained from such studies may be used to calculate binding isotherms to heterogeneous DNA sequences (Sturm, 1981), providing insight into sequences particularly favorable to drug binding.

The interaction of daunomycin (or the closely related adriamycin) with synthetic polynucleotides has been examined by several groups, with conflicting results. Tsou & Yip (1976) reported binding of adriamycin to a variety of polynucleotides and found that the binding constant decreased in the order poly[d(G-C)]·poly[d(G-C)] > poly[d(A-T)]·poly[d(A-T)] > poly(dG)·poly(dC) > poly(dA)·poly(dT). In contrast, Phillips et al. (1978) reported that daunomycin bound with approximately equal affinity to poly[d(A-T)]·poly[d(A-T)], poly[d(G-C)]·poly[d(G-C)], and poly(dG)·poly(dC) but less tightly to poly(dA)·poly(dT). They noted differences in the number of available binding sites for the various polynucleotides. DuVernay et al. (1979) reported tight binding of adriamycin to poly[d(G-C)]·poly[d(G-C)] but no detectable binding to poly[d(A-T)]·poly[d(A-T)]. Plumbridge & Brown (1977) reported that daunomycin will not bind to poly[d(I-C)]·poly[d(I-C)] and inferred from the result that daunomycin prefers B-form DNA over the A form. Finally, Krugh and co-workers (Graves & Krugh, 1982; Krugh et al., 1981; Krugh & Young, 1977) have reported binding of daunomycin to a variety of polynucleotides and have reported positive cooperativity in drug binding to some polynucleotides under certain ionic conditions.

In the present report, I describe experiments designed to explore the sequence specificity of the daunomycin–DNA interaction. The long-range goal of this work is to elucidate possible sequences within native genomes with particularly high affinity for anthracycline antibiotics. Particular care has been taken to prepare deoxypolynucleotide samples with defined physical properties for use in the binding studies. Fluorescence, absorbance, and equilibrium dialysis methods were used to study the interaction of daunomycin with selected deoxypolynucleotides. The optical properties of the duanomycin–polynucleotide complexes were found to differ, in particular in the extent to which the intrinsic fluorescence of the drug was quenched upon binding. Equilibrium binding data were analyzed in terms of the neighbor-exclusion model (Crothers, 1968; McGhee & von Hippel, 1974) and revealed that the

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affinity of daunomycin for the polynucleotides followed the order poly[d(A-T)]·poly[d(A-T)] > poly[d(G-C)]·poly[d(G-C)] > poly(dG)·poly(dC) > poly(dA)·poly(dT). Striking differences in the values for the exclusion parameters were found for the four polynucleotides. Thermal denaturation experiments in the presence of the drug, analyzed according to the theory of McGhee (1976), provided an independent confirmation of the estimates of the binding constant and the exclusion parameters. Poly(dA)·poly(dT) was unusual in that its binding isotherm indicated a cooperative binding mode. Finally, sedimentation velocity experiments showed unusual behavior for poly[d(A-T)]·poly[d(A-T)], poly[d(G-C)]·poly[d(G-C)], and poly(dA)·poly(dT) in the presence of daunomycin, indicative of either unusual solution structures for these deoxypolynucleotides or cooperative drug binding.

Materials and Methods

Materials. Poly[d(A-T)]·poly[d(A-T)], poly(dA)·poly(dT), poly[d(G-C)]·poly[d(G-C)], and poly(dG)·poly(dC) samples were obtained from Sigma Chemical Co. (St. Louis, MO), P-L Biochemicals (Milwaukee, WI), and Boehringer Mannheim (Indianapolis, IN). Similar binding results were obtained regardless of the source of the polynucleotides. Daunomycin was obtained from Sigma Chemical Co. (lot 22F-0593) and was used without further purification.

Preparation of Polynucleotides. Polynucleotides were dissolved in S1 digestion buffer (30 mM sodium acetate, 50 mM NaCl, and 1 mM $ZnCl_2$, pH 5.2), and S1 nuclease (Sigma, lot G9C-85103) was added to give a final ratio of 500 units of S1/mg of polynucleotides. Samples were incubated for 30 min at 37 °C, and the digestion was stopped by the addition of ethylenediaminetetraacetic acid (EDTA) to a final concentration of 10 mM and proteinase K (Boehringer Mannheim, lot 1170319) to a concentration of 50 units/mL. Polynucleotides were then dialyzed overnight against a large volume of BPES buffer (6 mM Na_2HPO_4, 2 mM NaH_2PO_4, 1 mM Na_2EDTA, and 0.185 M NaCl, pH 7.0). The dialyzed polynucleotides were fractionated on a Sepharose 4B column equilibrated with BPES buffer, the central portions of the major elution peak pooled, and the samples characterized by UV absorption, thermal melting, and sedimentation velocity.

The concentration of the deoxypolynucleotides was determined by using the following wavelengths and extinction coefficients: poly[d(A-T)]·poly[d(A-T)], λ_{max} = 262 nm, ϵ = 13 200 M^{-1}; poly(dA)·poly(dT), λ_{max} = 260 nm, ϵ = 12 000 M^{-1}; poly[d(G-C)]·poly[d(G-C)], λ_{max} = 254 nm, ϵ = 16 800 M^{-1}; poly(dG)·poly(dC), λ_{max} = 253 nm, ϵ = 14 800 M^{-1}.

Binding Studies. Equilibrium dialysis, absorbance titration, and fluorescence titration were used to determine the concentration of free and bound drug as previously described (Chaires et al., 1982).

