Adriamycin and Daunorubicin Bind in a Cooperative Manner to Deoxyribonucleic Acid†

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ABSTRACT: Phase partition techniques have been used to measure the binding of the antitumor drugs adriamycin (NSC-123127) and daunorubicin (NSC-82151) to various DNAs. These methods provide reliable equilibrium binding data at the low levels of drug binding that may be expected in vivo. Both adriamycin and daunorubicin exhibit positive cooperativity (and/or allosterism) in their equilibrium binding to DNA as indicated by the positive slope in the initial region of the binding isotherms (Scatchard plots) under conditions simulating physiological ionic strengths. The cooperative binding (i.e., the appearance of initial positive curvature in the binding isotherms) is dependent upon the ionic strength, which suggests a role for DNA flexibility in the cooperative binding process. An analysis of the slope of the initial portion of the binding isotherms for the interaction of adriamycin with synthetic deoxypolynucleotides shows that the degree of cooperative binding decreases in the order poly(dGdT)·poly(dAdC) ≥ poly(dAdT)·poly(dAdT) > poly(dGdC)·poly(dGdC). Marky and Breslauer [Marky, L. A., & Breslauer, K. J. (1982) *Biopolymers 21*, 2185–2194] found that the average base stacking enthalpies of these synthetic polynucleotides were in the same order, which also suggests that the properties of the DNA influence the cooperative binding (and/or allosteric effects). Adriamycin binds with a higher degree of cooperativity than daunorubicin (0.1 M NaCl); although this correlates with the effectiveness of the drugs as antitumor agents, the exact relationship between the observation of cooperative binding and pharmacological activity is yet to be determined.

The binding interaction of the potent anthracycline antibiotics adriamycin and daunorubicin (Figure 1) to nucleic acids has been extensively studied (e.g., Zunino et al., 1972, 1977, 1980; Gabbay et al., 1976; Henry, 1979; Plumbridge & Brown, 1977; DuVernay et al., 1979; Neidle & Taylor 1979; Patel et al., 1981; Chaires et al., 1982. The high-affinity mode of binding to nucleic acids was shown to involve intercalation of the planar chromophore, as illustrated to atomic resolution in the structure of the cocrystalline complex of daunorubicin with d(CpGpTpApCpG) reported by Quigley et al. (1980). In the previous equilibrium binding studies the analysis of the data led the authors to conclude that both adriamycin and daunorubicin bind to DNA in a noncooperative manner. However, in 1977 Krugh & Young demonstrated that low levels of either adriamycin or daunorubicin would facilitate the binding of actinomycin D to poly(dAdT)·poly(dAdT), a duplex to which actinomycin does not bind in the absence of the anthracycline antibiotics. This cooperative binding was interpreted as resulting from an adriamycin-induced distortion of the poly(dAdT)·poly(dAdT) helix which facilitates the binding of actinomycin D. Interestingly, other intercalating drugs such as ethidium and acridine orange were shown to be ineffective in eliciting this synergistic effect (Krugh & Young, 1977; Krugh et al., 1979). Due to their profound value as chemotherapeutic agents and their previously demonstrated property of facilitating the binding of other drugs to DNA, a closer examination of the detailed binding properties of both adriamycin and daunorubicin at low r values is of considerable interest.

Numerous studies concerning the binding properties of these antibiotics with nucleic acids have provided insight into the molecular orientations, binding affinities, and conformational states of these complexes. However, many of these previous studies have been limited to the $r > 0.1$ (drugs per base pair) region as a result of experimental constraints. The antitumor activities associated with adriamycin and daunorubicin in in vivo systems are elicited at very low drug concentrations (Sinha & Sik, 1980). Therefore, it is of considerable importance to examine the biophysical properties of these drugs at low concentrations. Utilization of the phase partition method (Albertsson, 1971; Waring et al., 1975; Davanloo & Crothers, 1976; Dattagupta et al., 1980; Krugh et al., 1981; Winkle & Krugh, 1981) has allowed accurate binding isotherms to be obtained at very low levels of bound drug ($r < 0.01$), thus providing a means of examining the interactions of adriamycin and daunorubicin with DNA at concentrations comparable to pharmacological conditions and at physiological ionic strengths. This report describes the nucleic acid binding properties of these antitumor antibiotics with special interest in the binding at low levels of bound drug as well as the effects of ionic strength and base pair composition on the binding isotherms. From these studies, insight into such phenomena as cooperative binding, preferential binding to certain sequences, and propagation of DNA structural perturbations can be obtained.

