Thermodynamic Characterization of Daunomycin–DNA Interactions: Microcalorimetric Measurements of Daunomycin–DNA Binding Enthalpies†

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ABSTRACT: We report the first direct determination of binding enthalpies for the complexation of *monomeric* daunomycin with a series of 10 polymeric DNA duplexes. These measurements were accomplished by using a recently developed stopped-flow microcalorimeter capable of detecting reaction heats on the microjoule level. This enhanced sensitivity allowed us to measure daunomycin–DNA binding enthalpies at monomeric drug concentrations (e.g., 10–20 μM), thereby precluding the need to correct for daunomycin self-association, as has been required in previous batch calorimetric studies [Remeta, D. P., Marky, L. A., & Breslauer, K. J. (1984) *Abstracts of Pittsburgh Conference and Exposition on Analytical Chemistry and Applied Spectroscopy*, 838a; Breslauer, K. J., Remeta, D. P., Chou, W. Y., Ferrante, R., Curry, J., Zaunczkowski, D., Snyder, J. G., & Marky, L. A. (1987) *Proc. Natl. Acad. Sci. U.S.A. 84*, 8922–8926]. We correct the published daunomycin–DNA binding enthalpies measured by batch calorimetry at higher drug concentrations (e.g., 0.5–1.0 M) for the enthalpy contribution associated with the binding-induced disruption of drug aggregates. The requisite correction term was obtained from a van't Hoff analysis of temperature-dependent NMR measurements on daunomycin solutions. We find remarkable agreement between the net binding enthalpies derived from these corrected batch calorimetric data and the corresponding binding enthalpies measured directly by stopped-flow microcalorimetry. The enhanced sensitivity of the stopped-flow instrument also allowed us to evaluate the influence of drug binding density on the daunomycin–DNA binding enthalpies. This assessment was accomplished by conducting stopped-flow calorimetric measurements over a range of seven different drug-to-phosphate ratios (r). For most of the 10 DNA host duplexes studied, we find that the daunomycin binding enthalpies exhibit small but significant r dependencies. The sensitivity of the stopped-flow instrument also enabled us to detect significant dilution enthalpies for several of the drug-free DNA duplexes, a quantity generally assumed to be negligible in previous studies. We discuss the binding enthalpies, their dependence on binding density, and the duplex dilution enthalpies in terms of the influence of base composition, sequence, conformation/hydration, and binding cooperativity on the sign and the magnitudes of the daunomycin–DNA binding enthalpy data reported here.

Advances in solution NMR (Van de Ven & Hilbers, 1988, and references cited therein) and X-ray crystallographic techniques (Wang, 1987, and references cited therein), coupled with the development of rapid and efficient methods of oligonucleotide synthesis (Miller, 1990, and references cited therein), have resulted in the elucidation of an increasing number of DNA structures in their free and drug-bound states. These structures are beginning to allow us to define the intra- and intermolecular interactions that drive the formation of DNA structures and their drug complexes. However, as has been shown, a more complete picture emerges when parallel thermodynamic studies also are conducted (Marky & Breslauer, 1987). The results of such studies allow one to characterize the nature of the forces that drive complexation, as well as to define the stability and temperature-dependent melting properties of the DNA structures in their free and drug-bound states. In short, parallel macroscopic (e.g., thermodynamic) and microscopic (e.g., X-ray, NMR) studies on DNA duplexes and their drug complexes yield insights that neither approach alone could provide. In recognition of this advantage, we have pursued a program in which a combination of spectroscopic and calorimetric techniques have been used to characterize thermodynamically the forces that stabilize DNA structures and their drug complexes, with particular emphasis on those systems for which structural data exist (Marky & Breslauer, 1987; Breslauer et al., 1987; Senior et al., 1988; Lee et al., 1989a,b; Snyder et al., 1989; Vesnaver et al., 1989).

Most previous efforts to obtain thermodynamic data for drug–DNA complexes have focused primarily on the determination of binding constants, K, and their associated binding free energies, $\Delta G°$. However, we have shown that enthalpy–entropy compensations can result in nearly identical $\Delta G°$ values for drug binding events that are driven by entirely different molecular forces (Breslauer et al., 1987). Thus, $\Delta G°$ data alone do not provide an adequate window for macroscopically characterizing the forces that control the affinity and specificity of drug–DNA interactions. Such a characterization requires the corresponding binding enthalpy, $\Delta H°$, entropy, $\Delta S°$, and heat capacity, ΔC_p, data. Until recently, such data have been rather limited due, in part, to problems associated with the use of model-dependent van't Hoff analyses

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to extract indirectly from temperature-dependent studies the desired thermodynamic data. For the case of daunomycin binding to calf thymus DNA, this problem is dramatically illustrated by the disparity in the enthalpy and entropy data reported in the literature (Huang & Phillips, 1977; Chaires et al., 1982b). Specifically, Huang and Phillips measured a binding enthalpy of −5.3 kcal/mol and a binding entropy of 11.1 cal/(mol·deg), whereas, under nearly identical conditions, the corresponding enthalpy and entropy values reported by Chaires and co-workers are −12.8 kcal/mol and −16.2 cal/(mol·deg). To address such disparities, we have pursued a program in which isothermal batch calorimetry has been used to measure directly the desired binding enthalpies (Marky et al., 1983a,b, 1985; Remeta et al., 1984; Snyder et al., 1984, 1989; Snyder, 1985; Marky & Breslauer, 1987; Chou et al., 1987; Breslauer et al., 1987; Vesnaver et al., 1989; Lee et al., 1989a,b; Jin & Breslauer, 1988; Remeta, 1990). These $\Delta H°$ values then have been used in conjunction with binding free energies, $\Delta G°$, derived either spectroscopically or calorimetrically, to calculate binding entropies, $\Delta S°$. This approach has been quite successful for many of the ligands we have studied to date. However, for drugs that strongly self-associate, DNA binding enthalpies measured by conventional batch calorimetry require a significant correction term for drug dissociation, thereby potentially compromising the final binding enthalpy data (Marky et al., 1983b; Breslauer et al., 1987). Two drugs of immediate interest to us that exhibit such behavior are daunomycin and actinomycin D, although many other drugs also self-associate to varying degrees, depending on the specific solution conditions.

In this paper, we describe how a recently developed stopped-flow microcalorimeter with enhanced sensitivity (a detection limit of ±2.0 μJ) (Mudd & Berger, 1988) was used to measure directly daunomycin–DNA binding enthalpies at monomeric drug concentrations (~10–20 μM), thereby precluding, for the first time, the need to correct for daunomycin self-association (Remeta et al., 1988, 1991; Remeta, 1990). We compare our stopped-flow microcalorimetric data with the corresponding binding enthalpies measured previously by batch calorimetric techniques (Remeta et al., 1984; Breslauer et al., 1987). We correct the latter enthalpy values for binding-induced disruption of aggregated drug species using a correction term derived from temperature-dependent NMR measurements. Comparison of the corrected batch calorimetric data with the directly measured stopped-flow microcalorimetric data reveals excellent agreement. The resultant enthalpy data are discussed in terms of the influence of base composition and sequence, as well as duplex conformation and hydration, on the sign and relative magnitudes of the daunomycin–DNA binding enthalpies at 25 °C. The enhanced sensitivity of the stopped-flow calorimeter also enabled us to conduct measurements over a range of drug-to-DNA phosphate ratios (r), thereby allowing us to evaluate, for the first time, the influence of drug binding densities on the daunomycin binding enthalpies. These data are discussed in terms of various binding models, including binding cooperativity.

