Site Selectivity of Daunomycin†

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ABSTRACT: We have reexamined the binding properties of the antitumor drug daunomycin using double-helical oligonucleotides 16 base pairs long that were designed to contain preferred binding sites for the drug. The preferred sites are contained in a six base pair core which is flanked on the 5′ and 3′ ends by tracts of adenines. The flanking sequences, which augment helix stability and reduce end effects, were chosen because daunomycin is known to bind poorly to poly(dA)·poly(dT). Four major sequences were examined in the six base pair core: CGTACG, TAGCTG, TCATCC, and $(TA)_3$ and compared with calf thymus DNA. A randomly generated 16 bp sequence containing no A tracts and a sequence containing only tracts of As and Ts were also used. Fluorometric, absorption, calorimetric, and stopped-flow techniques were used to examine the binding. The affinity of the drug for oligomers containing known binding sites was comparable to or enhanced relative to that for calf thymus bulk DNA. Association constants ranged from 1.0×10^8 to 3.0×10^7 M^{-1}. The strongest core binding site found was CGTACG, but its affinity is only 2-fold larger than that of other core sequences. Appreciable binding to the flanking A tracts was observed. An oligonucleotide which incorporates the CGTACG sequence in a short hairpin helix binds an order of magnitude more weakly. Complex lifetimes measured by stopped flow generally increase with equilibrium stability; the kinetics confirm the existence of a set of weaker sites. The exothermic binding enthalpy for daunomycin with the CGTACG core sequence is more than twice as large as for the TATATA sequence. Binding to dA_{20}·dT_{20} is endothermic, and a less exothermic component can be detected in the calorimetric binding curve of the oligomers containing flanking A tracts.

Anthracyclines have been extensively used as antineoplastic agents [for reviews see Lown (1988) and Arcamone (1981)], although the mode of action by which this class of drugs induces cytotoxicity is still unclear. The interaction of anthracyclines with DNA and with other cellular components has been extensively studied for several representative compounds, one of which is daunomycin.

Because the interaction of daunomycin with DNA is thought to contribute to the molecular steps by which this class of drugs interferes with cell growth, the drug–DNA complex has been studied using several different approaches. Binding of the drug to DNAs of different GC content (Müller & Crothers, 1975), binding to polynucleotides of alternating and nonalternating sequence, (Chaires, 1983; Xodo et al., 1988; Krishnamoorthy et al., 1986) and DNase footprinting (Chaires et al., 1987, 1990; Fox & Waring, 1987) are some of the methods that have been used to establish the affinity, the kinetics, and the site selectivity of the drug. These methods, in addition to theoretical calculations (Chen et al., 1985; Cieplak et al., 1990), have led to tables of preferred sites, with only partial agreement. The sequence selectivity of daunomycin thus remains an open question.

We describe here an experimental approach to examine the site selectivity of daunomycin which is based on the synthesis of oligonucleotides containing a six base pair core flanked on the 5′ and 3′ ends by tracts of adenines. The rationale behind this approach is 3-fold. First, the preferred daunomycin sites that have been reported are short sequences, containing fewer than six nucleotides. Second, oligonucleotides that are six base pairs long have a tendency to dissociate unless experimental conditions, such as concentration, temperature, and ionic strength, are adjusted to extremes that favor duplex formation. It is therefore advantageous to use oligonucleotides that are longer than six base pairs so that these parameters can be held within reasonable values. Finally, it is a known experimental fact that poly(dA)·poly(dT) binds intercalating drugs more weakly than other sequences, evidenced by the lower binding constant to this polymer relative to other polynucleotides (Chaires, 1983; Herrera & Chaires, 1989; Remeta et al., 1988). This polymer is known to have an unusual structure which is simulated by A-tracts that are four or longer [Nadeau & Crothers, 1989; Crothers et al. (1990) and references within]. Advantage is taken of this experimental fact by extending the helix beyond the six base core using A-tracts, in the expectation that new binding sites of competing affinity will not be introduced, while at the same time the helix will be stabilized against strand separation and opening of the binding site base pairs by fraying of the helix ends.

The sequence of the internal core was chosen to include base pairs that were cited by other authors as binding sites, using the methods mentioned above (Chaires et al., 1987, 1990; Fox & Waring, 1987). The core encompassed more than one site in some cases, and a Scatchard-type (Scatchard, 1949) analysis was used to determine whether there was multiple binding to each oligonucleotide and to assess the effect of having the A-tracts at the end of the molecule. The

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[1] Abbreviations: TLC, thin-layer chromatography; HPLC, high-pressure liquid chromatography; TBE, 0.089 M Tris–borate buffer and 0.002 M EDTA, pH 8.0; TEAAc, 0.01 M triethylamine acetate buffer, pH 6.8; SDS, sodium dodecyl sulfate; CD, circular dichroism; NMR, nuclear magnetic resonance.

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information so obtained is used to determine whether daunomycin binds preferentially to any particular sequence. Calf thymus DNA was also studied and the overall binding constant was compared with that obtained by other authors using similar experimental conditions (Chaires, 1985).

MATERIALS AND METHODS

Drug Preparation. Daunomycin was purchased from Sigma, or obtained from NCI, and used without any further purification. Stock solutions were prepared in absolute ethanol and stored at −20 °C. Solutions of the drug were tested periodically by TLC to ascertain purity.

DNA Preparation. Oligonucleotides were synthesized on an Applied Biosystems synthesizer and purified by either gel electrophoresis or HPLC. Crude preparations were run on 15–20% denaturing polyacrylamide gels (19:1), in TBE, at 60 A, until the oligonucleotides had traversed greater than 60% of the length of the gel. Bands were located by UV shadowing, cut out, and eluted in an electrophoresis apparatus fixed with membranes that selectively inhibited transfer of the oligonucleotides, using a low ionic strength buffer. These samples were then desalted using SepPak C_{18} filters.

Samples that were tritylated were purified on a Vydac C_4 column run at 35 °C. The column was eluted with a gradient of 0.01 M TEAAc/CH_3CN. Samples were evacuated to remove the solvent, detritylated with 75% acetic acid, and desalted on the same column, by SepPak C_{18} filters or by Sephadex G25 columns equilibrated in a low ionic strength buffer.

Single strands were purified individually, and the concentration was determined spectrophotometrically, using extinction coefficients that were calculated from tabulated values of dinucleotides (Fasman, 1975). Equimolar quantities of single strands and their complements were mixed in standard reaction buffer (sodium phosphate, 0.1 M in Na^+, and 0.001 M Na_2EDTA, pH 7.3) and heat-annealed. A melt was performed on all samples to check the T_m and the overall quality.

Binding Assay. Fluorescence assays were performed on an SLM 8000C fluorometer (SLM Aminco, Urbana, IL). The binding assays were performed in a 1-mL cuvette thermostated at 20 °C. Excitation was set at 480 nm and emission was set at 590 nm. The baseline fluorescence of the buffer alone was recorded and subtracted from all subsequent readings. All measurements were recorded against an internal reference solution of rhodamine, which was used to account for any fluctuations in lamp intensity.