The cooperative binding property discussed in this paper refers to the initial positive slope of the data in the Scatchard plots. The cooperative binding process may result from a cooperative binding of the drugs to the DNA and/or an allosteric transition of the structure of the DNA helix. Recently, Chaires et al. (1982) reported that daunorubicin binds to calf thymus DNA in a noncooperative manner. This conclusion was based upon data obtained by direct titration methods using fluorescence and absorption spectroscopies as well as equilibrium dialysis. As expected, their most reliable data were obtained at relatively high sodium chloride concentrations (1.0 and 0.5 M) where no evidence for cooperative binding was observed. This contrasted with our previously published results in which we reported that adriamycin and daunorubicin bind to calf thymus DNA in a highly cooperative manner when the sodium chloride concentration is 0.1–0.2 M (Krugh et al., 1981; Graves & Krugh, 1982; Rosenberg et al., 1982). These

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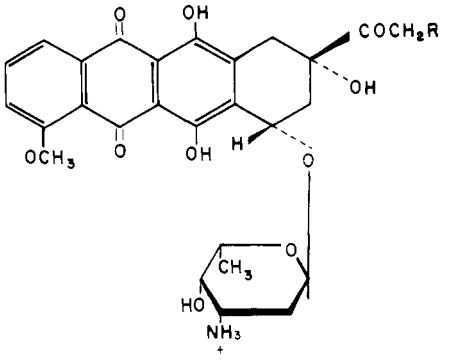

ADRIAMYCIN R = OH
DAUNORUBICIN H

FIGURE 1: Chemical structures of adriamycin and daunorubicin.

data were obtained by using the phase partition method, which provides reliable data at the very low levels of binding where the cooperative binding is observed at moderate ionic strengths. We resolve the apparent discrepancy between these previous results in this paper and report, as an interesting new phenomenon, that the cooperative binding process is dependent upon the ionic strength.

Experimental Procedures

Materials

Adriamycin (NSC 123127) and daunorubicin (NSC 82151) were supplied by the Natural Products Branch, National Cancer Institute. The purity of these compounds was confirmed by NMR spectroscopy and by TLC using methylene chloride–methanol–H_2O (100:20:2) as the solvent (Arcamone et al., 1972). Stock drug concentrations were determined spectrophotometrically (Cary 219) by using $\epsilon_{477} = 11\,500$ M^{-1} cm^{-1} (Gabbay et al., 1976). Calf thymus DNA (type I) was purchased from Worthington Biochemicals and purified by two separate procedures. Initial DNA purifications followed the method of Muller & Crothers (1975). A second and more extensive preparation (Chaires et al., 1982) was utilized for later experiments. The data obtained from binding experiments using DNA from either preparation were equivalent. The synthetic copolymers poly(dAdT)·poly(dAdT) and poly(dGdC)·poly(dGdC), purchased from P-L Biochemicals, Inc., and poly(dAdC)·poly(dGdT), purchased from Boehringer Mannheim, were filtered through a 0.47-μm Millipore filter and dialyzed prior to use. Nucleic acid concentrations are stated in terms of base pairs by using the molar absorptivities $\epsilon_{260} = 13\,200$ M^{-1} cm^{-1} for both calf thymus DNA and poly(dAdT)·poly(dAdT), $\epsilon_{254} = 14\,200$ M^{-1} cm^{-1} for poly(dGdC)·poly(dGdC) (Pohl & Jovin, 1972), and $\epsilon_{260} = 13\,000$ M^{-1} cm^{-1} for poly(dAdC)·poly(dGdT) (Wells et al., 1970). DNA solutions were prepared in 0.01 M sodium phosphate, pH 7.0, and 0.001 M EDTA[1] buffer with added sodium chloride concentrations as specified under Results and Discussion.

Methods

Single Cell Partition Analysis System. The interactions of adriamycin and daunorubin with various DNAs were examined by utilizing the "single cell partition analysis" technique. This method allows binding isotherms to be obtained by three independent measurements from a single experiment, thus allowing a direct comparison of the data obtained by fluorescence quenching, single cell partition analysis, and direct phase partition analysis. With this method, a series of fluorometer cells are used, each representing a single binding point on the isotherm. This feature allows such drugs as adriamycin and daunorubicin, which tend to adsorb to glass and plastic, to be examined with minimal error generated by pipetting. The drug concentrations in both phases were determined by measuring calibration curves in which the fluorescence intensity was plotted as a function of the antibiotic concentration in the absence of DNA; these plots were linear over the concentration range used. The calibration plots were measured for the aqueous phase, the aqueous–Me_2SO mixture (1:1 v/v), and the organic phase. The single cell partition analysis method requires a modification of the spectrofluorometer cell holder such that measurements of both the aqueous and organic phases can be obtained independently, by adjusting the height of the cell in the turret. This system allows both the bound and free drug concentrations to be determined by direct measurements, thus allowing accurate binding isotherms to be obtained at very low r values. A more detailed discussion of the single cell partition technique is presented in a separate paper (Graves & Krugh, 1983).