Experimental Procedures

Materials

Deoxypolynucleotides. The synthetic deoxypolynucleotides employed in this study were obtained from PL Biochemicals (Piscataway, NJ). These DNA polymers were used without further purification and dissolved directly in the 10 mM sodium phosphate buffer system described below. The concentrations of the DNA standard solutions were determined spectrophotometrically at the indicated subscripted wavelengths (nm) by using the following molar extinction coefficients, ϵ ($M^{-1}\cdot cm^{-1}$), expressed in terms of DNA phosphates: ϵ_{260} of poly[d(AT)]·poly[d(AT)] = 6650; ϵ_{260} of poly[d(A)]·poly[d(T)] = 6000; ϵ_{254} of poly[d(GC)]·poly[d(GC)] = 8400; ϵ_{253} of poly [d(G)]·poly[d(C)] = 7400; ϵ_{258} of poly [d(AC)]·poly[d(GT)] = 6500; ϵ_{258} of poly[d(AG)]·poly[d(CT)] = 5700; ϵ_{260} of poly[d(AU)]·poly[d(AU)] = 6900; ϵ_{257} of poly[d(A)]·poly[d(U)] = 6330; ϵ_{251} of poly[d(IC)]·poly[d(IC)] = 6900; and ϵ_{260} of salmon testes DNA = 6550.

Daunomycin. The hydrochloride salt of daunomycin was obtained from Sigma Chemical Co. (Milwaukee, WI). Standard solutions of the drug were prepared and used on a daily basis to avoid photodegradation. Concentrations were determined spectrophotometrically by measuring the absorbance of the monomer at 477 nm using a molar extinction coefficient of 11 500 $M^{-1}\cdot cm^{-1}$. The purity of the daunomycin standards was monitored by thin-layer chromatography. Nuclear magnetic resonance spectra revealed no evidence of drug degradation.

Buffer System. Standard solutions of the DNA polymers and daunomycin were prepared in a buffer consisting of 3.9 mM monobasic sodium phosphate, 6.1 mM dibasic sodium phosphate, and 1.0 mM EDTA. These buffer solutions typically fell within the pH range of 6.95–6.98, requiring minimal adjustment to pH 7.00 by the addition of microliter aliquots of 1.0 N NaOH. The total sodium ion concentration of the final buffer system was calculated to be 15 mM.

Methods

Stopped-Flow Microcalorimetry. Daunomycin–DNA binding enthalpies were measured using an all tantalum, differential, stopped-flow, heat conduction microcalorimeter (model DSFC-100, Commonwealth Technology, Inc., Alexandria, VA), which was developed at the National Institutes of Health (Mudd & Berger, 1986, 1988; Mudd et al., 1988). This instrument was designed specifically to surmount limitations normally encountered in conventional batch microcalorimeters by optimizing the effective differential sensitivity and maximizing sample throughput. In this regard, the stopped-flow microcalorimeter is nearly two orders of magnitude more sensitive than the batch calorimeter employed in previous drug–DNA binding studies (Remeta et al., 1984; Breslauer et al., 1987). The measured sensitivity of 1.595 J/(V·s) is achieved primarily through use of a differential measurement scheme and optimization of the common mode rejection ratio. The enhanced sensitivity of the stopped-flow microcalorimeter relative to its batch counterpart permits significant reductions in the total reaction volume (i.e., 160 versus 1000 μL), limiting reagent concentration (i.e., 20 versus 500 μM), and total run time (i.e., 200 versus 1800 s). Furthermore, minimization of the reequilibration time between sample sets affords a throughput of 120–150 runs per day, compared with 3–4 runs typically achievable with conventional batch microcalorimeters. One of the unique features of the microcalorimeter is its differential measurement scheme that incorporates the use of sample and reference mixing chambers to monitor *net* reaction enthalpies. The literature contains a complete description of the stopped-flow microcalorimeter system, including an extensive discussion of design specifications and operational considerations (Mudd et al., 1988), as well as applications to drug–DNA binding studies (Breslauer et al., 1991).

Following an equilibration period of 30 min or less, the reaction is initiated by a microprocessor-controlled stepping motor that activates a syringe drive which delivers, within 0.6

Table I: Chemical Calibration of the Stopped-Flow Microcalorimeter Employed for the Determination of Daunomycin–DNA Binding Enthalpies at 25.0 °C

chemical reaction	conc of reactants (m)	measured response (μV·s)	reaction enthalpy (μJ/mol)	microcalorimeter calibration const [J/(V·s)]
hydrochloric acid dilution	0.010 → 0.005	−26.10 ± 1.44	−41.78[a]	1.601 ± 0.088
sodium chloride dilution	0.010 → 0.005	−21.59 ± 1.41	−36.65[b]	1.697 ± 0.110
		−21.59 ± 1.41	−32.31[c]	1.496 ± 0.097
sucrose dilution	0.040 → 0.020	−22.40 ± 1.61	−35.55[d]	1.587 ± 0.114

[a] Weast (1976). [b] Robinson (1932). [c] Gulbransen and Robinson (1934). [d] Gucker et al. (1939).

s, precisely 80 μL of each reagent into tantalum mixing chambers. Heat sensors containing thermopile assemblies surround each mixing chamber and detect the reaction heat that is either liberated or absorbed. The heat dissipated is directly proportional to the output voltage of the thermopiles:

$$dQ/dT = kV$$

where dQ/dT is the rate of heat either liberated or absorbed by the mixing chamber contents, V is the thermopile output voltage, and k is a proportionality constant. In the differential measurement scheme, the total reaction heat evolved (Q_T) is obtained by subtracting the measured heat response of the reference chamber from that of the sample chamber, and integrating the area under the resultant voltage versus time curve:

$$Q_T = k(\text{area})$$

The sensitivity of the instrument is determined from the magnitude of the proportionality constant (k), which may be evaluated by generating a known amount of heat and measuring the area under the calibration curve, as described below.