A drug solution, usually 10^{-7} M, in the standard reaction buffer was titrated with increasing amounts of oligonucleotide in the same buffer. Adjustments were made in concentration to account for dilution. At least three readings were recorded at each addition and averaged.

In early experiments a reference cell containing a solution of the drug was used and exposed to the same amount of light to account for any degradation of the sample. As an additional control, the degradation of the drug was measured independently as well by exposing the same aliquot of drug in buffer for an hour or more to the excitation beam. Total degradation never exceeded 3–5%.

Absorption measurements were conducted in the same manner using Varian (Sugarland, TX), Cary 19, and Perkin-Elmer (Norwalk, CT) λ 4C spectrophotometers.

Calorimetry. Titration calorimetry was performed on an Omega titration calorimeter from MicroCal, Inc. (Northampton, MA). Oligonucleotides were prepared in the concentration range of 0.1 mM, and drug solutions in the range of millimolar. Approximately once every 4 min a 10-μL aliquot of the drug was added to the oligonucleotide solution thermostated at 20 °C, and the change in enthalpy was measured. A 250-μL total volume syringe was used in the stirring assembly. [See Wiseman et al. (1989) for a description of the instrument and optimization of experimental design.]

Stopped Flow. Dissociation kinetic measurements were taken on an Applied Photophysics stopped-flow apparatus. An equilibrated sample of oligonucleotide and drug was placed in one syringe, and a sample of 0.4% SDS prepared in the reaction buffer was placed in the other syringe. Samples were mixed in a thermostated cell, 120 μL in volume, and traces were automatically recorded. Signals from at least three traces were averaged to determine each kinetic constant. In some cases, where the signal was low, more than three traces were used.

Mathematical Treatment. The data were analyzed in a manner similar to previous authors (Chaires et al., 1982b; Blake & Peacocke, 1968). A fixed concentration of antibiotic was aliquoted into a cuvette, and the absorbance or fluorescence change was recorded. The mathematical treatment is based on equations derived from absorption studies and adapted to the fluorescence readings (Bloomfield et al., 1974).

Addition of the oligonucleotide in solution to the drug solution results in a drop in extinction and fluorescence. The absorption measured, A_{ob}, reflects the free drug in addition to all of the bound species:

$$A_{ob} = \epsilon_F C_F + \epsilon_B C_B \quad (1)$$

where F and B represent free and bound species, respectively, ϵ is the extinction coefficient, and C is the concentration. The extinction coefficient change upon binding is $\Delta\epsilon = \epsilon_B - \epsilon_F$, and the apparent extinction coefficient change is $\Delta\epsilon_{ap} = \epsilon_{ap} - \epsilon_F$, where $\epsilon_{ap} = A_{ob}/C_T$ and C_T is the total ligand concentration. With the assumption that $\Delta\epsilon$ does not depend on the number of ligands bound, the fraction of the drug that is bound is

$$f_B = \frac{\Delta\epsilon_{ap}}{\Delta\epsilon} \quad (2)$$

and the amount, r, of drug bound per unit of oligonucleotide is

$$r = f_B \frac{C_T}{C_N} \quad (3)$$

where C_N is the concentration of oligonucleotides.

For fluorescence, the analog of eq 1 is

$$I_{ob} = i_F C_F + i_B C_B$$

where i is the fluorescence intensity (in arbitrary units) divided by drug concentration; i is the fluorescence analog to the extinction coefficient ϵ. I_{ob} is the observed fluorescence intensity, and i_{ap} is I_{ob}/C_T. The fluorescence analog to eq 2 is

$$f_B = \frac{\Delta i_{ap}}{\Delta i} \quad (4)$$

where $\Delta i_{ap} = i_{ap} - i_F$ and $\Delta i = i_B - i_F$.

Determining $\Delta\epsilon$ or i_B requires an extrapolation to high oligonucleotide concentrations at which all of the drug is bound. This we did by assuming a simple binding isotherm with n binding sites per oligonucleotide and approximating the concentration of free binding sites by the total sites minus the total drug concentration, $(nC_N - C_T)$, for small values of r.

The result, written for fluorescence, is

$$i_{ap} = i_B + \frac{i_F - i_{ap}}{K(nC_N - C_T)} \quad (5)$$

where K is the binding constant. i_B is determined from the intercept of a plot of i_{ap} vs $(i_F - i_{ap})/(nC_N - C_T)$. In principle this method requires an estimate of n, but because of the large excess of C_N over C_T, the resulting value of i_B is insensitive to n. Furthermore, subsequent analysis of the binding data by the Scatchard method leads to a value for n, which can be used in a second round of refinement of the analysis if necessary.

Data Analysis. The fluorescence method was used to generate a graph of the Scatchard parameters r and r/C_F. The data points were fit to a curve using a simple two-state model, for which the isotherm is given by (Bloomfield et al., 1974)

$$\frac{r_T}{C_F} = K_1 n_1 + K_2 n_2 - K_1 r_T - r_2(K_2 - K_1) \quad (6)$$

which allows for two classes of binding sites with n_i binding sites of binding constant K_i per oligonucleotide. Occupancies of the individual sites are given by r_i, with $\sum r_i = r_T$. Initial values for the four parameters defined by the isotherm were calculated by drawing two slopes for the Scatchard plot and using a linear regression to obtain values for the slopes and intercepts of the two portions of the curve. Equations for the slopes and the intercepts of plots of this type are defined by Dougherty and Pigram (1982). Using their definitions, the four parameters K_1, K_2, n_1, and n_2 are obtained. These values were then modified by curve fitting using the isotherm in eq 6, which, although appearing linear, is not because of the quadratic relationship of r_1 and r_2 to r_T (Bloomfield et al., 1974). Values for the four parameters were considered valid if they fit both the values obtained from the slopes and the values obtained from the curve fit in eq 6 within 10%. Given the scatter in the Scatchard plots, binding equilibrium constants K_1 are subject to an estimated error of 25%. The estimated error in K_2 is about a factor of 2.

To verify the results obtained by the method cited above the analysis of Bujalowski and Lohman (1987) was used to examine binding at low values of r. For the systems tested, namely, the first three oligonucleotides listed in Table 1, the points determined at low degrees of binding compared well with the intercept on the r/C_F axis, giving confidence in the value of the intercept.

RESULTS

Oligonucleotides. The oligonucleotides synthesized are listed in Table 1. In each case, the top strand of the sequence is recorded in the 5′ to 3′ direction. After heat annealing with the opposite strand, the integrity of the resulting 16-mers was assessed by examining melting curves of the samples in the same phosphate buffer used in the experiments. There was no evidence of any biphasic melting in any of the samples. All the samples were double-stranded at 20 °C and were stable to 30 °C. Results from gel electrophoresis also indicated that the molecules are double-stranded and free from any contaminating single strands. The melting temperature of each oligomer is listed in Table 1.