The aqueous phase and the organic phase were preequilibrated with each other prior to the start of the experiments. Samples consisting of equal volumes of the aqueous phase containing drug and DNA and an equal volume of an appropriate organic solvent were equilibrated in a 1-cm Teflon-stoppered fluorometer cell (type 23, Savant Cells, Inc.) by shaking the cells for 2 h on a mechanical side-arm shaker (New Brunswick). The organic solvents used were chloroform, 1-pentanol, 1-octanol, or 1-nonanol. Equilibration was achieved under subdued light conditions at 22 °C. Utilization of fluorometer cells as equilibration chambers was essential due to the glass-adsorptive properties of these drugs (Schultz et al., 1979; Tomilson & Malspeis, 1982). After equilibration, the phases were separated by low-speed centrifugation, and the organic and aqueous phase drug concentrations were determined by direct spectrofluorometric measurements. Aqueous phase concentrations were determined by fluorescence measurement both before and after dissociation of the bound complex with Me_2SO.

Spectrofluorometric Titration. Measurements of the fluorescence quenching of adriamycin and daunorubicin with various DNAs were performed prior to addition of the organic solvent. The fluorescence was measured on a Perkin-Elmer MPF-44A spectrofluorometer equipped with a Haake constant temperature bath maintained at 22 °C. The excitation and emission wavelengths were 475 and 595 nm, respectively. Binding isotherms were obtained by using a series of fluorometer cells, calculating each point from an individual cell, rather than as serial additions of drug or DNA to a single cell. This technique allowed the drug–DNA solution to be used as the aqueous phase in the subsequent "single cell phase partition" method (described above), thus providing data for comparing the two different techniques.

Data Analysis. Binding isotherms obtained by the fluorescence titration method were calculated by using standard fluorescence analysis [see LePecq & Paoletti (1967) and Blake & Peacocke (1968)]. The single cell partition analysis utilized the fluorescence quenching of the drug upon binding to determine the bound drug concentration (C_b); the free drug concentration (C_f) was measured directly from the organic phase as described below. Phase partition data were

[1] Abbreviations: Me_2SO, dimethyl sulfoxide; DHAQ, 1,4-dihydroxy-5,8-bis[[2-[(2-hydroxyethyl)amino]ethyl]amino]-9,10-anthracenedione; EDTA, ethylenediaminetetraacetic acid.

Table I: Equilibrium Binding Parameters for the Interaction of Adriamycin and Daunorubicin with DNA as Derived from the Allosteric Binding Model of Dattagupta et al. (1980)

DNA	ionic strength	K_1 (M^{-1})	K_2 (M^{-1})	K_2/K_1	S^a	σ^b	n_1	n_2
			Adriamycin					
calf thymus	1.012		5.5×10^5 [c]					3[c]
calf thymus	0.212	4.5×10^5	2.2×10^6	5	0.985	0.005	17	3
calf thymus	0.112	4.0×10^5	3.2×10^6	8	0.985	0.007	17	3
calf thymus	0.022	1.3×10^7	1.3×10^7	1	0.999	0.010	3	3
poly(dAdT)	0.112	9.2×10^5	4.6×10^6	5	0.994	0.010	20	3
poly(dGdC)	0.112	1.9×10^6	3.7×10^6	2	0.997	0.004	17	3
poly(dAdC)·poly(dGdT)	0.112	4.0×10^5	3.2×10^6	8	0.985	0.003	17	2
			3.4×10^6 [c]					2.5[c]
			Daunorubicin					
calf thymus	1.012		2.3×10^5 [c]					3[c]
calf thymus	0.195	1.9×10^5	5.7×10^5	3	0.987	0.007	17	3
			7.8×10^5 [c]					
calf thymus	0.112	5.8×10^5	2.2×10^6	4	0.987	0.007	17	3
calf thymus	0.062	3.3×10^6	4.2×10^6	1.5	0.995	0.007	17	3
calf thymus	0.022	1.0×10^7	8.8×10^6	0.95	0.999	0.01	3	3
poly(dAdT)	0.112	1.6×10^6	3.2×10^6	2	0.993	0.007	17	3
poly(dGdC)	0.112	1.6×10^6	2.4×10^6	1.4	0.997	0.008	20	4

[a] S is defined as the equilibrium constant for the conversion of a base pair at the interface between form 1 and form 2 DNA. [b] The σ term is a reflection on the energy required to convert a base pair from form 1 to form 2 DNA. The overall equilibrium constant in the conversion of form 1 to form 2 DNA is $\sigma^2 S$ [see Dattagupta et al. (1980) for a complete explanation of terms]. [c] Denotes binding data obtained by the neighbor exclusion model (Crothers, 1968; McGhee & von Hippel, 1974).

used to calculate "directly" the C_b and C_f values. Organic and aqueous phase drug concentrations were determined from the standard concentration curves with direct spectrophotometric measurements. Free drug concentrations were then determined by dividing the concentration of the organic phase by the partition coefficient. Aqueous phase drug concentrations were determined by dissociating the bound drug with a 1:1 (v/v) Me_2SO addition, followed by spectrofluorometric determination of the total drug concentration. Subtracting the free drug concentration from the total drug concentration yields the concentration of bound drug.