Fluorescence polarization was determined by using a Perkin-Elmer 650-40 spectrofluorometer equipped with an ordinate processor, which allows for the automatic determination of both polarization and anisotropy.

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). Experimental data were fit to the neighbor-exclusion model of McGhee & von Hippel (1974):

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

where K_i is the intrinsic binding constant and n is the exclusion parameter in base pairs. Fitting was performed by using a nonlinear least-squares fitting routine available in the PROPHET computer resource, which allows data to be fit directly to eq 1 and returns estimates of K_i and n.

Table I: Physical Properties of the Polynucleotides Used in These Studies[a]

	λ_{max} (nm)	s_{app} (S)
poly(dA)·poly(dT)	258	6.0 ± 0.2
poly[d(A-T)]·poly[d(A-T)]	262	12.0 ± 1.0
poly(dG)·poly(dC)	252	14.0 ± 0.5
poly[d(G-C)]·poly[d(G-C)]	252	10.9 ± 1.0

[a] Commercial deoxypolynucleotides were treated with S1 nuclease, as described in the text, dialyzed exhaustively against BPES buffer (6 mM Na_2HPO_4, 2 mM NaH_2PO_4, 1 mM Na_2EDTA, and 0.185 M NaCl, pH 7.0), and fractionated on a Sepharose 4B column. The apparent sedimentation coefficient, s_{app}, and the absorbance maximum, λ_{max}, of the polynucleotides prepared in this manner are shown. The range in s_{app} for several preparations is indicated.

Fluorescence Quenching. Quenching of daunomycin fluorescence by iodide ion was measured by monitoring changes in the fluorescence intensity (λ_{ex} = 480 nm, λ_{em} = 555 nm) upon addition of microliter volumes of a 5 M KI stock solution containing 0.1 mM $Na_2S_2O_3$ to prevent I_3^- formation. Data were cast into the form of a Stern–Volmer plot:

$$F_0/F = 1 + K_{sv}[Q] \quad (2)$$

where [Q] is the molar concentration of quencher, F_0 the intensity of fluoresence in the absence of quencher, F the intensity of fluorescence at quencher concentration Q, and K_{sv} the Stern–Volmer quenching constant (Eftink & Ghiron, 1981).

Melting Curves. Thermal denaturation studies were performed in stoppered quartz cuvettes in a Cary 219 spectrophotometer, as previously described (Chaires et al., 1982). Polynucleotides were dialyzed overnight against a buffer containing 1.5 mM Na_2HPO_4, 0.5 mM NaH_2PO_4, and 0.5 mM Na_2EDTA, pH 7.0, prior to melting experiments. Melting profiles were digitized by using procedures available through the PROPHET computer resource (Kuhlmann & Perry, 1982), and differential melting curves were calculated as previously described (Chaires et al., 1983).

Sedimentation Velocity. Sedimentation velocity measurements were made on a Beckman Model E analytical ultracentrifuge equipped with an electronic speed control, a RTIC temperature control, and a UV scanner. Sedimentation experiments typically were at 32 000 rpm, 21 ± 1.0 °C, using an AnF four-place rotor. Sedimentation coefficients were determined by standard procedures, assuming the radial position to be the 50% concentration point.

Results

Physical Properties of Polynucleotide Preparations. Synthetic polynucleotides such as those used in the present study may, under some conditions, adopt unusual solution structures (Michelson et al., 1967; Zimmerman, 1982; Felsenfeld & Miles, 1967). It is, therefore, important to characterize the preparations as fully as possible to ensure that the polynucleotides are in a defined double-helical state. Table I summarizes the optical properties and sedimentation coefficients observed after the S1 nuclease treatment described under Materials and Methods. The range in s values is representative of the values found for several preparatations. All four polynucleotide preparations showed melting curves, T_m values, and hypochromicities characteristic of a duplex to coil transition.

Table II: Optical Properties of Daunomycin-Polynucleotide Complexes[a]

	λ_{max} (nm)	$\Delta\epsilon_{480}$ (M^{-1})	rel fluorescence[b]
poly(dA)·poly(dT)	505	4150 ± 150	0.77 ± 0.04
poly[d(A-T)]·poly[d(A-T)]	505	4375 ± 50	0.78 ± 0.04
poly(dG)·poly(dC)	505	4250 ± 250	0.05 ± 0.02
poly[d(G-C)]·poly[d(G-C)]	505	4100 ± 100	0.05 ± 0.02

[a] Data obtained by difference spectroscopy using samples in dialysis equilibrium with known concentrations of daunomycin, as described in the text. The absorbance maximum of the complex (λ_{max}), the change in extinction ($\Delta\epsilon$), as defined by eq 3, and the relative fluorescence are shown. [b] Relative to free daunomycin with λ_{ex} = 480 nm and λ_{em} = 555 nm.

Table III: Additional Fluorescence Properties of the Daunomycin-A-T Polynucleotide Complexes[a]

	P	K_{sv}
free daunomycin	0.103 ± 0.003	13.4
poly[d(A-T)]·poly[d(A-T)] + daunomycin	0.430 ± 0.003	~0
poly(dA)·poly(dT) + daunomycin	0.4	~0

[a] P is the fluorescence polarization; K_{sv} is the Stern-Volmer quenching constant, defined by eq 2.

The S1 treatment used was designed to eliminate possible single-stranded regions in the polynucleotides. Binding isotherms determined on untreated polynucleotides were in rough accord with those determined after S1 treatment but were in some cases shifted toward lower r values. This is diagnostic of an overestimate of potential binding sites and would be expected if there were single-stranded regions in the untreated polynucleotides. The S1 treatment was, therefore, adopted and provided material that yielded consistent, reproducible binding isotherms independent of supplier.