CD of Oligonucleotides. CD spectra of the oligonucleotides used in this study (Figure 1) cannot in general be generated by a simple sum of the spectra of GC homopolymers and poly(dA)·poly(dT). The CD spectrum of dA_{20}·dT_{20} closely resembles the spectrum of the homopurine–homopyrimidine polymer, having similar crossover points and similar maxima and minima (Figure 1, middle panel). The CD spectra of the oligomers composed of GC base pairs with flanking A tracts are altered from the spectra of oligomers composed of only GC base pairs referenced in the literature [for example, see Kastrup et al. (1978)], as might be expected simply from the inclusion of A-tracts. The spectra are shifted to the red in the positive region and contain alterations of the signal at 260 nm relative to the spectra of oligomers containing only GC base pairs. Instead of reaching a minimum at 250 nm and a crossover point at about 260 nm, with a maximum peak at 270 nm, the spectra of the oligonucleotides containing the A-tracts are shifted to the red and contain a shoulder at 265 nm. This change in the CD in this wavelength region has been reported for longer polymers with runs of As (Jayasena & Behe, 1991). The CD spectra do not eliminate the possibility that portions of the sequence are in a non-B-form structure.

In summary, the CD spectra of the oligomers used in this study contain qualitative characteristics of both A-tracts (Figure 1, middle panel) and random sequence oligomers (Figure 1, top panel), indicating that the oligomer conformation is the result of a summation of the internal core, which is presumed to be B-form, and the A-tracts. The spectral changes are especially evident in the sequences containing an internal core consisting of the alternating AT sequence and the alternating CI sequence (Figure 1, middle panel). The spectra of both of these sequences more closely resemble the spectra of homopurine–homopyrimidine polymer sequences than the molecules consisting of other core sequences.

Table 1: Equilibrium Binding Properties of Oligonucleotide Sequences for Daunomycin[a]

oligonucleotide	name	T_m	K_1	n	K_2	n	% rf
$A_5CGTACGA_5$	CG	44	9.8×10^7	1	3.5×10^5	5	2
$A_5TAGCTGA_5$	AG	41	4.0×10^7	1	3.0×10^5	4	10
$A_5TCATCCA_5$	TC	40	4.0×10^7	1			10
$A_5CGCGCGA_5$	CS	51	5.0×10^7	2			2
$A_5TATATAA_5$	TA	38	5.0×10^7	1			40
$A_5CITACIA_5$	CI	30	5.0×10^7	2			40
CGTACGTTTTCGTACG	HA	68	5.0×10^6	1			3
ACATGCTCATTAGCAA	RA	48	3.0×10^7	3			6
A_{20}·T_{20}	AT	44					40
calf thymus DNA			2.6×10^6				5

[a] All measurements were made in sodium phosphate buffer, 0.1 M Na^+, 0.001 M Na_2EDTA, pH = 7.3. K_1 here is the affinity of the primary site, taken at 20 °C. K_2 is the affinity of the secondary site(s). Fits to the data were done by examining the two slopes of the linear portions of the Scatchard graph by regression analysis. Equations defined by Dougherty and Pigram for these quantities were used to fit the data. These initial values were then fit to eq 6 as explained in the text. Only whole numbers were used for the number of sites, as fractional numbers were not reasonable. % rf is the percent residual fluorescence left at the end of the titration in the limit of high nucleotide concentration.

Binding Studies. The affinity of daunomycin was tested initially for binding to calf thymus DNA, using the same buffer system. Fluorescence quenching, absorbance and equilibrium dialysis were used to quantify the results. The values for the total binding constant and the r intercept (Table 1) agree well with previously published values (Chaires et al., 1982b; Chaires, 1985; Müller & Crothers, 1975). Note that the binding constants obtained in the presence of bulk DNA are expressed in base pair concentration units, whereas those for the oligomers are expressed in terms of double helix concentration. Converting the oligonucleotide K values to the same scale as bulk DNA requires dividing the oligomer values by the number of base pairs in the oligomer, or 16. This

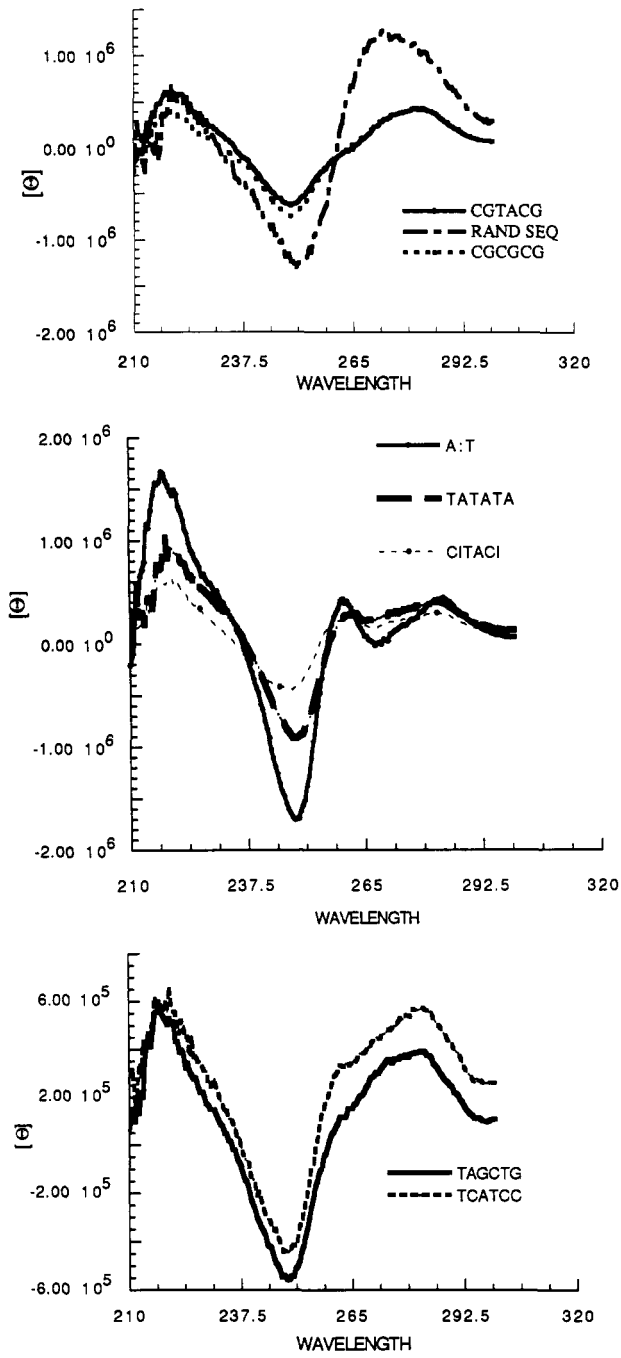

FIGURE 1: CD spectra for all oligonucleotides. Concentration of samples was 5.0×10^{-6} M, in reaction buffer.

yields, for example, K values of 6.1×10^6 M^{-1} for the CG oligomer and 2.5×10^6 M^{-1} for the AG oligomer. These values are reasonably close to the result for thymus DNA (2.6×10^6 M^{-1}), for which the r intercept is between 0.2 and 0.3, as expected for this system (Chaires et al., 1982b).