Chemical Calibration of Stopped-Flow Microcalorimeter. The stopped-flow microcalorimeter was calibrated initially by applying an electrical pulse through heaters situated in the calorimeter block proximate to the mixing chambers, yielding an overall sensitivity of 1.56 J/(V·s). Since chemical calibration provides a more realistic evaluation of the instrument's effective usable sensitivity, several standard chemical reactions were selected for measuring the actual microcalorimeter constant. These included the 1:1 dilutions of standard solutions of sucrose (i.e., 0.040 m → 0.020 m), hydrochloric acid (i.e., 0.010 m → 0.005 m), and sodium chloride (i.e., 0.010 m → 0.005 m). In conjunction with established literature values, these measured dilution enthalpies were used to calculate a calibration constant for the instrument. Inspection of the relevant data in Table I reveals a discrepancy between the two sodium chloride reaction enthalpies reported in the literature. Specifically, dilution enthalpies of −36.65 μJ/mol (Robinson, 1932) and −32.31 μJ/mol (Gulbransen & Robinson, 1934) were determined for the 1:1 dilution of an 0.010 m sodium chloride standard. From our measured heat response of 21.59 μV·s for this standard dilution, values of 1.697 and 1.496 J/(V·s) were calculated for the stopped-flow microcalorimeter constant in accordance with the different reaction enthalpies. In the absence of additional data to provide a more definitive determination of the actual reaction enthalpy for the sodium chloride dilution, we elected to average these two calibration constants and compare the resultant value [i.e., 1.596 J/(V·s)] with that obtained for the dilution of hydrochloric acid [i.e., 1.601 J/(V·s)] and the dilution of sucrose [i.e., 1.587 J/(V·s)]. Utilizing these three values, an average microcalorimeter constant of 1.595 J/(V·s) was employed in all subsequent calculations of daunomycin–DNA binding enthalpies. It should be noted that this value is in close agreement with that of 1.600 J/(V·s) determined previously from multiple chemical calibrations of the instrument (Mudd et al., 1988).

Measurement of Daunomycin–DNA Binding Enthalpies. The microjoule detection capabilities of the stopped-flow microcalorimeter enabled direct determination of daunomycin–DNA binding enthalpies at extremely dilute drug concentrations. Since the self-association of daunomycin is well documented (Barthalemy-Clavey et al., 1973, 1974; Eksborg, 1978; Schutz et al., 1979; Martin, 1980; Chaires et al., 1982a), a combination of UV/Vis spectrophotometric and NMR techniques were employed to evaluate the extent of drug aggregation under the conditions of our calorimetric studies. Analysis of the spectroscopic data revealed drug aggregation to be negligible for daunomycin concentrations of 50 μM or less under the low ionic strength conditions used in these studies (Remeta, 1990). On the basis of the sensitivity characteristics of the stopped-flow microcalorimeter and the desire to determine binding enthalpies in the absence of drug aggregation effects, all of the drug–DNA reactions reported here were conducted at a daunomycin concentration of 20 μM.

For each of the 10 daunomycin–DNA complexation reactions investigated, eight sets of runs were conducted with a minimum of 25 usable measurements per set. The first of the eight sets was essentially a control for that series of drug–DNA experiments and consisted of identical 1:1 dilutions of a 20 μM daunomycin standard in both the sample and reference mixing chambers. The concentration of the DNA standard (i.e., 100 μM) for the second set of runs was selected to yield the highest drug–DNA binding density (i.e., one drug per five phosphate residues). Since the daunomycin standard concentration remained constant throughout the series of experiments, the concentration of DNA was increased in subsequent sets (i.e., 140, 200, 280, 400, 800 μM) until the lowest drug–DNA binding density (i.e., 1 drug per 80 phosphate residues) was achieved at a deoxypolynucleotide concentration of 1.6 mM. This protocol of increasing the DNA concentration during a single series of drug–DNA mixes ensured that any residual or excess DNA remaining in the tantalum tubing and/or mixing chamber was adequately absorbed by the next DNA standard of higher concentration without appreciably altering the concentration of that standard.

Binding enthalpies were measured at seven different drug binding densities for daunomycin complexation to the duplexes formed by six alternating copolymers, three homopolymers, and one native DNA sequence. The resulting binding enthalpies listed in Table II are expressed as the mean value and standard deviation for a basis set of 25 measurements conducted at each of the specified drug:phosphate ratios, corresponding to a total of 1750 individual determinations. The binding enthalpies were calculated according to

$$\Delta H_B = (KQ)/(VC_B)$$

where K is the calorimeter calibration constant [i.e., 1.595 J/(V·s)], Q is the measured heat response in μV·s, V is the volume of limiting reagent (i.e., 80 μL), and C_B is the concentration of *bound* daunomycin. It is important to note that the concentration of the limiting reagent employed in deriving

Table II: Summary of Daunomycin–DNA Binding Enthalpies Reported as the Mean Value and Standard Deviation for Several Drug:Phosphate Ratios at 25.0 °C[a]

DNA host polymer	daunomycin–DNA binding enthalpies (kcal/mol) measured as a function of the drug:phosphate (D:P) ratio						
	1:80	1:40	1:20	1:14	1:10	1:7	1:5
poly[d(AT)]·poly[d(AT)]	−6.18 ± 0.26	−6.08 ± 0.28	−5.94 ± 0.28	−5.95 ± 0.33	−5.54 ± 0.30	−5.30 ± 0.37	−5.11 ± 0.30
poly[d(A)]·poly[d(T)]	+3.97 ± 0.38[b]	+3.54 ± 0.26[b]	+2.53 ± 0.21[b]	+2.15 ± 0.31	+1.94 ± 0.19	+1.50 ± 0.28	+1.45 ± 0.40
poly[d(GC)]·poly[d(GC)]	−9.40 ± 0.35	−8.65 ± 0.32	−8.27 ± 0.38	−8.19 ± 0.40	−7.98 ± 0.38	−7.95 ± 0.23	−7.76 ± 0.37
poly[d(G)]·poly[d(C)]	−7.10 ± 0.36	−6.80 ± 0.32	−6.68 ± 0.37	−6.52 ± 0.32	−6.46 ± 0.46	−6.31 ± 0.34	−6.24 ± 0.38
poly[d(AC)]·poly[d(GT)]	−10.76 ± 0.40	−10.64 ± 0.30	−10.17 ± 0.38	−9.67 ± 0.39	−9.45 ± 0.45	−8.83 ± 0.39	−8.42 ± 0.37
poly[d(AG)]·poly[d(CT)]	−5.87 ± 0.30	−5.78 ± 0.27	−4.90 ± 0.30	−4.30 ± 0.18	−4.14 ± 0.10	−3.94 ± 0.18	−3.89 ± 0.21
poly[d(AU)]·poly[d(AU)]	−6.31 ± 0.27	−5.98 ± 0.29	−5.57 ± 0.30	−5.50 ± 0.32	−5.18 ± 0.31	−5.14 ± 0.30	−4.81 ± 0.24
poly[d(A)]·poly[d(U)]	+4.11 ± 0.30[b]	+3.64 ± 0.26[b]	+2.69 ± 0.23[b]	+2.27 ± 0.21	+2.11 ± 0.24	+1.56 ± 0.34	+1.00 ± 0.32
poly[d(IC)]·poly[d(IC)]	−6.86 ± 0.41	−6.79 ± 0.48	−6.72 ± 0.40	−6.49 ± 0.29	−6.48 ± 0.32	−6.44 ± 0.30	−6.36 ± 0.29
salmon testes DNA	−9.52 ± 0.39	−9.24 ± 0.36	−9.15 ± 0.32	−8.57 ± 0.34	−8.23 ± 0.37	−7.55 ± 0.34	−6.99 ± 0.34