Optical Properties of the Daunomycin–Polynucleotide Complexes. The optical properties of the daunomycin–polynucleotide complexes were determined by difference spectroscopy. Absorbance spectra of samples in dialysis equilibrium with known concentrations of free drug were recorded by using the dialysate as the reference solution. The recorded spectrum represents the spectrum of the bound drug, allowing for the direct determination of the λ_{max} of the complex. The change in extinction coefficient, $\Delta\epsilon$, may then be calculated by using the known concentrations of total and bound drug (C_T and C_B, respectively):

$$\Delta\epsilon_{480} = \epsilon_f - \epsilon_B = (C_T[\epsilon_f] - A_{480}^{obsd})/C_B \quad (3)$$

As seen in Table II, slight differences in $\Delta\epsilon_{480}$ were found for the various polynucleotides.

The relative fluorescence of bound daunomycin was examined by the sequential addition of polynucleotide to a solution of daunomycin, until a plateau in the observed fluorescence was reached. Table II shows that binding of the drug to G-C-containing polynucleotides results in essentially complete quenching of the intrinsic daunomycin fluorescence. The A-T-containing polymers quench the daunomycin to a considerably lesser extent.

Two additional fluorescence properties were examined for the complexes of daunomycin with the A-T-containing polynucleotides, to ensure that the estimated relative fluorescence truly represented the properties of the bound, intercalated drug. Table III shows the results of these experiments. First, the fluorescence polarization of daunomycin increases significantly in the presence of both polynucleotides, consistent with the binding of the smaller fluorophore to the high molecular weight polynucleotide. Second, while free daunomycin fluorescence may be readily quenched by the addition of iodide ion, no quenching by iodide is observed in the presence of excess polynucleotide. This suggests that the daunomycin is inaccessible to the added quencher, as would be expected if the drug is intercalated. Daunomycin is thus likely to be intercalated in the A-T-containing polynucleotides, and the greater fluorescence of these complexes relative to the G-C-containing polynucleotide probably reflects differences in the molecular interactions at the binding site.

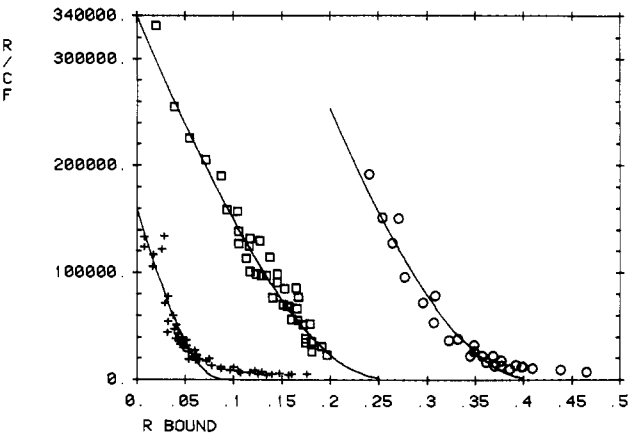

FIGURE 1: Scatchard plots for the binding of daunomycin to deoxypolynucleotides. Results are shown for at least four separate fluorescence/absorbance titrations in BPES buffer, 20 °C, for poly[d(A-T)]·poly[d(A-T)] (○), poly[d(G-C)]·poly[d(G-C)] (□), and poly(dG)·poly(dC) (+). The solid lines are the least-squares fits of the data to eq 1 with the parameters shown in Table IV.

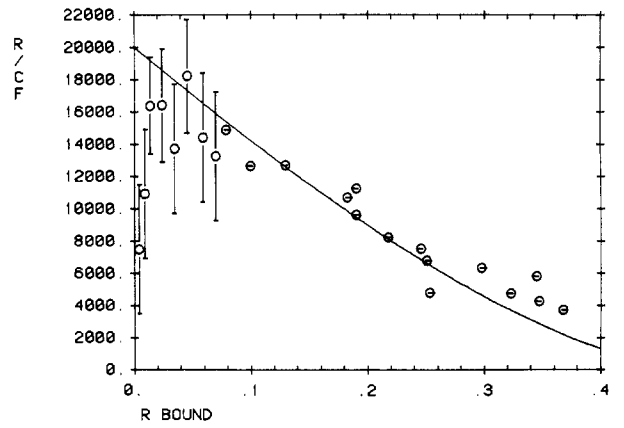

FIGURE 2: Binding of daunomycin to poly(dA)·poly(dT). Solution conditions are BPES buffer, 20 °C. The solid line is the fit of the data for $r_b > 0.05$ to eq 1.

Table IV: Summary of Binding Data[a]

	K (M^{-1})	n (bp)
poly(dA)·poly(dT)[b]	2.0 (±0.1) × 10^4	2.0
poly[d(A-T)]·poly[d(A-T)]	7.6 (±0.6) × 10^5	2.4 ± 0.1
poly(dG)·poly(dC)	1.6 (±0.1) × 10^5	9.8 ± 0.5
poly[d(G-C)]·poly[d(G-C)]	3.4 (±0.1) × 10^5	3.6 ± 0.1

[a] Values are the results from nonlinear least-squares fits of the experimental data of Figures 1 and 2 to eq 1 and refer to 20 °C, BPES buffer. K is the intrinsic binding constant for the binding of a drug molecule to an isolated site, and n is the exclusion parameter in base pairs. [b] For $r > 0.05$.