Figure 2 illustrates the plot used to determine the residual drug fluorescence i in the limit of infinite oligonucleotide concentration. Illustrative results of the fluorescence experiments are reported in Figure 3. In this series of experiments, the drug concentration was the same in each case (10^{-7} M), and the raw fluorescence, corrected only for buffer and lamp fluctuation, is plotted as a function of the nucleotide concentration, in moles of double-stranded oligomer per liter. By plotting the data in this manner, the difference in the intensity of the residual fluorescence for each oligonucleotide can be seen (Table 1).

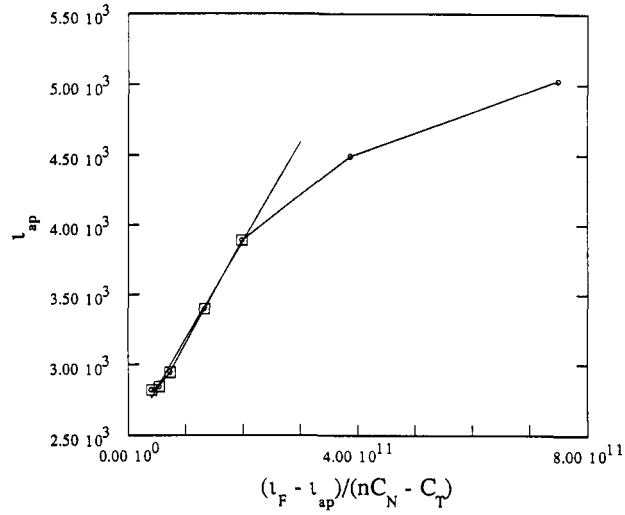

FIGURE 2: Determination of the limiting fluorescence for fully bound drug. As suggested by eq 5, i_{ap} is plotted against $(i_F - i_{ap})/(nC_N - C_T)$, with $n = 1$. The intercept on the y-axis is the limiting fluorescence of bound drug, as determined by the best linear fit to the data points indicated by squares. The concentration of drug was 2×10^{-7} M, and the final concentration of oligonucleotide was 3.0×10^{-5} M; all measurements were made in reaction buffer. The limiting slope of 7.0×10^{-9} M gives a binding constant of 1.4×10^8 M^{-1}, in reasonable agreement with the value of 1.0×10^8 M^{-1} from the Scatchard analysis (Table 1).

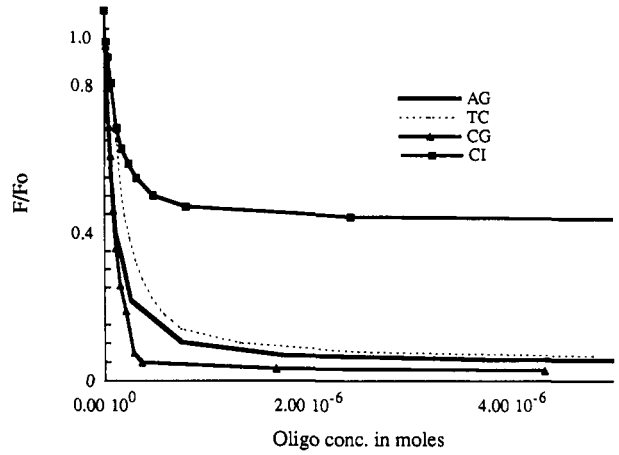

FIGURE 3: Plot of the raw fluorescence F/F_o against nucleotide concentration in moles of oligomer per liter; drug concentration was 10^{-7} M. Note the differing residual fluorescence for the various core sequences.

Binding affinities of the oligonucleotides are presented in Table 1 in terms of the parameters fitted to a Scatchard analysis of the data, as illustrated in Figure 4. As discussed below, the data do not unambiguously distinguish models in which there are $n_1 = 1$ or 2 strong binding sites per oligomer. The values given refer to the best fit found for integer values of the number of sites n_1 and n_2. In some cases n_2 could not be determined. The product $K_1 n_1$ is, however, determined with better accuracy (~25%) because the intercept on the y-axis of a Scatchard plot is equal to $\sum K_i n_i$, which is approximately equal to $K_1 n_1$ when the secondary sites are weak.

The oligomer which contained the sequence CGTACG as the internal core has the highest overall affinity for daunomycin, and fluorescence quenching was relatively complete, leaving a residual of about 2%. In the next five sequences in Table 1, overall binding was about 2-fold lower than in the CGTACG sequence. The differences among these sequences are not experimentally significant. The oligomers do, however, differ strongly in residual fluorescence of the bound drug

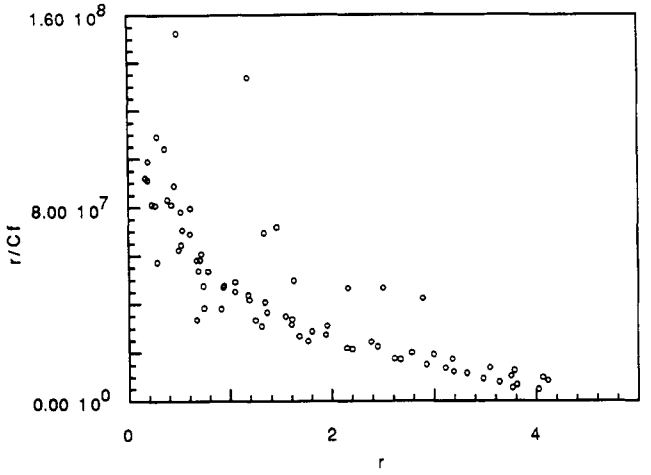

FIGURE 4: Scatchard data for the CG sequence. r is the number of drug molecules bound per oligonucleotide, and C_f is the free drug concentration. All measurements were made in sodium phosphate buffer, 0.1 M Na^+, and 0.001 M EDTA, pH = 7.3, 20 °C.

(Table 1). Only the hairpin sequence HA and the homopolymeric sequence dA_{20}·dT_{20} bind significantly more weakly than the others. These results indicate that daunomycin has only a marginal DNA sequence selectivity.

A Scatchard plot is linear if the site or sites are identical and independent. The x-intercept reflects the number of sites on the oligonucleotide and the y-intercept reflects the affinity (Scatchard, 1949). As seen in the typical Scatchard plot in Figure 4, the graph is not linear but curved, indicating that one of these conditions was not satisified. Drug aggregation is not the cause of the curvature as the drug concentration was kept below the level leading to dimerization in this buffer system (Chaires et al., 1982a). Either the binding sites are not identical or the sites are not acting independently, i.e., binding to the oligonucleotides follows a neighbor exclusion model or is anticooperative for some other reason. We have analyzed the results for the CG and AG oligomers in terms of the heterogeneous site model (Table 1), rather than invoking neighbor exclusion effects. However, it should be realized that this distinction cannot in general be made unambiguously on the basis of equilibrium binding data. In both cases, the secondary site appears to be weaker by about 2 orders of magnitude, but the number of sites, 4–5, partially compensates and there is appreciable binding in the weaker mode.

To assess the effect of the A-tracts at the ends of the molecule and to determine whether the A-tracts were responsible for the shape of the Scatchard plots, binding studies were conducted with an oligomer, dA_{20}·dT_{20}, that had been previously examined by NMR (Nadeau & Crothers, 1989). Binding to this sequence was reduced by a factor of about 100 relative to any other sequence tested, and the residual fluorescence was about 40%.