[a] Daunomycin–DNA binding enthalpies are corrected for the amount of *bound* drug per DNA base pair at each of the specified drug:phosphate ratios. [b] Daunomycin–DNA binding enthalpies corrected for dilution of the host polymer.

the daunomycin–DNA binding enthalpies is that of the *bound* drug (C_B) as opposed to the *total* drug (C_T). These concentrations are not equivalent (e.g., $C_B < C_T$) due to the *fractional* saturation of available binding sites in the DNA duplex, thereby resulting in the presence of *free* drug (C_F) species in solution. This difference is magnified in the presence of multiple binding equilibria, cooperative ligand–ligand interactions, and at higher drug–DNA binding densities. Since the ratio r is defined as the moles of *bound* daunomycin per mole of DNA base pair, it is tempting to tabulate the binding enthalpies accordingly. However, the binding affinity of daunomycin varies considerably among these DNA host duplexes. Thus, measurements conducted at each of the seven drug–DNA binding densities correspond to different r values. Consequently, we have adopted the convention of reporting the binding enthalpies as a function of the actual daunomycin (D) to phosphate (P) ratio expressed in terms of the total molar concentrations of the two reagents. Nevertheless, it must be emphasized that these binding enthalpies are corrected for the concentration of *bound* daunomycin at each of the D:P ratios, as deduced from equilibrium binding data.

Isothermal Batch Calorimetry. Previous studies have employed the use of an isothermal batch calorimeter (model DBC-100, Commonwealth Technology, Inc., Alexandria, VA) to characterize the binding of daunomycin to poly[d(AT)]·poly[d(AT)], poly[d(GC)]·poly[d(GC)], poly[d(A)]·poly[d(T)], and salmon testes DNA (Remeta et al., 1984; Breslauer et al., 1987; Remeta, 1990). Compared with conventional batch calorimeters (Pennington & Brown, 1969; Prosen, 1973), this instrument was modified to accommodate larger reagent volumes (i.e., 1000 versus 200 μL), thereby affording an overall improvement in terms of sensitivity (Mudd et al., 1982). In contrast with the reagent delivery system in the stopped-flow microcalorimeter, the mixing chamber of the batch calorimeter consists of a bicompartment cell that requires manual loading of each reagent with a calibrated micropipet. The reaction cells are manufactured from Kel F, a translucent and highly inert plastic material. The interior faces of each cell are coated with a thin layer of tantalum to augment their chemical resistivity characteristics and to enhance mixing. Although the mixing chambers are capable of holding 1000 μL of reagent on each side of the bicompartment cell, optimal performance is achieved when limiting the individual reactant volumes to 500 μL. Following reagent loading, both sample and reference cells are inserted into their respective compartments within the calorimeter block, allowed to equilibrate (~2 h), and manually rotated to initiate mixing of the reactants. The resultant reaction heats are detected by thermopiles that are strategically situated on the cell compartment faces.

Nuclear Magnetic Resonance Spectroscopy. The self-association of daunomycin was monitored by NMR spectroscopy to determine both an equilibrium constant and a van't Hoff enthalpy for drug aggregation. Derivation of these parameters was essential since published batch calorimetric measurements of the daunomycin–DNA binding enthalpies were conducted at reagent concentrations requiring correction of the reported values for disruption of aggregated drug species. Proton NMR experiments were performed on a Varian XL-400 spectrometer equipped with a 32-bit microprocessor for data acquisition and analysis. One-dimensional NMR spectra were recorded over a sweep width of 4000 Hz using a standard two-pulse sequence and typical acquisition parameters. Constant temperature was maintained at either 1.0 or 25.0 °C through use of a Varian temperature control unit interfaced to the spectrometer. The digital temperature readout was calibrated through measurement of the difference in chemical shifts between the two resonances in a neat solution of ethylene glycol (Senior, 1988).

A total of 12 daunomycin standards ranging in concentration from 5 μM to 5 mM were prepared in the 10 mM sodium phosphate buffer system described above. Aliquots of 500 μL of each standard were frozen in liquid nitrogen and lyophilized. The samples were redissolved in 99.96% D_2O (Sigma Chemical Company, St. Louis, MO) and relyophilized to remove residual water. This procedure was repeated in triplicate for each of the daunomycin standards. Following the final lyophilization step, the samples were redissolved in 500 μL of 99.96% D_2O and transferred to 5-mm o.d. NMR tubes for spectroscopic analysis. The number of transients collected varied according to the daunomycin standard concentration, and all free induction decays (FIDs) were acquired in the double-precision mode to improve accuracy. Proton chemical shifts in the daunomycin solutions were measured relative to an internal TMSP standard.

Derivation of Daunomycin Aggregation Parameters. Analysis of the daunomycin proton NMR data requires derivation of expressions for the free monomer concentration, assuming both monomer–dimer and indefinite association models. In the case of the former model, the equilibrium constant can be expressed as

$$K_D = (C_T - C_M)/2(C_M)^2 \quad (1)$$

where K_D is the dimerization constant, C_T is the total daunomycin concentration, and C_M represents the concentration of monomeric drug species. Recasting this relationship into

the form of the familiar quadratic equation and solving for the monomer concentration, one obtains

$$C_M = [1 + 8K_DC_T]^{1/2}/(4K_D) - 1/(4K_D) \quad (2)$$

On the basis of a previous multiparameter analytical investigation (Chaires et al., 1982a), it was concluded that an indefinite association model accurately describes the daunomycin aggregation process. Consequently, the applicable equilibrium may be written as

$$K_N = (C_N)/[(C_{N-1})(C_M)] \quad (3)$$

$$K_1 = K_2 = \ldots = K_N = K_I$$

where K_I is the intrinsic association constant, and C_M, C_N, and C_{N-1} are the concentrations of the monomer, Nth, and Nth − 1 aggregated drug species, respectively. On the basis of the formalism of Meyer and van der Wyk (1937), one may derive the following expression for the monomer concentration of the drug:

$$C_M = (1/K_I)[2K_IC_T + 1 - (4K_IC_T + 1)^{1/2}]/(2K_IC_T) \quad (4)$$

We now will describe the relationship between the equations derived above, as well as the actual calculation of the daunomycin aggregation parameters from the NMR chemical shift data. One of the primary underlying assumptions in the interpretation of the daunomycin chemical shift data is that the monomer and the aggregate resonances are in *fast exchange* on the NMR time scale, thereby causing one to observe an *average* chemical shift rather than separate signals for the free and aggregated drug species. Thus, the observed chemical shift (S_O) for a specific total drug concentration (C_T) is a weighted linear combination of the monomer chemical shift (S_M) and the infinite aggregate chemical shift (S_A), as reflected in the expression

$$S_O = X_MS_M + X_AS_A \quad (5)$$

where X_M and X_A are the mole fractions of the monomer and aggregated drug species, respectively. The monomer and aggregate mole fractions are defined as

$$X_M = C_M/C_T = C_M/(C_M + C_A) \quad (6)$$

$$X_A = C_A/C_T = C_A/(C_A + C_M) \quad (7)$$

where C_M and C_A are the molar concentrations of the monomer and aggregated drug species, respectively. Expressing the aggregate concentration in terms of the monomer and total drug concentrations, one can derive the following relationship for the observed chemical shift:

$$S_O = S_A + (S_A/C_T)(S_M - S_A) \quad (8)$$

The measured drug concentration (C_T) for each daunomycin standard and the observed chemical shift (S_O) for the individual proton resonances were entered as x,y data pairs into a nonlinear least-squares *three-parameter* fitting routine that was written in BASIC. The fitting routine incorporated eqs 2 and 8 for the dimerization model, and eqs 4 and 8 for the indefinite association model. Expected ranges and search intervals for the aggregation constant (K_D or K_I), monomer chemical shift (S_M), and aggregate chemical shift (S_A) were entered into the program. All possible combinations of these three parameters were tested iteratively to derive a set of self-consistent values.