Data obtained by fluorescence titrations and phase partition techniques were plotted as r/C_f vs. r (Scatchard, 1949). Theoretical curves, shown as the solid lines through the binding isotherms, were generated as the best fit of the experimental data by using two cooperative binding models. The "two-site" binding model of L. S. Rosenberg and T. R. Krugh (unpublished results) is used to describe the cooperative binding observed in Figure 2. This model assumes two distinct types of binding sites on the DNA; one set of sites binds the drug cooperativity, while the other set of sites binds the drug in a noncooperative manner. Individual parameters for the two types of binding (i.e., K_1 and K_2, binding constants; n_1 and n_2, site exclusion parameters; ω_1 and ω_2, cooperativity parameters) are used to generate composite-calculated binding isotherms. These parameters are varied to provide our estimates of the best match between the experimental and calculated binding isotherms as noted in the legend of Figure 2. An alternative model used to examine the cooperative binding is the allosteric model which was initially used by Crothers and co-workers (Dattagupta et al., 1980) to characterize the binding of distamycin to DNA. According to the allosteric binding model, DNA may exist in two structural forms designated form 1 and form 2. The values designated as K_2 and n_2 reflect the binding parameters of the drugs to form 2 DNA and are comparable to those obtained by the neighbor exclusion model. The equilibrium constant for the binding of the drug to form 1 DNA is K_1 and corresponds to the intercept on the r/C_f axis. Qualitative measurements of the degree of cooperativity can be obtained by comparing the K_2/K_1 ratios; a K_2/K_1 ratio of 1 corresponds to no positive cooperativity while a K_2/K_1 ratio greater than 1 reflects positive cooperativity. Parameters obtained from an analysis of the data by using the allosteric binding model are given in Table I.

Results

Cooperative Binding of Adriamycin and Daunorubicin to Nucleic Acids. The binding isotherms obtained for the interactions of adriamycin and daunorubicin with calf thymus DNA at 0.1 M sodium chloride are shown in Figure 2. These plots extend previously reported binding information (Gabbay et al., 1976; Tsou & Yip, 1976; DuVernay et al., 1979; Schultz et al., 1979; Zunino et al., 1980; Pachter et al., 1982) to very low r values ($r \leq 0.01$ concentration of bound drug per base pairs), thus providing a novel examination of the binding properties of these anticancer antibiotics under conditions comparable to physiological drug levels. Clearly, both drugs show a cooperative binding process as illustrated by the positive slope in the low r region of the binding isotherm. The curves reach a maximum at a value of $r \approx 0.04$. A decreasing slope is observed at higher r values, consistent with the neighbor exclusion model for drug binding. The smooth line drawn through the data in Figure 2 represents a "best fit" obtained by using the two-site model of L. S. Rosenberg and T. R. Krugh (unpublished results). The data above $r = 0.04$ show adriamycin to have a higher binding affinity than daunorubicin, which is consistent with previous results (Zunino et al., 1980; Schultz et al., 1979). Adriamycin exhibits a much greater degree of positive cooperativity, as illustrated both by the shapes of the binding isotherms (Figure 2) and by the cooperativity parameter, ω_1, of 100 for adriamycin as compared to an ω_1 value of 38 for daunorubicin. Both drugs show n_1 and n_2 values of 3 (base pair per drug), respectively. This n_2 value is in agreement with previously reported values ($1/n_2$) of 0.30 to 0.36 obtained at comparable salt conditions (Gabbay et al., 1976; DuVernay et al., 1979; Zunino et al., 1980; Chaires et al., 1982).

Figure 3 illustrates the binding of daunorubicin to calf thymus DNA at 0.185 M sodium chloride. Binding isotherms were obtained by the single cell partition analysis method which allows a comparison of data obtained by fluorescence quenching and the phase partition systems. Binding isotherms