Calorimetric Analysis. The curvature of the Scatchard plots indicated that the oligonucleotides possessed multiple binding sites that were either inequivalent or interacting. Titration calorimetry was used to help assess which condition of the Scatchard model was not being satisfied. A plot of the heat of binding as a function of added titrant can be obtained for any ligand/macromolecule system for which the binding enthalpy is not zero. A sigmoidal curve will result if there is only one ligand binding site per macromolecule or if multiple sites exist that, although not necessarily independent, are identical. If multiple sites with differing binding affinities exist, then the resulting plot will be a composite of multiple curves. Typical results of these experiments are reported in

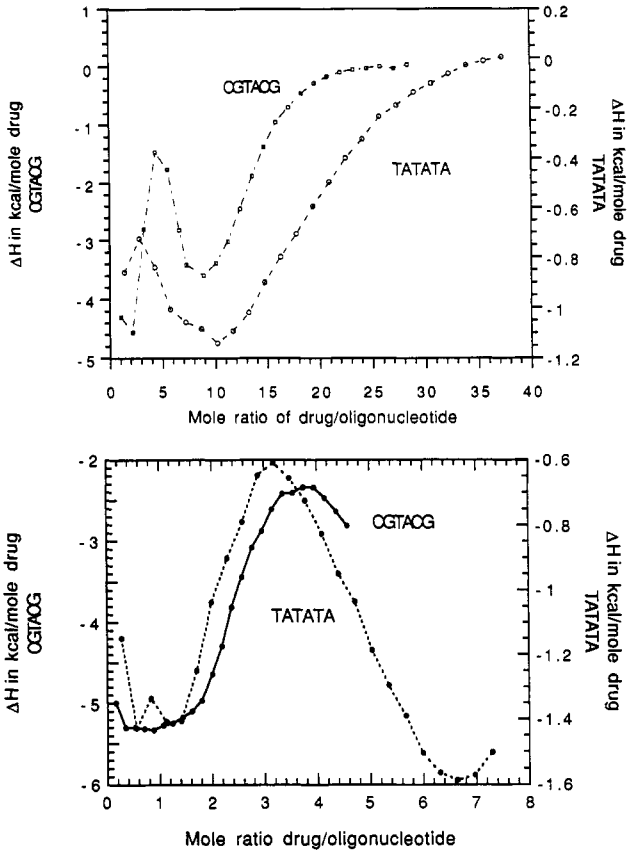

FIGURE 5: Calorimetry data for the CG and TA sequences. The enthalpy values refer to the heat absorbed per incremental addition of drug, expressed in kilocalories per mole of added drug, shown as functions of the molar ratio of total drug to oligonucleotide. Note that as a consequence of instrument assembly and diffusion of drug and oligonucleotide across the injection port during the preequilibrium period, the heat of injection observed for the first injection is typically less than expected on the basis of subsequent injections. (A, top panel) Results at a concentration of oligonucleotide that demonstrates the saturation of sites; initial concentration of CG is 4.9×10^{-5} M and that of TA is 3.8×10^{-5} M. The negative sign indicates an exothermic binding process. (B, bottom panel) Results when the drug is added in smaller increments. The concentration of CG is 1.2×10^{-4} M and that of AT is 8.4×10^{-5} M.

Figures 5 and 6. It should be noted that these results have not been corrected for the heat of dilution of the added daunomycin.

Figure 5A reports the progressive addition of daunomycin in relatively large increments (greater than 1 mol of drug/mol of oligonucleotide). It can be seen with the CGTACG core sequence that saturation of all possible binding sites is not achieved until the drug concentration is more than 20-fold that of the oligonucleotide concentration, as monitored by leveling of the binding profile to near zero enthalpy. Disruption of the sigmoidal character of the binding profile suggests that daunomycin binds to this oligonucleotide with at least three different binding modes. The heats of binding for the highest and lowest affinity sites are clearly exothermic, but these processes are separated by a binding event that contributes a less exothermic (possibly endothermic) component which is maximum at about 4–5 drug molecules added per oligonucleotide. However, it should be pointed out that due to the complex nature of the binding profile and additional complications arising from dissociation of the added daunomycin, an absolute determination of the values of the binding enthalpies and binding constants is not possible. Thus, although the binding enthalpy associated with the intermediate binding affinity is clearly less exothermic, we cannot be sure

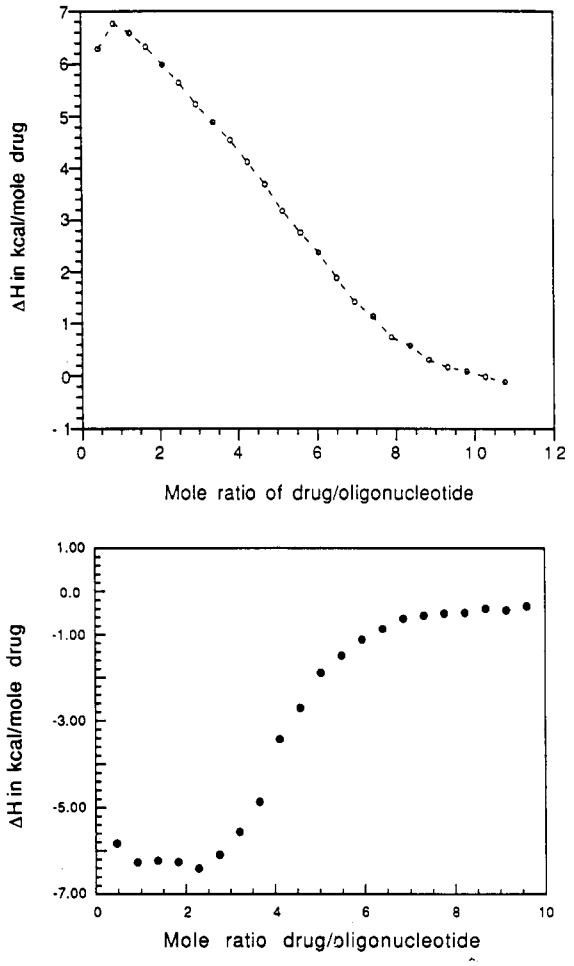

FIGURE 6: (A, top panel) dA_{20}·dT_{20} (AT in Table 1) titrated in the same manner as the previous figure, indicating the endotherm involved in the binding; the initial concentration of oligonucleotide is 3.0×10^{-5} M. (B, bottom panel) A randomly generated sequence (RA in Table 1) titrated in the same manner as the previous figure, indicating an exotherm and no evidence of an endotherm; the concentration of oligonucleotide is 2.9×10^{-5} M.