Binding Isotherms. The optical parameters determined in Table II were used to determine binding ratios by using absorbance and fluorescence titration procedures previously

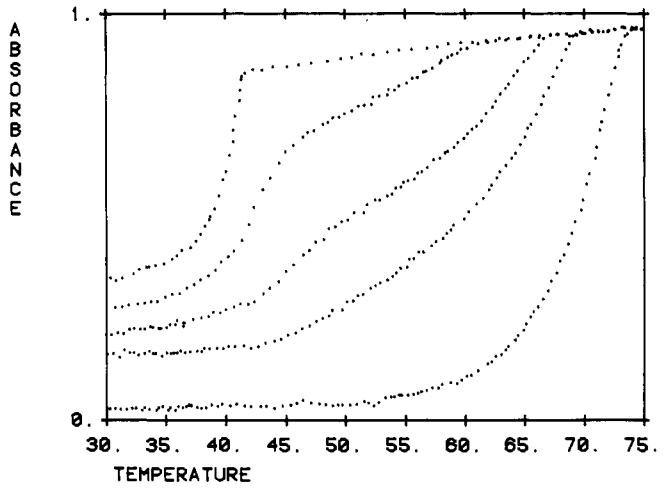

FIGURE 3: Thermal denaturation of poly[d(A-T)]·poly[d(A-T)] in the presence of daunomycin. From left to right, the ratio of drug/base pairs is 0.0, 0.03, 0.077, 0.154, and 0.308. The buffer was 1.5 mM Na_2HPO_4, 0.5 mM NaH_2PO_4, and 0.5 mM Na_2EDTA, pH 7.0. The heating rate was ~0.8 °C/min.

described (Chaires et al., 1982). The results are presented in Figures 1 and 2 in the form of Scatchard plots (Scatchard, 1949). The data in these figures were fit to the neighbor-exclusion model, eq 1, by using a nonlinear least-squares fitting routine, with the results shown in Table IV. Tightest binding was found to poly[d(A-T)]·poly[d(A-T)]; the binding constants for remaining polynucleotides followed the order poly[d(G-C)]·poly[(G-C)] > poly(dG)·poly(dC) > poly(dA)·poly(dT). The most striking difference in the four polynucleotides is in the value of the exclusion parameter, n, required to fit the data. For the A-T-containing polynucleotides, n is approximately 2, but increases to approximately 4 for poly[d(G-C)]·poly[d-(G-C)] and to approximately 10 for poly(dG)·poly(dC).

Systematic deviations from the calculated neighbor-exclusion model may be seen in Figure 2 for poly(dG)·poly(dC) and poly[d(A-T)]·poly[d(A-T)] near saturating values of r_b. This may result from a weaker, ionic binding mode or from incomplete neighbor exclusion.

The binding isotherm for poly(dA)·poly(dT) (Figure 2) shows a positive slope at low r values. This indicates a cooperative binding mode, which may be interpreted by using the allosteric binding model of Dattagupta et al. (1980). In this model, the polynucleotide is assumed to exist in two conformations, each with distinct drug binding properties. The binding constant to form 1 of the polynucleotide, K_1, may be obtained from the intercept on the r/C_f axis of Figure 3 and is estimated to be 6000 M^{-1}. The binding parameters to form 2 of the polynucleotides may be obtained by fitting the data of Figure 3 for $r_b > 0.05$ to the standard neighbor exclusion, eq 1, yielding $K_2 = (2.0 \pm 0.1) \times 10^4$ M^{-1} and $n = 2.0$. The ratio K_2/K_1 is a measure of the cooperativity and was found to be 3.3, a value similar to that reported for daunomycin binding to poly[d(A-T)]·poly[d(A-T)] and poly[d(G-C)]·poly[d(G-C)] (Graves & Krugh, 1982).

Thermal Denaturation. The influence of daunomycin on the thermal denaturation profiles of the four deoxypolynucleotides studied was examined. In all cases, addition of daunomycin stabilized the polynucleotides, with an increase in the T_m dependent upon both the extent of bound drug and the type of deoxypolynucleotide. All four deoxypolynucleotides exhibited biphasic melting curves at certain ratios of drug/base pair. Figure 3 shows a representative melting experiment for poly[d(A-T)]·poly[d(A-T)]. Differentiation of melting curves such as illustrated in Figure 3 permits the accurate estimation

Table V: Observed Melting Temperatures in the Presence and Absence of Daunomycin[a]

polynucleotide	r_{add} (mol of daunomycin/mol of bp)	T_m^1 (°C)	T_m^2 (°C)
poly(dA)·poly(dT)	0.0	40.0	
	0.011	42.2	58.6
	0.022	45.0	64.5
	0.044	51.3	67.7
	0.165		71.3
poly[d(A-T)]· poly[d(A-T)]	0.0	32.7	
	0.03	33.4	67.6
	0.077	34.4	66.8
	0.154	38.1	68.8
	0.308		75.7
poly(dG)·poly(dC)	0.0	73.7	
	0.012	75.2	81.0
	0.024	77.9	84.5
	0.048	78.7	84.5
	0.180		91.1
poly[d(G-C)]· poly[d(G-C)]	0.0	75.2	
	0.053	77.0	82.2
	0.106	79.8	84.1
	0.25	86.9	90.2
	0.375		96.25

[a] Indicated are the observed T_m values, determined from derivative melting curves as described in the text, as a function of the molar ratio of added daunomycin. T_m^1 and T_m^2 refer to the low- and high-temperature melting transitions, respectively.

of T_m values even in the case of biphasic melting. Table V summarizes the estimates for the T_m values made for all four deoxypolynucleotides at various ratios of added drug. A difference may be seen in the greater extent to which the A-T-containing polynucleotides are stabilized relative to the G-C-containing polynucleotides.