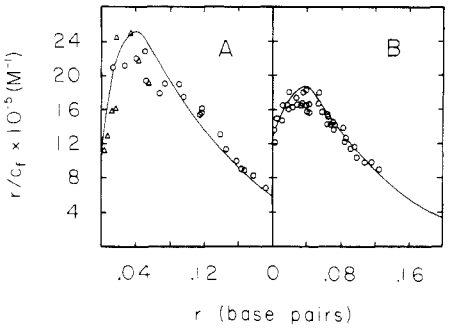

FIGURE 2: Scatchard plots of the binding of (A) adriamycin and (B) daunorubicin to calf thymus DNA in phosphate buffer plus 0.1 M sodium chloride. Binding isotherms were obtained by direct phase partition analysis. The aqueous phase (equilibrated with the appropriate organic solvent) was added to individual fluorometer cells. Additional volumes of drug and/or DNA were added to each cell. The cells without DNA present were used in standard curve and partition coefficient calculations. The final volume in the aqueous phase was 1.0 mL. An equal volume of the organic solvent aqueous buffer saturated) was added to each cell. Equilibration between the phases were achieved on a side-arm shaker at 22 °C in the dark. After equilibration, the phases were separated by centrifugation at 2000 rpm for 2 min, and the fluorescence intensity of the organic phase was measured. Subsequently, the organic phase was removed by aspiration. An equal volume of Me_2SO was added to the remaining 1-mL aqueous phase, allowing the fluorescence intensity of the drug in the aqueous phase to be determined. Open circles (○) and triangles (△) represent phase partition data with 1-nonanol and chloroform as the organic solvents, respectively. The aqueous buffer consists of 0.01 M sodium phosphate, pH 7.0, 0.001 M Na_2EDTA, and 0.1 M sodium chloride. Drug concentrations ranged from 0.1 to 0.7 micromolar while DNA ranged from 0.15 to 150 micromolar. Fluorescence emission was measured at 595 nm with excitation at 475 nm. Slit widths for both the excitation and emission were 20 nm. Parameters used to provide the "best fit" for the "two-site" binding model of adriamycin (A) include K_1 equal to 7×10^5 M^{-1} and K_2 equal to 2.2×10^6 M^{-1} with n_1 and n_2 values of 3 and 2.75, respectively. The cooperativity parameters ω_1 and ω_2 are 100 and 0.998. The daunorubicin binding isotherm (B) is best fit with the parameters K_1 and K_2 equal to 1.5×10^6 M^{-1}, n_1 and n_2 values of 3, and ω_1 and ω_2 equal to 20 and 0.995, respectively.

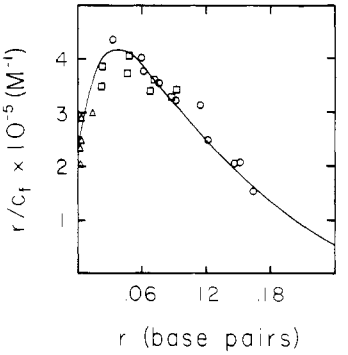

FIGURE 3: Scatchard plot for the binding of daunorubicin to calf thymus DNA using the single cell partition analysis technique. Aqueous buffer (0.006 M Na_2HPO_4–0.002 M NaH_2PO_4, pH 7.0; 0.002 M Na_2EDTA; 0.185 M NaCl) equilibrated with organic solvent was added to each fluorometer cell. Appropriate volumes of buffer, DNA, and drug were added to each cell such that the total volume in the aqueous phase was 1.0 mL. The cells which contained no DNA were used to calculate the standard curves and partition coefficients. The fluorescence intensity measurements obtained prior to addition of the organic solvent were used to determine the binding data based upon the fluorescence quenching technique. The single cell phase partition technique required an equal volume of organic solvent (equilibrated with buffer) to be added to each cell, and the phases were equilibrated with a side-arm shaker. Phases were then separated by centrifugation (2000 rpm for 2 min). The cells were positioned on the modified cell holder such that the organic and aqueous phases could be measured independently. Fluorescence intensities obtained from the aqueous phase were used to calculate Cb by the fluorescence quenching technique. After the fluorescence intensity was measured in each phase, the cells were opened, and the organic phase was carefully removed. An equal volume of Me_2SO was added to the aqueous phase to effectively dissociate the bound drug complex from the DNA in the aqueous phase. Final fluorescence intensity measurements were then used to calculate the drug concentration in the aqueous phase. Data were calculated according to the methods described in the text. Data obtained by the fluorescence quenching technique are represented by the open circles (○); the single cell partition analysis data are shown by the open squares (□); direct phase partition analysis data are shown by open triangles (△). The organic solvents used were 1-pentanol (partition coefficient of 20) and 1-nonanol (partition coefficient of 6). The solid line drawn through the points represents the "best fit" obtained by using the allosteric binding model of Crothers and co-workers (Dattagupta et al., 1980). Parameters used to fit this curve are given in Table I.

obtained by these methods showed excellent agreement over a wide range of r values. At r values less than 0.05, however, data obtained by the fluorescence quenching method showed considerable scatter, primarily due to the lack of sensitivity in measuring the value of C_f (Deranleau, 1969). The single cell partition and direct phase partition techniques allow direct measurement of C_f, thus providing reliable data to be obtained at lower r values. These data are included to allow a direct comparison of the present results to that of Chaires et al. (1982).

Effects of Ionic Strength on the Slope of the Binding Isotherm. The data obtained for adriamycin and daunorubicin binding to calf thymus DNA at several ionic strengths are presented in Figures 4 and 5, respectively. These data show that the observation of cooperative binding (i.e., a positive slope) in the initial portion of the binding isotherm is a function of the ionic strength of the solution. Adriamycin (Figure 4) binds in a highly cooperative manner at a salt concentration of 0.1 M sodium chloride, which decreases only slightly upon increasing the salt concentration to 0.2 M. However, no evidence for an initial cooperative binding process was observed either at high salt concentrations or at very low salt concentrations (Figures 4 and 5). Analous results were observed with daunorubicin (Figure 5) where cooperative binding was observed at 0.185 and 0.1 M sodium chloride concentrations. However, at 0.05 M sodium chloride, the initial portion of the binding isotherm is relatively flat, while at 0.01 M sodium chloride, no evidence of positive cooperativity is observed. Daunorubicin exhibits a lower degree of cooperative binding than adriamycin under identical conditions.