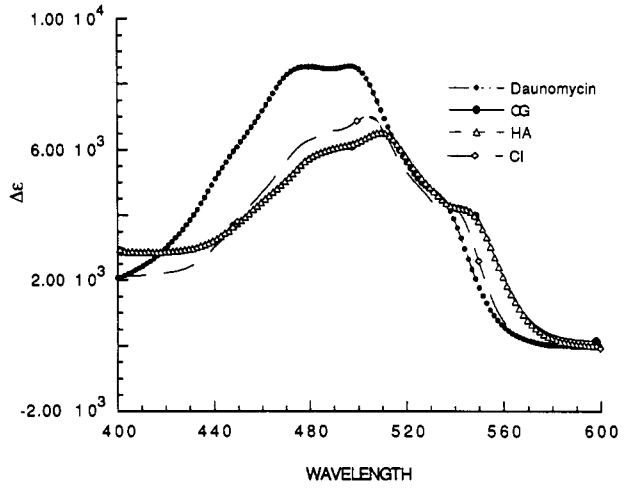

FIGURE 7: Comparison of the absorption spectra of daunomycin at saturating levels of CG, CI, and HA; the concentration of daunomycin is 5.6×10^{-6} M. The final concentration of nucleotide ranged from 1.5×10^{-5} to 1.9×10^{-5} M for the different samples.

whether or not the binding enthalpy associated with this event is endothermic.

When an alternating A-T base core sequence is substituted for the CGTACG core sequence, similar results are obtained. However, daunomycin binds with a lower net affinity in the case of the alternating A-T sequence, as indicated by the more gradual approach to saturation.

The experiment was repeated using a lower molar ratio of injected drug to oligonucleotide in order to observe the binding of the drug to the highest affinity site(s) (Figure 5B). In this case the saturation of the highest affinity sites is more readily observed for both sequences. Both appear to possess 1–2 high-affinity binding sites as indicated by the relatively constant enthalpy generated by the addition of daunomycin until the molar ratio of drug to oligonucleotide reaches about 1–2. The main difference between these two oligonucleotides is the magnitude of the apparent binding enthalpy of the high-affinity sites, with the CGTACG core having a significantly larger observed heat of reaction with the drug than is observed for the TATATA sequence. (Note the difference in the vertical scale for the two sequences).

An estimate of the enthalpy of binding of daunomycin to the highest affinity sites can be calculated from the titration data, assuming that for any given injection essentially all of the daunomycin titrated into the cell binds to oligonucleotide. This assumption is valid if the binding constant is large compared to the reciprocal drug concentration and if the number of sites is sufficient to accommodate the added drug. This condition will result in a constant heat of injection, as is observed in Figures 5B and 6B for the early injections (except the first injection, which is compromised by dead volume effects).

To calculate the enthalpy of binding the contribution of the heats of dilution of the ligand and the macromolecule must be subtracted from the observed heats. The heat of dilution of the macromolecule is obtained by repeating the titration experiment with buffer substituting for the daunomycin solution. Similarly, the heat of dilution of the ligand is obtained by substituting the oligonucleotide with buffer. Whereas the heats of dilution of the oligonucleotides were found to be relatively constant, the heat of dilution of the daunomycin varied markedly with both initial drug concentration and with the concentration of the drug already present in the cell (data not shown). This is typical for a highly self-associated molecule.

The observed enthalpy upon mixing concentrated drug with oligonucleotide is given by the sum of the heat required to dissociate the drug and the heat of binding, or

$$\Delta H_{\text{observed}} = \Delta H_{\text{dissociation}} + \Delta H_{\text{binding}}$$

In most cases the observed heat is negative (exothermic), whereas the heat of dilution, which produces full or partial dissociation, is positive (endothermic). Therefore the heat of binding, $\Delta H_{\text{binding}} = \Delta H_{\text{observed}} - \Delta H_{\text{dissociation}}$, is more negative than the observed heat. Chaires et al. (1982a) have studied the self-association of daunomycin and concluded that aggregation beyond a dimeric state occurs, but they could not definitively conclude whether the aggregated state could best be described as a tetramer or by an indefinite association model. Without this knowledge the precise contribution of the heat of dissociation of the daunomycin cannot be determined. However, the first injection of the daunomycin dilution experiments can be used as a lower limit for the magnitude of $\Delta H_{\text{dissociation}}$, and thus a lower limit for the magnitude of the (exothermic) binding enthalpy can be obtained. The results are −6.9, −2.5, and −8.0 kcal/mol, respectively, for the CG, TA, and RA sequences. Utilizing a recently developed differential, stopped-flow, high-sensitivity calorimeter (Mudd & Berger, 1988), Remeta et al. (1991)

have obtained comparable results for the binding of daunomycin to various polymeric DNA duplexes.

To determine which, if any, of the three calorimetrically detected binding events is associated with the flanking A tracts, two control sequences were utilized. One oligonucleotide, $dA_{20}{\cdot}dT_{20}$, possessed no internal core sequence, and the other oligomer was a 16-mer of "random" (meaning arbitrarily selected) sequence possessing no flanking A tracts. The titration results are presented in Figure 6. As can be seen, binding to the "pure" A-tract DNA is entirely endothermic (Figure 6A), confirming the earlier report of Herrera and Chaires (1989). Conversely, binding to the sequence lacking A tracts is entirely exothermic (Figure 6B). Thus the binding event with intermediate affinity can reasonably be attributed to the presence of the flanking A tracts, as both of the other two binding events are exothermic.

The third binding event, observed for both the CGTACG and TATATA core sequences, appears to be unique to the oligonucleotides composed of both an internal core sequence and flanking A tracts. As already mentioned, binding of daunomycin to $dA_{20}{\cdot}dT_{20}$ does not display any exothermic binding component, and (exothermic) daunomycin binding to the random sequence appears to saturate at a molar ratio of drug to oligonucleotide much lower than that seen for either of the other two oligonucleotides. For this reason, and because the binding affinities of this third site are different for the two sequences, it would appear that this binding involves both the internal core sequence and the flanking A tracts, with the net binding affinity and exothermic component being influenced by the nature of the internal sequence. Cooperative coupling between the conformation of the core sequence and the structure of the flanking A tracts could be the source of this behavior.

Absorbance Spectral Data. To investigate the hypothesis that daunomycin binds to C-G base pairs, an oligomer was synthesized with a core sequence CITACI (CI), substituting inosine for guanine. A hairpin sequence (HA) was also synthesized that contained the sequence CGTACGTTTTCG-TACG. Both of these sequences were titrated into a solution of drug until the absorption spectrum reached a minimum and no longer changed. The results were compared to the oligomer with the CGTACG internal core, titrated in the same manner. The final spectrum for each oligonucleotide with daunomycin was recorded at the end of the titration and overlaid. As can be seen in Figure 7, the two spectra of oligonucleotides that contain guanine in the sequence CG-TACG are the same, but the spectrum with inosine substituted for guanine is shifted. This is not unexpected since by removing the exocyclic amine from guanine, the electronic state of the base and its interaction with the drug are changed. The results of this experiment support the hypothesis that daunomycin binds to at least one CG base pair in this sequence. However, it does not identify the particular CG base pair.

Surprisingly, the binding affinity to the hairpin is an order of magnitude lower than any of the oligomers tested (Table 1). Possibly this is due to the proximity of the drug binding site to the helix ends or to anomalous effects of the adjacent A tracts. Fraying of the terminal base pairs might modify the binding constant but is not likely to account for the observed order of the magnitude difference. There might be some anomalous structure introduced at the helix end or at the juncture with the loop which limits binding. Another possibility is the contribution of long-range electrostatic effects to binding in the larger oligonucleotides. It remains uncertain why the hairpin has a markedly lower affinity than the other sequences.