Results and Discussion

Daunomycin–DNA Binding Enthalpies Determined by Stopped-Flow Microcalorimetry

Table II summarizes the enthalpy values we have measured over a range of binding densities for the complexation of daunomycin with 10 different DNA duplexes. Inspection of these data, at a given binding density, reveals a broad range

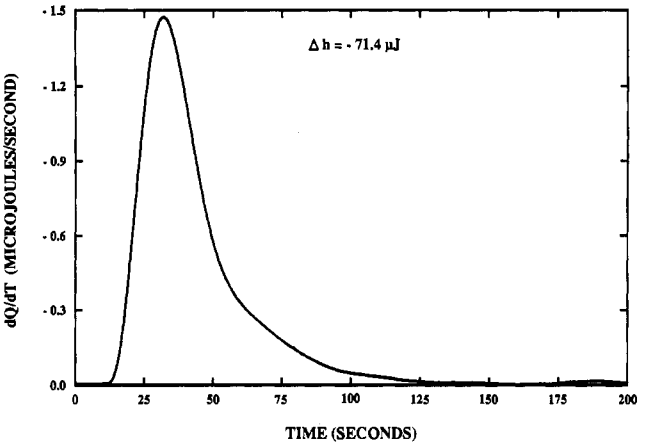

FIGURE 1: Stopped-flow microcalorimetric thermogram delineating the exothermic heat evolved for the binding of daunomycin to poly[d(AC)]·poly[d(GT)] at a ratio of 1 drug molecule per 40 phosphate residues (D:P = 1:40) at 25.0 °C.

of sequence-specific daunomycin binding enthalpies. For example, at a ratio of 1 drug molecule per 40 DNA phosphate residues, we measure an exothermic binding enthalpy (Figure 1) of −10.64 kcal/mol for the interaction of daunomycin with the alternating copolymer poly[d(AC)]·poly[d(GT)]. By contrast, at an identical drug-to-phosphate ratio, we measure an endotherm (Figure 2, panel C) of +3.54 kcal/mol for daunomycin binding to the homopolymer poly[d(A)]·poly[d(T)]. We previously have observed such large differences in association enthalpies for ligand binding to alternating versus homopolymeric host duplexes (Marky et al., 1985; Breslauer et al., 1987). These differences have been interpreted by us (Marky et al., 1985; Breslauer et al., 1987) and others (Wilson et al., 1985; Herrera & Chaires, 1989) as reflecting binding-induced changes in the conformation/hydration of the homopolymeric duplexes. For example, in the case of poly(dA)·poly(dT), it has been proposed that the duplex assumes a hyperhydrated non-B conformation in its drug-free state (Pilet et al., 1975; Leslie et al., 1980). Upon drug binding, it has been suggested that this altered conformation is converted to a "normal" B conformation (Marky et al., 1985; Breslauer et al., 1987; Herrera & Chaires, 1989). Thus, for poly(dA)·poly(dT), the intrinsic drug-binding enthalpy appears to be coupled with the transition enthalpy of an underlying conformational change in the host duplex.

Further inspection of the enthalpy data listed in Table II reveals that for the nonhomopolymeric sequences, which are believed to form duplexes that assume "normal" B-form conformations, the magnitudes (although not the signs) of the binding enthalpies vary appreciably, from −5.78 to −10.64 kcal/mol, at a drug-to-phosphate ratio of 1:40. Such a range of values may reflect sequence-dependent differences in the enthalpic contributions and/or even the nature of the specific daunomycin–DNA interactions that drive the overall binding event. In support of this contention, several general features of the data listed in Table II are worthy of note:

(1) The magnitude and even the sign of the enthalpy change for daunomycin binding is strongly dependent on the base composition and sequence of the host duplex. As noted above, daunomycin binding at 25 °C to the duplexes formed by homopolymeric sequences comprised of either A-T or A-U base pairs is characterized by *endothermic* enthalpies, whereas daunomycin binding to duplexes formed by alternating and mixed copolymeric sequences is characterized by *exothermic* enthalpies. Ranking the 10 DNA host duplexes in order of decreasing daunomycin binding *exothermicity* at 25 °C, one

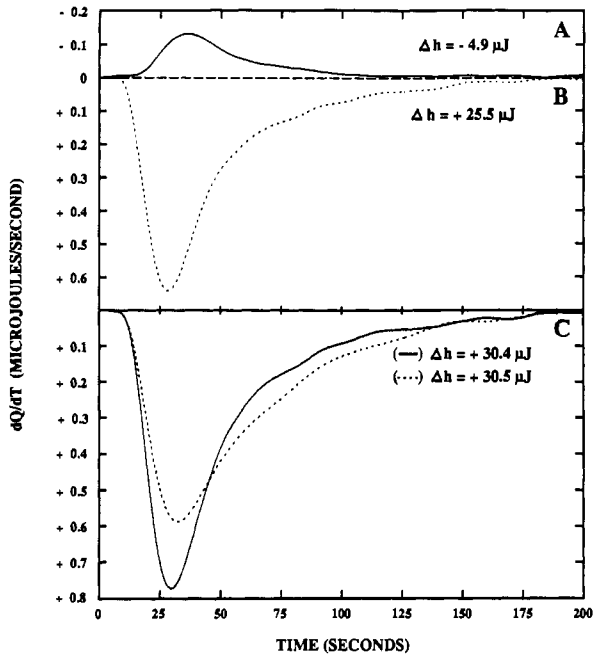

FIGURE 2: Stopped-flow microcalorimetric thermograms delineating the exothermic heat evolved upon the 1:1 dilution of an 8.0×10^{-4} M standard solution of poly[d(A)]·poly[d(T)] in panel A and the endothermic heat absorbed upon binding daunomycin to this homopolymer at a ratio of 1 drug molecule per 40 phosphate residues (D:P = 1:40) in panel B. The net endotherm characterizing formation of the daunomycin–poly[d(A)]·poly[d(T)] complex may be obtained by subtracting the thermograms (i.e., panel B − panel A) resulting in the solid line in panel C. Alternatively, the differential heat may be measured directly in a single mixing experiment by monitoring binding of the drug to the homopolymer in the sample channel and dilution of the host duplex in the reference channel, as represented by the dashed line in panel C. Comparison of the resultant thermograms for drug–DNA complex formation reveals that the reaction heats calculated via both techniques are equivalent.

obtains poly[d(AC)]·poly[d(GT)] > salmon testes DNA > poly[d(GC)]·poly[d(GC)] > poly[d(G)]·poly[d(C)] > poly[d(IC)]·poly[d(IC)] > poly[d(AU)]·poly[d(AU)] > poly[d(AT)]·poly[d(AT)] > poly[d(AG)]·poly[d(CT)] > poly[d(A)]·poly[d(T)] > poly[d(A)]·poly[d(U)].