The dependence of the equilibrium binding constant upon the ionic strength has been well established for a variety of drugs (LePecq & Paoletti, 1967; Record et al., 1978; Manning, 1978; Wilson & Lopp, 1979; Zunino et al., 1980; Chaires et al., 1982). Such studies have demonstrated that increasing the ionic strength of the buffer decreases the drugs binding affinity for DNA. Both adriamycin and daunorubicin exhibit the expected ionic strength dependence of the noncooperative equilibrium binding constant, K_2, as shown in a plot of log K_2 vs. -log [Na^+] (Figure 6). Slopes corresponding to $-m\psi$ of 0.82 and 0.88 were obtained for adriamycin and daunorubicin, respectively. When a value of 0.88 was used for the fraction of counterions released by B-DNA upon binding a charged ligand, the number of charges carried by adriamycin and daunorubicin was calculated to be 0.93 and 1.0, corresponding to a single charge carried by both drugs. We note, however, that we have not used the Manning and Record equations to analyze the initial portions of the binding isotherms where cooperative binding is observed at intermediate salt concentrations.

Effects of Base Composition on the Binding of Adriamycin and Daunorubicin to DNA. Influences exerted by both the

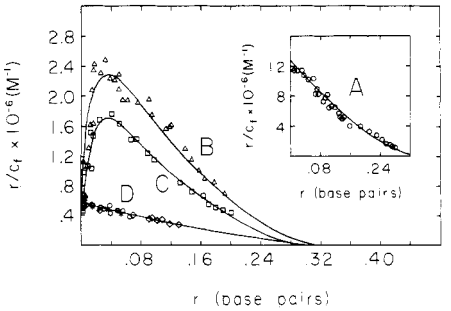

FIGURE 4: Scatchard analyses showing the effects of varying the salt concentration of the binding properties of adriamycin to calf thymus DNA. Data shown in curves labeled A, B, and C were obtained by direct phase partition analysis. Curve A corresponds to 0.01 M sodium chloride, curve B, 0.1 M sodium chloride, and curve C, 0.2 M sodium chloride. Experimental procedures and instrument parameters used to obtain this data are given in figure legend 2. Curve D was obtained by the single cell partition analysis technique (details of this procedure given in figure legend 3). The symbols represent data obtained by the three different measurements of this technique. The open circles (○) represent data obtained by the direct phase partition technique, while the data shown by asterisks were obtained by single cell partition analysis, and the diamonds (◇) represent data obtained from the fluorescence quenching method. Buffer concentrations were 0.01 M sodium phosphate, pH 7.0, and 0.001 M Na_2EDTA, and sodium chloride concentrations are varied as stated above. Parameters used to fit these data to the allosteric binding model (shown by the solid lines) are given in Table I.

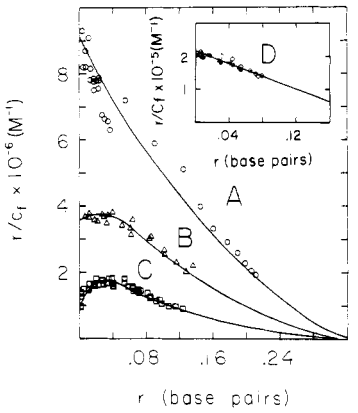

FIGURE 5: Scatchard analyses of the binding of daunorubicin to calf thymus DNA with varying sodium chloride concentrations. Data shown in curves A–C were obtained by direct phase partition techniques as described in the legend of Figure 2. Curves A, B, C, and D correspond to sodium chloride concentrations of 0.01, 0.05, 0.1 M, respectively. The binding of daunorubicin to calf thymus DNA at 1.0 M sodium chloride is shown in the inset as curve D. Binding data for (D) was obtained by using the single cell partition analysis technique. Data obtained by direct phase partition techniques are represented by the open circles (○), single cell partition analysis data are shown by the asterisks, and data obtained by fluorescence quenching are represented by the open diamonds (◇). Buffer concentrations were 0.01 M sodium phosphate, pH 7.0, and 0.001 M Na_2EDTA, with the added sodium chloride concentrations, stated above. The allosteric binding model represented by the solid lines was used to fit the binding data. Parameters are listed in Table I.