The behavior illustrated in Figure 3 and Table V is entirely consistent with the theoretical predictions made by Crothers (1971) and McGhee (1976) for the effect of intercalating drugs on the thermal denaturation of nucleic acids. The large difference between the A-T and the G-C polynucleotides in the extent of stabilization by added daunomycin may be understood in terms of these theories primarily as an effect of the differences in the neighbor-exclusion values reported in Table III. At saturating values of bound drug, the shift in T_m may be specified by

$$1/T_m^\circ - 1/T_m = R/\Delta H \ln (1 + KL)^{1/n} \quad (4)$$

where ΔH is the enthalpy change for the melting of a base pair, K and n are the binding parameters for drug binding according to the neighbor-exclusion model, and L is the free ligand activity (McGhee, 1976). This expression assumes binding of the ligand only to the double helix. Thus, at a constant KL, a substance with a larger exclusion parameter will shift the T_m less than one with a smaller exclusion parameter. In a separate series of melting experiments, ΔT_m was measured for each polynucleotide under conditions where the number of sites was nearly saturated and the concentration of free ligand was known. The results are shown in Table VI, along with calculated values for $1/T_m^\circ - 1/T_m$ according to eq 4. For the calculation, the values of ΔH for base-pair melting were taken from Marky & Breslauer (1982); K values for the ionic conditions used in the melting experiment were estimated by using the measured values shown in Table IV and assuming a value of $d \ln K/d \ln M^+ = -0.88$ (Chaires et al., 1982); n values were taken from Table III, and L values were measured as part of the experimental design. The inverse correlation between n and ΔT_m is shown in Table VI. The

Table VI: Summary of Melting Experiments[a]

	n (bp)	ΔT_m^{max} (°C)	$1/T_m^\circ - 1/T_m$ ($\times 10^4$) obsd	predicted[c]
poly(dA)·poly(dT)[b]	2.0	34.6	3.2	3.2
poly[d(A-T)]·poly[d(A-T)]	2.5	41.0	3.8	5.9
poly[d(G-C)]·poly[d(G-C)]	3.6	21.1	1.6	1.3
poly(dG)·poly(dC)	9.8	17.4	1.3	0.4

[a] Polynucleotides were dialyzed against daunomycin solutions of sufficient concentration to saturate all possible binding sites. The free ligand concentration was determined, and the melting temperatures of the drug–polynucleotide complex and the free polynucleotide were determined as described in the text. ΔT_m is the difference in these two values. [b] Not completely saturated. [c] Calculated according to eq 4 as described in the text.

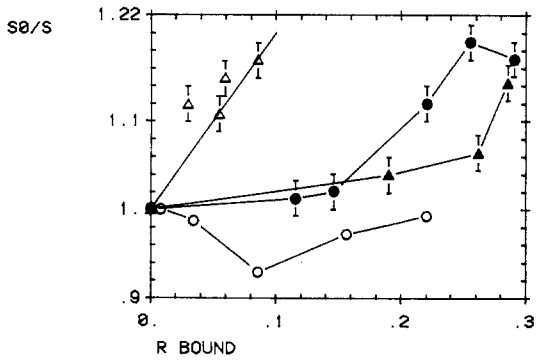

FIGURE 4: Effect of daunomycin on the sedimentation coefficient of the deoxypolynucleotides. (△) Poly(dG)·poly(dC); (▲) poly[d(G-C)]·poly[d(G-C)]; (○) poly(dA)·poly(dT); (●) poly[d(A-T)]·poly[d(A-T)]. Sedimentation was at 32 000 rpm, 21 ± 1.0 °C. S^0/S is the ratio of the sedimentation coefficient of the polynucleotide in the absence of bound drug to that observed at a binding ratio of r_b.

observed and predicted values for $1/T_m^\circ - 1/T_m$ are in remarkably good agreement, given the neglect of the enthalpy of drug binding (which may differ for each polynucleotide) in the theoretical treatment and the uncertainty in the values of ΔH used. Most importantly, the theory matches the overall trend seen in the experimental data. The data of Table VI provide independent confirmation of the equilibrium data of Table III, particularly in the differences in the values of the exclusion parameters reported.

Sedimentation Velocity. The effect of daunomycin binding on the hydrodynamic properties of the four deoxypolynucleotides was examined by sedimentation velocity, with the results shown in Figure 4. Poly[d(A-T)]·poly[d(A-T)] and poly[d(G-C)]·poly[d(G-C)] both show marked deviation from the expected linear behavior. Poly(dA)·poly(dT) shows even more unusual behavior, while poly(dG)·poly(dC) appears to follow the behavior expected for an intercalating drug. Thus, with the exception of poly(dG)·poly(dC), the deoxypolynucleotides studied seem to show structural changes not expected from the normal intercalation model, indicative of either nonstandard conformational changes upon intercalation or allosteric effects upon drug binding. Cooperative binding was observed in the binding isotherm for poly(dA)·poly(dT). No cooperativity was observed in the binding isotherms for poly[d(A-T)]·poly[d(A-T)] and poly[d(G-C)]·poly[d(G-C)], although it has been reported to be present by Krugh and co-workers. The effects seen in Figure 4 for these two polynucleotides suggest that such cooperativity may indeed exist, although the data presented here cannot distinguish between that possibility and the possibility of an unusual conformational change resulting from drug intercalation.