(2) Daunomycin binding to alternating purine–pyrimidine sequences that contain G-C base pairs is characterized by large *exothermic* enthalpies [e.g., $\Delta H_b = -10.64$ kcal/mol for poly[d(AC)]·poly[d(GT)]; $\Delta H_b = -8.65$ kcal/mol for poly[d(GC)]·poly[d(GC)]].

(3) Removal of a pyrimidine methyl group that projects into the major groove [poly[d(AT)]·poly[d(AT)] versus poly[d(AU)]·poly[d(AU)]] does not significantly alter the daunomycin binding enthalpy. This observation is consistent with available structural data, which reveal the absence of interactions between daunomycin and the major groove domain of DNA host duplexes.

(4) Removal of a purine amino group that projects into the minor groove [poly[d(GC)]·poly[d(GC)] versus poly[d(IC)]·poly[d(IC)]] results in a significant decrease in the magnitude of the daunomycin binding enthalpy. This observation also is consistent with available structural data, which reveal that the daunomycin sugar ring lies in the minor groove and the planar aglycon ring system interacts with the purine residue.

In the aggregate, these enthalpy data suggest sequence/conformation-specific daunomycin–DNA interactions. As noted above, this possibility is consistent with available structural data (Quigley et al., 1980; Wang et al., 1987; Remeta et al., 1987a,b; Remeta, 1990), as well as footprinting results (Chaires et al., 1990). A detailed discussion of the specific drug–DNA interactions that may give rise to these enthalpy differences will be presented in a subsequent paper in which we report and compare *complete* thermodynamic binding profiles for daunomycin complexation with the same family of DNA host duplexes (Remeta et al., manuscript in preparation). The primary foci of this paper are to demonstrate how stopped-flow microcalorimetry can be used to measure daunomycin–DNA binding enthalpies at monomeric drug concentrations, thereby precluding the need to correct for drug aggregation; to assess if/how the measured ligand binding enthalpies depend on the sequence/conformation of the DNA host duplexes; and to evaluate the influence of ligand binding density on the measured daunomycin–DNA binding enthalpies. All of these assessments are feasible due to the enhanced sensitivity of the stopped-flow instrument employed in our studies.

Binding Enthalpies Depend on the Drug Binding Density. Examination of the calorimetric data listed in Table II reveals that several of the drug binding enthalpies exhibit a dependence on the daunomycin-to-phosphate ratio (r). For those DNA host duplexes in which daunomycin binding is characterized by an exothermic enthalpy, we observe that the magnitude of the *exothermicity* decreases at higher r values (as the fractional saturation of the helix increases). An example of such a trend appears in the fifth row of Table II for the complexation of daunomycin with poly[d(AC)]·poly[d(GT)]. In this case, the binding enthalpies range from −10.76 to −8.42 kcal/mol as the D:P ratio increases from 1:80 to 1:5. By contrast, the measured *endothermicity* of daunomycin binding to the duplexes formed by the homopolymeric sequences comprised of A-T and A-U base pairs increases as the drug–DNA binding density decreases. Since each of the binding enthalpies listed in Table II is corrected for the amount of *bound* drug per DNA base pair at each drug-to-phosphate ratio, the observed decrease in the absolute magnitude of the binding enthalpies is not simply due to saturation of the available DNA binding sites at the higher r values.

One possible explanation for the observed r dependence evokes a cooperative binding model at low r, in which the cooperative effect is manifested in the binding enthalpy term. In this connection, several investigators have reported that daunomycin binds cooperatively to some DNA duplexes at low r values (Chaires et al., 1982b; Graves & Krugh, 1983). Specifically, in sodium phosphate buffers of moderate ionic strength (e.g., 100–200 mM Na^+), cooperativity is observed over a narrow range of $r < 0.05$. Our stopped-flow microcalorimetric measurements conducted at D:P ratios of 1:80 and 1:40 fall within this binding density window (e.g., $r = 0.025$ and 0.05, respectively). Note that the measured endothermic and exothermic binding enthalpies are greatest in this binding density region and gradually decrease in magnitude as the DNA lattice is saturated with increasing amounts of drug. At higher r values, daunomycin binding is characterized by anticooperative behavior (Chaires et al., 1982b; Graves & Krugh, 1983). Consequently, if one assumes that the cooperative effects are manifested in the enthalpy term, it is tempting to suggest that the observed trends in the measured binding enthalpies reflect the presence of ligand–ligand interactions along the helix that are more pronounced, or propagated further, as the degree of fractional saturation is increased. Although primarily speculative in nature, such an interpretation is plausible and merits further investigation. In this regard, we currently are studying the influence of ionic strength on the thermodynamics of daunomycin binding to

Table III: Summary of Microcalorimetric Data Determined for the Interaction of Daunomycin with a Series of DNA Host Duplexes at 25.0 °C[a]

DNA host duplex	drug binding density (drug:phosphate ratio)	$\Delta H^\circ_{reported}$[b] (kcal/mol)	$\Delta H^\circ_{corrected}$[c] (kcal/mol)	$\Delta H^\circ_{observed}$[d] (kcal/mol)
poly[d(AT)]·poly[d(AT)]	1:10	−8.9	−6.0	−5.5
poly[d(A)]·poly[d(T)]	1:10	−2.1	+1.9	+1.9
poly[d(GC)]·poly[d(GC)]	1:10	−10.4	−7.6	−8.0
salmon testes DNA	1:10	−9.9	−7.3	−8.2

[a] Our published batch calorimetric binding enthalpies ($\Delta H^\circ_{reported}$) are corrected for drug aggregation, assuming an indefinite association model and a van't Hoff correction term derived from nuclear magnetic resonance measurements. The resultant daunomycin binding enthalpies ($\Delta H^\circ_{corrected}$) are compared with those measured directly via stopped-flow microcalorimetry ($\Delta H^\circ_{observed}$). [b] Published drug binding enthalpies corrected for daunomycin dimerization by using ΔH_{vH} = +16.5 kcal/mol of dimer species disrupted (Breslauer et al., 1987). [c] Drug binding enthalpies corrected for daunomycin aggregation by using ΔH_{vH} = +1.5 kcal/mol of aggregate species disrupted (Remeta, 1990). [d] Drug binding enthalpies measured directly via stopped-flow microcalorimetry.

DNA duplexes (Remeta et al., 1991). Our preliminary measurements reveal that the drug binding enthalpies exhibit a significant r dependence at a sodium ion concentration of 200 mM. Upon completion, these studies should provide insight into the thermodynamic origins of cooperative binding events.