base composition and sequence on the binding of adriamycin and daunorubicin to nucleic acids have been the subject of numerous studies with some conflicting results [e.g., see the review by Neidle (1978) and references therein; Phillips et al., 1978; DuVernay et al., 1979]. In an effort to examine the effects of base composition and sequence on the binding of adriamycin and daunorubicin to DNA at low r values, the alternating copolymers poly(dAdT)·poly(dAdT), poly(dGdC)·poly(dGdC), and poly(dAdC)·poly(dGdT) were examined. Scatchard analyses for the interactions of adriamycin and daunorubicin with poly(dAdT)·poly(dAdT) and poly(dGdC)·poly(dGdC) in the buffer with 0.1 M sodium chloride are shown in Figure 7. Binding affinities (K_2 values) of 4.6 × 10^6 M^{-1} for poly(dAdT)·poly(dAdT) and 3.7 × 10^6 M^{-1} for poly(dGdC)·poly(dGdC) were obtained for adriamycin binding. Similarly, binding constants of 3.2 × 10^6 M^{-1} and 2.4 × 10^6 M^{-1} were obtained for daunorubicin binding to poly(dAdT)·poly(dAdT) and poly(dGdC)·poly(dGdC), respectively. These values are comparable to those obtained with the calf thymus DNA interactions. Both drugs, however, exhibit a larger degree of curvature in the initial portion of the binding isotherms for binding to poly(dAdT)·poly(dAdT) as compared with the poly(dGdC)·poly(dGdC). Furthermore, adriamycin exhibits a much greater degree of cooperativity with poly(dAdT)·poly(dAdT) than does daunorubicin under identical conditions. The correlation of base sequence with a cooperative binding effect could be associated with the thermodynamic properties of the helix, as for example, the stability of poly(dGdC)·poly(dGdC) as compared with poly(dAdT)·poly(dAdT) (Davies & Baldwin, 1963; Arnott et al., 1974; Pohl, 1974), or the relative enthalpy of base pair stacking in the three synthetic polynucleotides studied.

The poly(dAdC)·poly(dGdT) copolymer retains the purine–pyrimidine sequence but alternates between A·T and G·C base pairs. The average base pair stacking enthalpy of 5.6 kcal/base pair per base–base stacking for poly(dAdC)·poly(dGdT) is comparable to that for poly(dAdT)·poly(dAdT) (7.1 kcal/base pair) and compares to a ΔH of base pair stacking of 12.8 kcal/base pair for poly(dGdC)·poly(dGdC). The

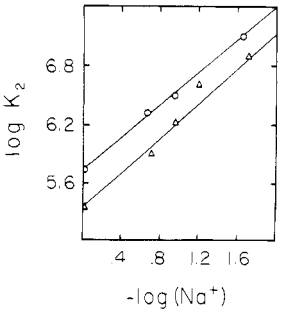

FIGURE 6: Dependence of K_2 (intrinsic equilibrium constant) of adriamycin (○) and daunorubicin (△) binding to calf thymus DNA on the ionic strength expressed and [Na^+].

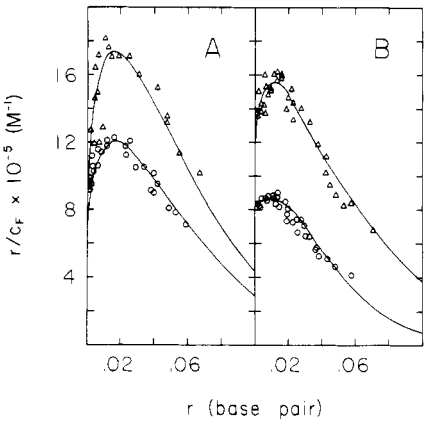

FIGURE 7: Scatchard plots of the binding data for the interaction of adriamycin (△) and daunorubicin (○) with (A) poly(dAdT)·poly(dAdT) and (B) poly(dGdC)·poly(dGdC). Binding isotherms were obtained by direct phase partition techniques as described in the legend of Figure 2. The buffer concentration was 0.01 M sodium phosphate, pH 7.0, 0.001 M Na_2EDTA, and 0.1 M sodium chloride. The solid lines drawn through the binding isotherms represent the "best fit" using the allosteric binding model. Parameters are given in Table I.

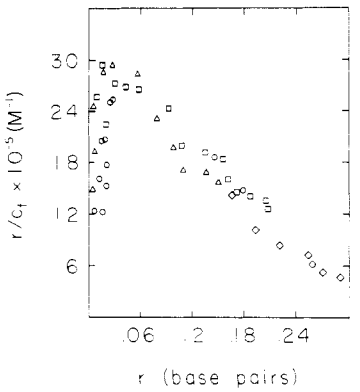

FIGURE 8: Binding isotherm of the interaction of adriamycin with the synthetic copolymer poly(dAdC)·poly(dGdT). Data were obtained by the single cell partition analysis method. The direct phase partition data are represented by the open circles and open triangles obtained with 1-nonanol (○) and 1-pentanol (△). The data obtained by the single cell partition technique are illustrated by the open squares (□). Fluorescence quenching data are shown by the open diamonds (◇).

binding isotherm for adriamycin with poly(dAdC)·poly(dGdT) in buffer plus 0.1 M sodium chloride is shown in Figure 8. This interaction is characterized at r values greater than 0.05 by the neighbor exclusion model with an intrinsic binding constant of 3.6×10^6 M^{-1} and n_2 of 2.6.