Discussion

Daunomycin binds tightly to native DNA of random sequence by intercalation, with binding isotherms fully accounted for at binding ratios greater than 0.05 by the neighbor-exclusion model (Chaires et al., 1982). The results presented here show surprising variances in the affinity of daunomycin for deoxypolynucleotides of defined sequence. Particularly striking is the variance among the four polynucleotides studied in the exclusion parameter required to fit the data. These results, above all, emphasize the fact that the binding isotherm previously found for the binding of daunomycin to calf thymus DNA is probably deceptively simple and must represent average binding properties that mask numerous specific binding events at specific sequences.

Generalizations about the Intrinsic Binding Constant. The results of Table IV show that daunomycin binds tightest to the deoxypolynucleotides with alternating pyrimidine–purine sequences. Krugh et al. (1975) have found a similar sequence preference for ethidium bromide. Ornstein & Rein (1979) have rationalized this observed sequence preference as primarily a reflection of the relative energy of conformational adjustment of the DNA upon intercalation and not necessarily indicative of differences in intermolecular interactions at binding sites. A general preference of daunomycin for alternating pyrimidine–purine sequences was proposed by Patel et al. (1981) on the basis of NMR studies of the daunomycin–poly[d(A-T)]·poly[d(A-T)] interaction, which the results presented here support. Daunomycin binds most tightly to poly[d(A-T)]·poly[d(A-T)]. This may be rationalized by assuming that, to intercalate into the helix, daunomycin must overcome the free energy of base stacking, which is probably less for A-T homopolynucleotides than for G-C homopolynucleotides (Marky & Breslauer, 1982). The overall free energy would thus be more favorable to the A-T polynucleotide.

Neighbor Exclusion. The neighbor-exclusion parameter required to fit the binding data was found to vary from approximately 2 for poly(dA)·poly(dT) and poly[d(A-T)]·poly[d(A-T)] to about 4 for poly[d(G-C)]·poly[d(G-C)] and to about 10 for poly(dG)·poly(dC). These differences may reflect differences in the secondary structures of the deoxypolynucleotides or may reflect differences in the extent of distortion of the double helix upon intercalation, triggered by specific interactions at the binding site. Quigley et al. (1980) have shown that the A ring of daunomycin may participate in specific interactions with adjacent guanine and cytosine bases in an alternating G-C intercalation site. Further, intercalation results in distortion of the double helix several base pairs distant from the actual intercalation site. These interactions, and presumably the extent of helix distortion, would be expected to differ upon substitution of A-T base pairs at the intercalation site or by changing the alternating G-C structure to a nonalternating G-G structure. Differences in the effect of intercalation on the polynucleotide structure were evident in Figure 4, supporting this idea.

Unusual Behavior of Poly(dA)·Poly(dT). The deoxypolynucleotide poly(dA)·poly(dT) showed unusual binding properties relative to the other polymers used in these studies. First, the affinity of daunomycin was considerably less for poly(dA)·poly(dT) than for the other polymers studied. Second, the binding isotherm for poly(dA)·poly(dT) (Figure 2) is suggestive of a cooperative interaction. Finally, the sedimentation coefficient varied upon addition of daunomycin in

an unusual manner. While unusual, this behavior is consistent with previous observations on the binding of daunomycin and other intercalators to poly(dA)·poly(dT).

In temperature-jump experiments on daunomycin–DNA complexes, we have consistently seen an increase in the absorbance at 480 nm upon increasing the temperature for calf thymus DNA and a variety of synthetic deoxypolynucleotides, corresponding to a dissociation of bound drug. In similar experiments, poly(dA)·poly(dT), in contrast, shows an absorbance *decrease*, corresponding to binding of drug at elevated temperature. This might reasonably be interpreted as a shift in the polynucleotide conformation to a state more favorable to binding at the higher temperature. The cooperative binding seen in Figure 2 would be consistent with this model. Bresloff & Crothers (1981) have reported weak binding of ethidium to poly(dA)·poly(dT), in contrast to the tight binding found for a variety of other synthetic polynucleotides. Sturm et al. (1981) found unusual binding properties for the interaction of tilorone with poly(dA)·poly(dT), including weak binding relative to other deoxypolynucleotides and temperature-jump behavior similar to the results mentioned above.

The results for the daunomycin–poly(dA)·poly(dT) interaction thus probably reflect some unusual structural properties of the polynucleotide. Indeed, whereas poly[d(A-T)]·poly[d(A-T)], poly[d(G-C)]·poly[d(G-C)], and poly(dG)·poly(dC) were all found to have a pitch of ~10.6 base pairs (bp)/turn in solution, poly(dA)·poly(dT) was found to have a pitch of 10.0 bp/turn (Peck & Wang, 1981; Rhoades & Klug, 1981; Behe et al., 1981). Further, poly(dA)·poly(dT) does not readily switch from the B to the A conformation, in contrast to the other deoxypolynucleotide used here which will readily make the transition (Arnott & Selsing, 1974).

G-C Specificity. Using competition dialysis, we have found that daunomycin shows a preference for native DNAs whose overall base composition is G-C rich, from which we inferred a preference for G-C base pairs as a daunomycin binding site (Chaires et al., 1982). At first glance, the results presented here seem to contradict this previous result. However, the binding to native DNA sequences must represent average properties, weighted by the relative frequency of the various types of binding sites. The results here show that daunomycin binds best to alternating A-T sequences but poorly to nonalternating A-T sequences. In contrast, the affinity of the drug for alternating and nonalternating G-C pairs is nearly the same. Overall, then, the binding constants for the two types of A-T sites must average to a lower value than the averaged binding constant for a G-C site, leaving an apparent preference for G-C-rich DNA.