Daunomycin Aggregates at the Drug Concentrations Used in Conventional Batch Calorimetric Studies

The self-association of daunomycin in aqueous buffer systems of varying ionic strength has been studied extensively by a variety of experimental techniques (Barthalemy-Clavey et al., 1973, 1974; Schutz et al., 1979; Martin, 1980; Chaires et al., 1982a). Although association constants and van't Hoff enthalpies have been reported for the disruption of daunomycin aggregates in these buffer solutions, there is considerable disagreement in the literature regarding the exact nature of the aggregated drug species (Chaires et al., 1982a, and references cited therein). In this regard, some investigators have derived the requisite thermodynamic data on the assumption of a monomer–dimer equilibrium, while others have employed an indefinite association model. The most thorough study of daunomycin self-association was conducted by Chaires et al. (1982a). This investigation provides a comparison of thermodynamic data gleaned from both models and concludes that an indefinite association model provides an optimum fit of both sedimentation and proton NMR data. The same study presents evidence that, in buffers containing 200 mM Na^+, daunomycin self-associates at concentrations exceeding 10 μM. In light of these results, we selected a buffer system containing a maximum sodium ion concentration of only 15 mM, so as to minimize the extent of aggregation at the elevated reagent concentrations required for batch calorimetry. At this low ionic strength, our concentration-dependent spectroscopic measurements revealed the onset of aggregation at a total drug concentration of 50 μM (Remeta, 1990). Consequently, batch microcalorimetric measurements conducted at reagent concentrations of 500 μM necessarily required correction for binding-induced disruption of aggregated drug species.

Invoking a simple monomer–dimer aggregation model and employing temperature-dependent UV/Vis spectroscopy, an initial estimate of +16.5 kcal/mol was obtained for the daunomycin dissociation enthalpy (Remeta et al., 1984; Marky et al., 1983b; Breslauer et al., 1987). Application of this van't Hoff correction term to our batch calorimetric measurements results in the drug–DNA binding enthalpies, $\Delta H^\circ_{reported}$, listed in the third column of Table III. These published values should be compared with the corresponding stopped-flow microcalorimetric data, $\Delta H^\circ_{observed}$, presented in the fifth column of Table III. Note that the daunomycin–DNA binding enthalpies determined at monomeric drug concentrations ($\Delta H^\circ_{observed}$) are more endothermic than those determined via batch calorimetric techniques ($\Delta H^\circ_{reported}$). This disparity

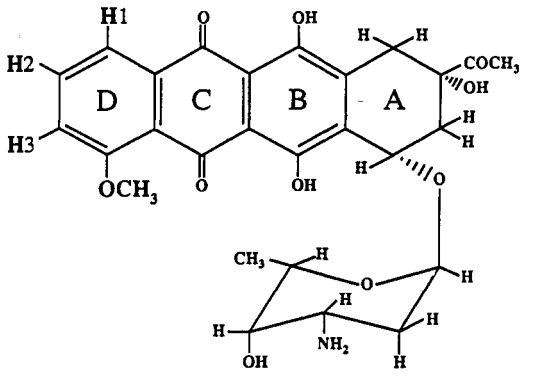

FIGURE 3: Chemical structure of daunomycin highlighting the methyl (OCH_3) and aromatic (H1, H2, and H3) proton resonances of ring D monitored in nuclear magnetic resonance studies of the drug aggregation process.

suggests that the van't Hoff enthalpy employed to correct the batch calorimetric data for binding-induced disruption of aggregated drug species may be in error. This error may be due to the inadequate sensitivity afforded by the spectrophotometric technique employed, and/or the assumption of a simple dimerization model for daunomycin aggregation.

Previous studies have demonstrated the utility of NMR spectroscopy for deriving the requisite drug aggregation parameters (Chaires et al., 1982a, and references cited therein). NMR analysis of the chemical shifts of selected daunomycin proton resonances as a function of the total drug concentration and temperature permits a determination of the daunomycin van't Hoff dissociation enthalpy. Measurements of chemical shift changes in the limit of infinite dilution also eliminate certain assumptions that are usually required to fit aggregation data, thereby reducing the overall uncertainty in the validity of the adopted model.

Initially, we conducted COSY and NOESY experiments to assign all of the daunomycin proton resonances in D_2O (Remeta et al., 1987a,b). Our assignments generally are consistent with those reported in the literature (Arcamone et al., 1968; Nuss et al., 1980; Patel, 1980; Chaires et al., 1982a), although the chemical shifts of specific proton resonances are not identical due to differences in solution conditions among these studies. Referring to the molecular structure of daunomycin shown in Figure 3, the OCH_3, H1, H2, and H3 proton resonances on ring D experience significant concentration-dependent chemical shifts. Routine one-dimensional NMR spectra were acquired for a series of 12 daunomycin standards spanning three orders of magnitude in concentration (i.e., 5 μM to 5 mM). The observed chemical shifts of the four proton resonances were recorded at temperatures of 1.0 and 25.0 °C and analyzed as described under Experimental Procedures.

Comparison of the daunomycin monomer concentrations calculated from eqs 2 and 4 reveals that for a given dimeri-

Table IV: Comparison of the Fraction of Daunomycin Monomer Species Predicted for Various Combinations of Total Drug Concentration and Aggregation Constant, Assuming Simple Monomer–Dimer and Indefinite Association Models

aggregation const (M^{-1})	tot daunomycin conc (M)	predicted monomer conc (M)		predicted monomer fract (%)	
		dimerization model	indefinite model	dimerization model	indefinite model
500	2.0×10^{-5}	1.962×10^{-5}	1.961×10^{-5}	98.1	98.0
	5.0×10^{-4}	3.660×10^{-4}	3.431×10^{-4}	73.2	68.6
	1.0×10^{-3}	6.180×10^{-4}	5.359×10^{-4}	61.8	53.6
1000	2.0×10^{-5}	1.926×10^{-5}	1.924×10^{-5}	96.3	96.2
	5.0×10^{-4}	3.090×10^{-4}	2.679×10^{-4}	61.8	53.6
	1.0×10^{-3}	5.000×10^{-4}	3.820×10^{-4}	50.0	38.2
1500	2.0×10^{-5}	1.893×10^{-5}	1.888×10^{-5}	94.6	94.4
	5.0×10^{-4}	2.743×10^{-4}	2.222×10^{-4}	54.9	44.4
	1.0×10^{-3}	4.343×10^{-4}	3.009×10^{-4}	43.4	30.1
2000	2.0×10^{-5}	1.861×10^{-5}	1.854×10^{-5}	93.1	92.7
	5.0×10^{-4}	2.500×10^{-4}	1.910×10^{-4}	50.0	38.2
	1.0×10^{-3}	3.904×10^{-4}	2.500×10^{-4}	39.0	25.0