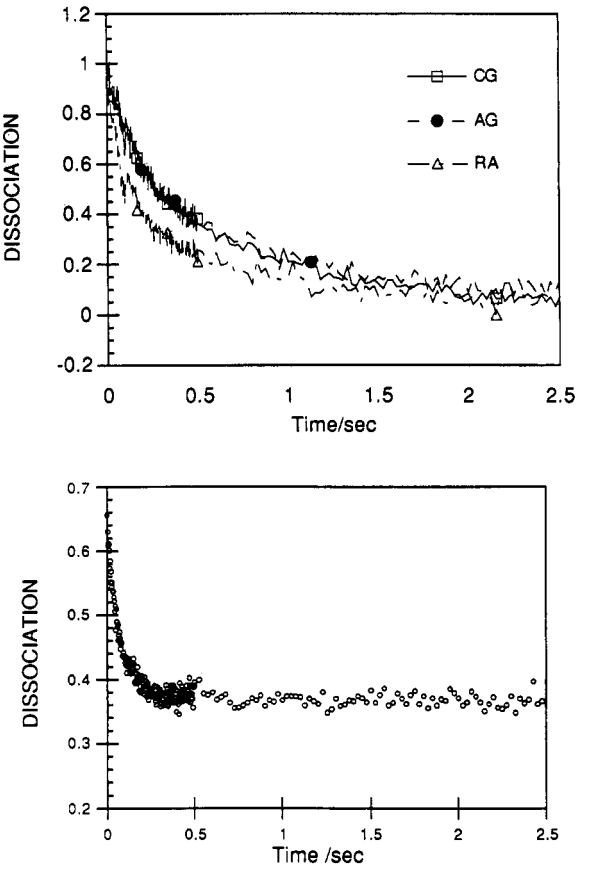

FIGURE 8: (A, top panel) Stopped-flow data for the SDS-induced dissociation of daunomycin. Comparison of the decay curves for three sequences indicates only a slight change in the dissociation profile. (B, bottom panel) The decay curve for dissociation from the hairpin sequence on the same time scale as the top panel, demonstrating the lack of a slower component in this case.

Stopped-Flow Experiments. To investigate the multiplicity of binding sites, stopped-flow experiments were conducted to measure the dissociation rate of the drug from a few selected oligonucleotides. An equilibrated sample was dissociated by addition of SDS, and the multiexponential curve was examined for the lifetimes of the species. The experiments were carried out as a function of added r, which was varied from a ratio of 1 to 0.25 drug molecules/oligonucleotide. This was done to peel away the less specific sites and examine the relative dissociation rates of the stronger binding sites. Comparative results are shown in Figure 8.

For the CG sequence (Figure 8A), the dissociation rate R_2 of the stronger binding site was about 1.2 s^{-1} (Table 2), with a comparable rate observed for the AG sequence. Analysis of the data for the hairpin (Figure 8B), which contains the same site as the CG sequence, seems to indicate a single site. However, the dissociation rate of the species in the hairpin approaches that of the faster site in the other oligonucleotides, in agreement with the weaker binding observed by equilibrium methods.

The random sequence also dissociates in two kinetic components, with rates similar to those of the CG sequence. As the ratio of drug to oligonucleotide is lowered, the rate of the more kinetically stable site decreases but remains comparable to that observed for the CG and AG oligomers. This is not surprising because the random sequence contains site similar to that in the AG sequence. Since the randomly

Table 2: Comparison of Dissociation Rates for Oligonucleotides[a]

oligo	r	R_1	R_2	A_1	A_2
CG	0.25	5.4	0.91	0.084	0.044
	0.50	11.6	1.21	0.060	0.166
	1.0	6.5	1.27	0.176	0.275
RA	0.25	10.2	0.99	0.082	0.078
	0.5	11.6	1.50	0.079	0.169
	1.0	12.0	1.47	0.239	0.203
AG	0.25	49.0	1.37	0.038	0.061
	0.5	5.9	1.03	0.061	0.107
	1.0	5.4	0.92	0.171	0.167
HA	0.5	14.5		0.246	
	1.0	14.3		0.274	

[a] The table reports a summary of stopped-flow data, using SDS dissociation of complexes. The molar ratio of added drug to oligonucleotide is r; R_1 and R_2 are the first-order rate constants (in seconds^{-1}) for the decay curve as resolved into two exponential components; A_1 and A_2 are the corresponding signal amplitudes (in arbitrary units). In the case of the hairpin sequence (HA), only the faster component was detected. All measurements were made in sodium phosphate buffer, 0.1 M Na^+, 0.001 M Na_2EDTA, pH = 7.3, T = 20 °C.

generated sequence has no terminal A tracts, the species with the faster dissociation rate must reflect weaker binding modes within the presumably normal B-DNA sequence.

DISCUSSION

Design of the Oligonucleotides. The oligonucleotides used in this binding study were designed with A-tracts at the ends of the molecule, primarily to stabilize the helix and prevent fraying of the internal core sequence. We reasoned that additional strong binding sites should not result from extending the helix beyond the six base pair core because, according to the literature (Chaires, 1983; Herrera & Chaires, 1989; Remeta et al., 1988), daunomycin does not bind well to poly(dA)·poly(dT). The binding constants published for this sequence are a factor of 10 lower than for alternating copolymers, presumably due to the anomalous structure of the molecule. Any fraying that does occur then is removed from the core into an area of the molecule that should not bind. However, in adding the A-tracts at the ends of these oligomers, a junction is introduced between the core sequence and the A-tracts at the ends of the molecule. Furthermore, the A-tract structure may by modified by its juxtaposition with the B-DNA core segment. These factors may affect the shape of the helix, just as helix ends might.

A comparison of the calorimetric data for the sequence that is homopurine–homopyrimidine, dA_{20}·dT_{20}, demonstrates that the binding of daunomycin to this sequence is endothermic. The sequence that does not contain any A-tracts, the randomly chosen sequence, shows no trace of an endothermic component in the calorimetry experiment. Only a sigmoidal exotherm is evidenced. This implies then that the change in the calorimetric binding profile is somehow associated with the presence of the A-tracts in the oligomers. A plausible explanation for the observations is that initial daunomycin binding to the junction/A-tract region in the molecules containing flanking A-tracts converts the A-tract structure in a cooperative, endothermic transition to a form that then binds the drug exothermically.