Comparison with Previous Work. Krugh and co-workers (Graves & Krugh, 1982; Krugh et al., 1981; Krugh & Young, 1977) have reported cooperative binding of daunomycin to poly[d(A-T)]·poly[d(A-T)] and poly[d(G-C)]·poly[d(G-C)]. They have fit their data to the allosteric model proposed by Dattagupta et al. (1980); the parameters K_2 and n_2 resultant from their analysis are directly comparable to the values reported here for these polynucleotides. They reported values of $K = 3.2 \times 10^6$ M^{-1} and $n = 3$ for the poly[d(A-T)]·poly[d(A-T)] daunomycin interaction and $K = 2.4 \times 10^6$ M^{-1} and $n = 4$ for the poly[d(G-C)]·poly[d(G-C)] interaction, at 0.112 M ionic strength. These values are in close agreement with those reported here that refer to higher ionic strength. The difference in the exclusion parameter is essentially the same as that reported here. Phillips et al. (1978) reported equal binding affinities for the interaction of daunomycin with the same series of deoxypolynucleotides used in this study. They did note, however, differences in the apparent site size, which roughly followed the trend reported here. They reported changes in ϵ_b ranging from 50% to 70% of ϵ_f, suggesting values of $\Delta\epsilon$ ranging from ~3300 to ~5800 M^{-1}, a considerably greater range than found here (Table II). These authors did not specify values of $\Delta\epsilon$ determined for the various polynucleotides, but the differences between this work and theirs can probably be explained by the differences in $\Delta\epsilon$. Finally, Tsou & Yip (1976) reported that the binding of daunomycin to deoxypolynucleotides followed the order poly[d(G-C)]·poly[d(G-C)] > poly[d(A-T)]·poly[d(A-T)] > poly(dG)·poly(dC) > poly(dA)·poly(dT). Their data show very low values for the apparent site size, which probably results from a considerable concentration of single-stranded regions in their material.

Sturm (1981) found the same order of binding constants for the deoxypolynucleotides used in this study for another intercalator, tilorone, although the differences in exclusion values were not as pronounced. The specificity of binding seen for tilorone and daunomycin may be primarily governed by the conformation of the intercalation site of the polynucleotide, rather than specific interactions of the ligand with the different base pairs. Theoretical predictions by Ornstein & Rein (1979) are in accord with this view.

Is Daunomycin Binding Cooperative? Krugh and co-workers have reported the cooperative binding of daunomycin to calf thymus DNA, poly[d(A-T)]·poly[d(A-T)], and poly[d(G-C)]·poly[d(G-C)] by using phase partition methods to obtain binding data (Graves & Krugh, 1982; Krugh et al., 1981; Krugh & Young, 1977). In addition, daunomycin was found to facilitate the binding of actinomycin to poly[d(A-T)]·poly[d(A-T)], suggestive of structural alteration of the polynucleotide promoted by daunomycin (Krugh & Young, 1977). We previously reported apparent cooperativity for the binding of daunomycin to calf thymus DNA by using a phase partition method, although the disagreement of the results between that procedure and optical titration methods and equilibrium dialysis led us to place less confidence in that data (Chaires et al., 1982).

In the present study, cooperativity is evident in the binding of daunomycin to poly(dA)·poly(dT) (Figure 2), where the drug binding is relatively weak and the optical methods used may accurately determine the free drug concentration. The ratio $K_2/K_1 = 3.3$ found for the nonalternating A-T polymer is larger than values of 1.4 and 2.0 reported by Graves & Krugh (1982) for poly[d(G-C)]·poly[d(G-C)] and poly[d(A-T)]·poly[d(A-T)], respectively. This suggests a stronger cooperative process for poly(dA)·poly(dT) relative to the other deoxypolynucleotides. For poly[d(A-T)]·poly[d(A-T)] and poly[d(G-C)]·poly[d(G-C)], drug binding is tight, and the optical methods used would be less reliable in the binding region ($r \leq 0.05$) where cooperativity has been reported. The results of the sedimentation experiments in Figure 4, however, may be interpreted as resulting from cooperative drug binding to these polymers, in accord with the results of Krugh and co-workers. However, the results of Figure 4 might also arise from unique conformational changes resulting from intercalation to these deoxypolynucleotides, more complex than the analogous conformational changes seen in native DNA. More detailed hydrodynamic studies are required to distinguish these possibilities.

Melting of DNA in the Presence of Daunomycin. The effect of daunomycin on the thermal denaturation of the polynucleotides used is entirely in accord with the theoretical predictions made by Crothers (1971) and McGhee (1976).

The quantitative agreement (Table VI) between the experimental data and the theoretical predictions lends confidence to the validity of the equilibrium binding parameters determined here.

The differences in ΔT_m among deoxypolynucleotides in the presence of daunomycin seen here and previously reported (Phillips et al., 1978; Zunnio et al., 1974) are primarily a result of differences in the neighbor-exclusion parameter.

Optical Properties of the Daunomycin–Polynucleotide Complexes. The differences in the extent to which the intrinsic fluorescence of daunomycin is quenched (Table II) reflect differences in the molecular interactions at the intercalation site. Quigley et al. (1980) have shown that several molecular interactions may occur in a G-C intercalation site that may be absent in an A-T site. The 2-amino group of guanine may hydrogen bond with intercalated daunomycin, which may contribute to fluorescence quenching (Manfait et al., 1982; Johnston et al., 1978). Chelation of di- or trivalent metals to daunomycin also quenches its intrinsic fluorescence (J. B. Chaires, unpublished results), suggesting that interaction of the phenolic residues of the chromophore with the metals or with constituents in the intercalation site may also contribute to the fluorescence quenching.