Discussion

The present studies have provided a unique insight into the interactions of adriamycin and daunorubicin with various nucleic acids. Examinations of the binding isotherms throughout the low r range have revealed that adriamycin and daunorubicin exhibit cooperative binding to both native DNA and synthetic copolymer systems. Both drugs were shown to bind cooperatively at ionic concentrations comparable to physiological levels; however, at ionic concentrations of 0.01 and 1 M NaCl, both drugs were shown to interact with DNA in a noncooperative manner. Recent studies have demonstrated that the antibiotics actinomycin D (Winkle & Krugh, 1981) and DHAQ (Rosenberg et al., 1982) bind cooperatively to various DNAs. In these experiments the degree of cooperativity appears to increase at low ionic strengths (0.01 M), thus exhibiting a salt effect markedly different from that observed for adriamycin and daunorubicin. The cooperative binding of distamycin was shown to be relatively insensitive to the ionic concentrations (Dattagupta et al., 1980). Cooperative binding of ethidium bromide to *Escherichia coli* DNA was observed throughout the range of salt concentrations studied (0.01–1 M sodium chloride) (Winkle et al., 1982).

Studies directed at examining the effects of base pair sequence on the interactions of adriamycin and daunorubicin showed that both drugs bound to poly(dAdT)·poly(dAdT) and poly(dAdC)·poly(dGdT) in a highly cooperative manner. Conversely, their binding to poly(dGdC)·poly(dGdC) showed a marked decrease in this cooperative binding effect. These results suggest that the properties of the DNA helix such as base pair stacking energies play a role in the cooperative binding phenomena.

In comparing the binding properties of adriamycin with daunorubicin, it was found that where conditions were conducive for cooperative binding to occur, adriamycin exhibited a higher degree of cooperativity than daunorubicin as evidenced by the steeper positive slopes in the initial portion of the Scatchard plots. The distinction between the effectiveness of adriamycin and daunorubicin as antitumor agents is well-known. It is interesting to speculate that the enhanced activity of adriamycin may be associated with the observation that adriamycin binds to DNA with a higher degree of cooperativity than does daunorubicin. The additional hydrogen bond formed by the C_{14} hydroxyl of adriamycin might provide enough of a stabilizing factor to the drug–nucleic acid complex to allow a more efficient allosteric process to take place along the DNA backbone. It remains to be demonstrated whether the enhanced cooperativity shown by adriamycin contributes to the drugs increased chemotherapeutic value when compared to daunorubicin. However, studies from this laboratory have shown a correlation between drugs exhibiting enhanced biological effectiveness in areas of mutagenesis, carcinogenesis, and antitumor activity and the observation of cooperative binding to DNA.

A recent report by Chaires et al. (1982) states that the binding of daunorubicin to calf thymus DNA at 0.185 M sodium chloride is noncooperative, based upon fluorescence, absorption, and equilibrium dialysis binding experiments. Their data obtained from phase partition experiments did indicate a slight degree of cooperative binding; however, due to the discrepancy of both the shape and magnitude of the binding isotherm obtained from phase partition experiments when compared with the other methods, the results from the phase partition data were concluded to be artifacts of the systems. The results presented in this report demonstrate the validity of data obtained by the phase partition analysis method and provide a comparison of data obtained simultaneously by three independent techniques: fluorescence quenching, single cell partition analysis, and direct phase partition analysis. As shown in Figure 3, the binding isotherm of daunorubicin to calf thymus DNA at 0.185 M sodium chloride demonstrates positive cooperativity. Furthermore, we note that the present data, from all three methods, agrees with the equilibrium dialysis and fluorescence quenching data of Chaires et al. (1982). The data in Figure 3 from the fluorescence quenching experiments and the two phase partition techniques show excellent agreement for r values larger than 0.05.

The phase partition system was employed because this is the only technique which will provide reliable data on the DNA binding properties of adriamycin and daunorubicin at low r values. The reproducibility of the experimental data obtained by these methods is shown to be quite good, as evidenced by the internal agreement of multiple experiments which must be used to construct each binding isotherm. By experimental design, the degree of error associated with the phase partition experiments is dictated by technique and the accuracy of the standard curve calibrations. The advantage of the phase partition method at the low r values is achieved through the direct measurement of C_f, in contrast to the classical titration technique whose difficulties in obtaining accurate C_f values at low r have been well documented [e.g., see Deranleau (1969) and Peters & Pingoud (1979)]. As additional support for the validity of these results we note that both adriamycin and daunorubicin were shown to bind calf thymus DNA in a noncooperative manner at 0.01 and 1.0 M sodium chloride, as shown in Figure 4 and 5. The combination of the results presented in this report with the previous drug–DNA binding studies of Winkle & Krugh (1981) on actinomycin D, Winkle et al. (1982) with ethidium, and Rosenberg et al. (1982) for DHAQ clearly demonstrates that the cooperative binding phenomenon are dependent upon the particular drug, the nucleic acid system, and the ionic concentration. Positive cooperative binding was observed for both adriamycin and daunorubicin only over an intermediate range of salt concentrations. Thus, the phenomenon of cooperative binding is an interesting property of the drug–DNA equilibrium whose

role in the activity of these compounds is being explored.

Acknowledgments

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Registry No. Adriamycin, 23214-92-8; daunorubicin, 20830-81-3.

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