CD spectra taken of the sequences indicate that some structural alteration due to the A-tracts may be present (Figure 1). The CD of an alternating GC helix does not extend above the baseline at 220 nm, nor is there a shoulder at 265 nm. However, in the molecules synthesized for this study, there is a positive signal at or below 220 nm and a red shift forming a shoulder at 265 nm. Presumably this is due to the presence

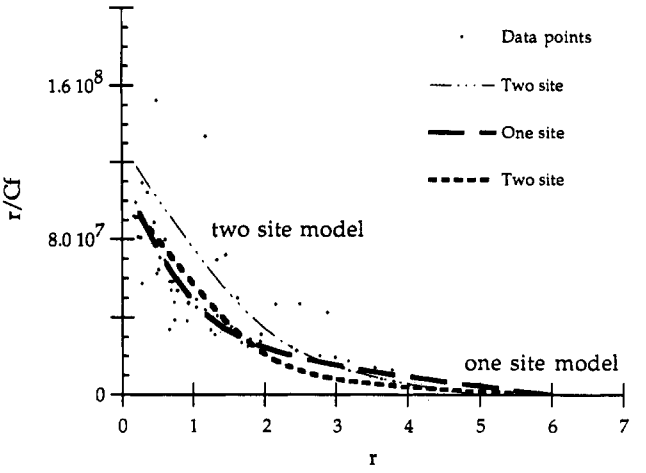

FIGURE 9: Fits to eq 6 overlaid on the raw data points from Figure 5 for the CG sequence. Fits for the one-site and two-site models are marked; alternate fits for the two-site model fall above and below the data points, depending on which range of r values is emphasized in the fit.

of the A-tracts. What effect the A-tracts have on the shape of the oligonucleotides is unclear.

Binding Model. The binding isotherm in eq 6 defines a two-state model in which there are two types of binding sites with binding affinities that are appreciably different from each other. This model would then assume that the core sequences represented a more select site for daunomycin than the A-tracts at the ends of the molecule. By comparing the results for the four parameters derived from best fits to this equation and fits to the equations defined by Dougherty and Pigram (1982), it was determined that one strong site in the CG oligomer is marginally more compatible with all the data than two sites. Using the isotherm to calculate a curve and varying the parameters results in the curve in Figure 9. If two strong sites are assumed for the first part of the isotherm, the calculated curve fits less well to the experimental data points. The steeper portion of the experimental curve seems to intersect the x-axis at a value between 1 and 2. This is the result expected for a single strong site and a substantial number of weaker sites. In addition, using the equations from Dougherty and Pigram (1982), a model with one strong site and a number of weaker sites fits the set of four simultaneous equations better than one with two strong sites. This pattern is evident for all of the Scatchard plots for the first three oligomers listed in Table 1. The results are compatible with the presence of a single strong site and a number of lesser sites. However, the scatter in the experimental data makes this conclusion tentative, and it furthermore has to be reconciled with the calorimetric results, as discussed below. We cannot rule out a model in which a second drug can bind to the core region with an affinity somewhat below that of the initial binding but above that of the set of weaker sites. The number of lesser affinity sites is difficult to determine accurately, since changing that parameter has only a modest effect on the fit to the binding isotherm at the higher values of r which reflect the weaker binding mode.

The calorimetric results show deviation from constant enthalpy of binding each additional drug when r is between 1 and 2 (Figure 5B). This result can be compared with predictions based on models that assume either one or two strong binding sites. The parameter r_1, the fraction of sites of type 1 occupied, is calculated from the isotherm and plotted as a function of total binding r_T in Figure 10. In the first case it is assumed that there is one strong site and a number of

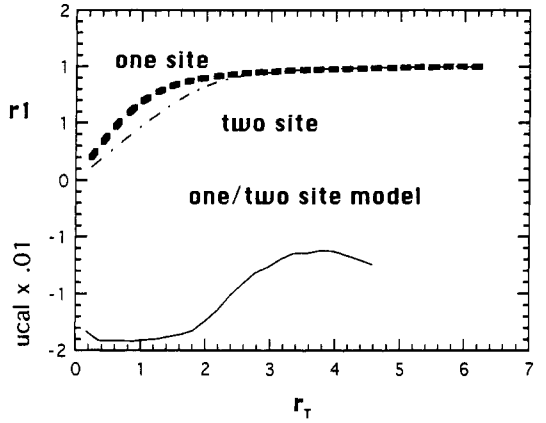

FIGURE 10: r_1, the calculated degree of binding to the strong site determined from the parameters in Table 1, plotted as a function of r_T (eq 6), the total degree of binding. The lower curve shows the calorimetry data for comparison. Assuming that all of the added drug is bound, the region of r values in which the enthalpy change is constant should coincide with the region in which the slope of the plot of r_1 vs r is constant.

lesser sites. Below that is plotted r_1 where two strong sites are assumed and a number of lesser sites. Plotted on the same graph are the calorimetric data from Figure 5. Assuming that all the added drug is bound, it is expected that the enthalpy change should be constant in the range of r values over which the slope of the curve of r_1 vs r is roughly constant. This criterion appears to be marginally better satisfied for the two-strong-site model than for the corresponding one-site model. However, keeping in mind that the calorimetry experiment is reported in terms of added r and the Scatchard data is bound r (not all of the added drug will bind, but at the level of nucleotide used in the experiment, greater than 90% is bound), we conclude that the calorimetry data cannot reliably distinguish models which assume one strong site or two. Again, a plausible model is one in which a second drug can bind to the core region, with a similar enthalpy change but with lesser affinity than the first.

A study on the six base pair internal core of the CG sequence was conducted by Rizzo et al. (1989), who found that one site in the six base pair oligomer exhibits higher affinity than a second site due to an exclusion effect by binding of the second drug molecule. The results were reported for both daunomycin and adriamycin. The study was conducted at high ionic strength, 1 M NaCl, to ensure association of the oligomers. Under these conditions aggregation of the drugs occurs, and corrections were made for this. Their conclusion, that there is a difference in affinity for the two CG sites, is in agreement with the findings in the present study. One site seems to bind better, and this model fits the data somewhat better than assuming two equivalent sites in the internal core. Other authors, such as Chaires (1990), surmise that if there is a preferred site for daunomycin the affinity would be $>10^7$ M^{-1}, which is in agreement with the findings here.

Sequence Dependence of the Binding Affinity and Dissociation Rate. The main conclusion from our studies is that daunomycin shows very little preference when presented with alternative binding sites identified as strong binders in other studies. From the data presented above, it can be seen that daunomycin binds with a small preference to the sequence containing the internal sequence CGTACG. Switching the sequence to the AG core diminishes binding by about 2-fold. If the number of sites n_1 on the CG molecule is taken to be 2, and $n_1 = 1$ on the other molecules, then the overall affinity for the other sequences is only slightly lower. The sample containing inosine in lieu of guanine binds well to daunomycin, albeit somewhat more weakly than the CG sequence.

Stopped-flow data indicate that the AG sequence does have a relatively strong site for the drug, at least comparable in kinetic stability to that of the CG sequence. If the site on the AG sequence is buried within the core and removed from the A-tract, then the tract of As next to the core sequence may not affect the binding. This may explain the difference between this sequence and the CG sequence. In the CG sequence, binding is presumably at the CpG step, and both of these sites occur at the end of the internal core, adjacent to the A-tracts, which may affect structure and drug binding affinity.

Weak Binding to the Hairpin Sequence. An unexpected result was the relatively low binding to the hairpin containing the same internal sequence as the CG oligonucleotide that binds so well. Possibly structural distortion due to the hairpin loop precludes binding to this molecule, or the helix may be too short to avoid end effects which alter binding properties.

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