Biological Implications. These results suggest that daunomycin, in the cell, may show a preference for sequences of an alternating purine–pyrimidine structure in general, and for sequences containing an alternating A-T sequence in particular. Since alternating A-T sequences have been consistently found near the putative mRNA start sites in a variety of eucaryotes (Breathnach & Chambon, 1981), this sequence preference may be related to the inhibition of transcription by daunomycin. Daunomycin may be photochemically cross-linked to DNA (J. B. Chaires, unpublished results). Cross-linking experiments are currently under way to test directly whether daunomycin will preferentially bind to particular regions in native genomes, such as these A-T-rich start sites.

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Registry No. Daunomycin, 20830-81-3; poly[d(G-C)], 36786-90-0; poly[d(A-T)], 26966-61-0; poly(dC)·poly(dG), 25512-84-9; poly(dA)·poly(dT), 24939-09-1.

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Interaction of Bleomycin A_2 with Poly(deoxyadenylylthymidylic acid). A Proton Nuclear Magnetic Resonance Study of the Influence of Temperature, pH, and Ionic Strength†

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ABSTRACT: The binding of bleomycin A_2 to poly(deoxyadenylylthymidylic acid) [poly(dA-dT)] has been monitored by proton nuclear magnetic resonance spectroscopy. This study includes an analysis of the effects of temperature, ionic strength, and pH. Sites of drug–nucleic acid interaction have been delineated on the basis of chemical shift perturbations of drug and nucleic acid resonances. The data indicate that the binding of the antibiotic occurs with partial intercalation of the aromatic bithiazole group and immobilization of the cationic dimethylsulfonium group. This complex dissociates as the nucleic acid is denatured to the single-stranded form. The absence of significant pH effects suggests that the N terminus of bleomycin A_2, which contains the titratable groups, does not contribute to the interaction of the drug molecule with poly(dA-dT). The problems associated with assigning a specific geometry to the drug–nucleic acid complex are discussed.

The bleomycins (Bleo;[1] Figure 1), a group of glycopeptide antibiotics used clinically in the treatment of various neoplastic diseases, cause the degradation of DNA [see reviews in Hecht (1979)]. It is this action that is generally believed to be responsible for their biological activity. In vitro, the degradation requires the presence of iron(II), dioxygen, and a reducing agent and is accompanied by the generation of various oxygen radicals, which may or may not be the ultimate reactive species (Burger et al., 1981). The Bleo molecule possesses two functional portions. One part comprised of the cationic C terminus and the bithiazole moiety appears to be responsible for the association of the drug with the target DNA (Chien et al., 1977; Chen et al., 1980; Glickson et al., 1981; Sakai et al., 1981). The other portion, containing the pyrimidine and adjoining residues, appears to be responsible for binding the necessary metal cofactor. The two regions of bleomycin appear to be essentially independent of each other: The binding of Bleo-A_2 (the most common congener) to poly(deoxyadenylylthymidylic acid) [poly(dA-dT)] is not influenced by and does not affect the binding of various metal ions to Bleo (Glickson et al., 1981). Similarly, the metal-binding residues are not influenced by the binding of the distal portion of the molecule to poly(dA-dT), although the valerate and threonine residues, which bridge the two functionally distinct regions, exhibit perturbations due to the presence of either the metal ion or the nucleic acid (Glickson et al., 1981).

The bifunctional nature of the Bleo-A_2 molecule has permitted the separate study of the nucleic acid and metal ion binding sites. Earlier fluorescence and proton NMR studies by Chien et al. (1977) monitored the interaction of Bleo-A_2 with calf thymus DNA. Quenching of the bithiazole fluoresence by DNA indicated involvement of that chromophore in the association process. Proton NMR spectra of the complex showed that the two aromatic bithiazole hydrogens and the methyl hydrogens of the sulfonium group of Bleo-A_2 exhibit the greatest degree of preferential broadening in the presence of DNA. These groups (and hence the C-terminal dipeptide) appear to be most extensively immobilized when the drug is bound to the nucleic acid. A preliminary proton NMR study of the interaction of Bleo-A_2 with poly(dA-dT) was reported by Chen et al. (1980). The utility of poly(dA-dT) as a model DNA in NMR studies has been amply demonstrated (Patel, 1979; Patel & Canuel, 1977, 1978). The extremely flexible and mobile structure of poly(dA-dT) allows well-defined proton NMR spectra to be obtained, in contrast with DNA, which exhibits only extremely broadened resonances due to its rigid rodlike structure. Proton resonances of the aromatic bithiazole group of Bleo-A_2 exhibited a temperature-dependent upfield shift upon complexation to poly(dA-dT), which suggested the presence of two modes of binding (Chen et al., 1980) and/or a preference of the drug for a partially opened helical structure (Sakai et al., 1981). Those studies also indicated that the drug is binding to the minor groove of the nucleic acid.

The present study examines in detail these chemical shift displacements, as well as the influence of temperature, pH, and ionic strength on the binding process, in an effort to further clarify the molecular geometry of the drug–nucleic acid complex.

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[1] Abbreviations: Bleo, bleomycin; Bleo-A_2, bleomycin A_2; pH_m, pH meter reading, uncorrected for isotope effects; BIT, bithiazole; THR, threonine; VAL, γ-amino-β-hydroxy-α-methylvaleric acid; HIS, β-hydroxyhistidine; PYR, pyrimidine; PRO, propionamide, ALA, β-aminoalanine; G, gulose; M, mannose; NMR, nuclear magnetic resonance.

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