Table V: Summary of Aggregation Parameters Derived for the Self-Association of Daunomycin in 10 mM Sodium Phosphate Buffer (pH 7.0) from Nuclear Magnetic Resonance Studies of the Concentration and Temperature Dependence of Selected Drug Proton Resonances[a]

daunomycin proton resonance	aggregation const (M^{-1})		monomer chemical shift (ppm)		aggregate chemical shift (ppm)	
	1.0 °C	25.0 °C	1.0 °C	25.0 °C	1.0 °C	25.0 °C
OCH_3	980	790	4.052	4.065	3.770	3.804
H1	985	790	7.945	7.960	7.180	7.160
H2	980	795	7.890	7.894	7.487	7.508
H3	975	785	7.610	7.630	7.170	7.150

[a] An indefinite association model was used to derive the aggregation parameters.

zation (K_D) or association (K_I) constant and a specific total drug concentration (C_T), the indefinite association model generally predicts a lower fraction of monomeric drug species than the dimerization model. Table IV provides a summary of daunomycin monomer concentrations and monomer fractions calculated for various combinations of total drug concentrations and aggregation constants, assuming either a simple dimerization or indefinite association model. These data suggest that both models adequately predict the fraction of monomeric drug in the dilute solutions routinely employed for stopped-flow microcalorimetric measurements. Assuming a total daunomycin concentration of 20 μM and an aggregation constant of 1000 M^{-1}, monomer fractions of 96.3% and 96.2% are calculated for the dimerization and indefinite association models, respectively. However, for reagent concentrations that are typically used in either batch microcalorimetry (e.g., 0.2–0.5 mM) or two-dimensional NMR (e.g., 1.0 mM), the simple dimerization model exaggerates the daunomycin monomer concentration. Specifically, for a total daunomycin concentration of 0.5 mM and an aggregation constant of 1000 M^{-1}, the simple dimerization model yields a monomer fraction of 61.8%, a value significantly greater than that of 53.6% calculated from the indefinite association model. This overestimate is further magnified for higher drug aggregation constants as evidenced by comparing the respective monomer fractions of 50.0% (i.e., dimer model) and 38.2% (i.e., definite model) that are predicted for a daunomycin association constant of 2000 M^{-1}. Consequently, one can readily appreciate the significance of identifying an appropriate model to describe accurately the daunomycin aggregation process. This is of critical importance for ensuring the validity of the experimentally derived drug aggregation constants and association enthalpies that are required for correcting the daunomycin–DNA binding enthalpies determined from batch microcalorimetric data.

Analysis of the Concentration-Dependent NMR Chemical Shift Data. Comparison of nonlinear least-squares fits derived for the simple dimerization and indefinite association models

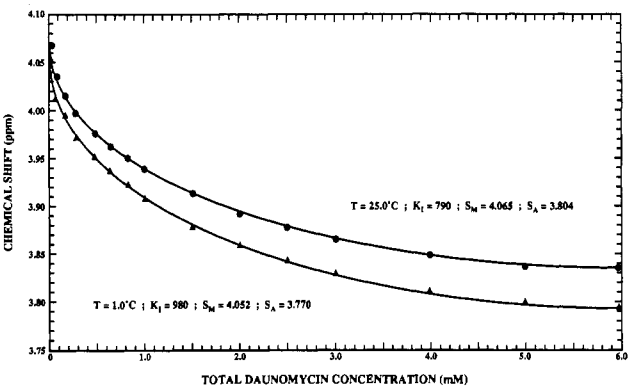

FIGURE 4: Nonlinear least-squares fits derived for the concentration-dependent NMR chemical shifts of the ring D methyl resonance of daunomycin at 1.0 °C (closed triangles) and 25.0 °C (closed circles).

reveals that the latter furnishes an optimal fit of the daunomycin aggregation data. A sample plot illustrating the fits for the concentration-dependent chemical shifts of the daunomycin methyl resonance at 1.0 and 25.0 °C is presented in Figure 4. A summary of the daunomycin aggregation parameters (i.e., K_I, S_M, S_A) determined from the indefinite association model appears in Table V. Note the agreement among the four determinations of the intrinsic association constants measured at each temperature. Consequently, an *average* daunomycin association constant may be calculated at each temperature and employed in the computation of the van't Hoff enthalpy for disruption of the aggregated drug species. Substituting K_1 = 790 M^{-1} and K_2 = 980 M^{-1} for the daunomycin association constants at temperatures T_1 = 298.15 K and T_2 = 274.15 K into the familiar van't Hoff equation

$$\ln (K_1/K_2) = (\Delta H°/R)[(1/T_1) - (1/T_2)]$$

we calculate a daunomycin *association* enthalpy of −1.5 kcal/mol of aggregate species formed, or conversely, a daunomycin *dissociation* enthalpy of +1.5 kcal/mol of aggregate species disrupted. This aggregation enthalpy is significantly lower than that of −8.0 kcal/mol determined previously via

NMR analysis of daunomycin chemical shift data, assuming an indefinite association model (Chaires et al., 1982a). It is important to note, however, that although both measurements were conducted in sodium phosphate buffer systems, the ionic strengths of the two solutions differed considerably. Specifically, the association enthalpy of −8.0 kcal/mol was determined in a buffer containing a total sodium ion concentration of 200 mM, whereas our van't Hoff enthalpy of −1.5 kcal/mol was measured under low ionic strength conditions (i.e., maximum sodium ion concentration of 15 mM). Since previous investigators have demonstrated that an increase in ionic strength is paralleled by a corresponding magnification of the daunomycin association constant and degree of aggregation (Schutz et al., 1979; Martin, 1980), the results reported here are not necessarily in conflict with the earlier NMR studies of Chaires et al. (1982a). In fact, one may reasonably expect to observe a significant decrease in the daunomycin dissociation enthalpy as the total sodium ion concentration is reduced from 200 to 15 mM.

We observe a large difference between the drug dissociation enthalpy derived here from daunomycin proton chemical shift data (ΔH_D = +1.5 kcal/mol) and that calculated from spectrophotometric measurements (ΔH_D = +16.5 kcal/mol) (Remeta et al., 1984; Marky et al., 1983b; Breslauer et al., 1987). One potential source of variability arises from the aggregation models selected to analyze the data sets; namely, the assumption of an indefinite association model for the NMR data and a simple dimerization model for the optical data. In addition, the NMR and optical methods exhibit different potentials for monitoring the aggregation equilibrium. The spectrophotometric approach involves conducting thermal denaturation experiments for a series of daunomycin standards and calculating van't Hoff enthalpies from these melting curves. Since these published measurements were performed with 1.0-cm path length quartz cuvettes, the minimum daunomycin concentration required to yield a measurable and reproducible absorbance within the linear range of the instrument is 1.0×10^{-5} M. The observed temperature-dependent changes in the absorbance of these low concentration drug standards are relatively small when compared to their absolute absorption. Hence, in this case, the effective sensitivity of the spectrophotometric technique is substantially inferior to that realized by NMR analysis of proton chemical shift data in which significant changes are measured for drug concentrations spanning the range of 5 μM to 5 mM. Thus, *in this particular case*, NMR may provide a more effective means of deriving the appropriate aggregation parameters for the self-association of daunomycin. Alternatively, the disparity in the drug dissociation enthalpies as measured by NMR and UV/Vis spectroscopy simply may reflect different association processes at the concentrations generally employed in these two techniques.

Correction of Published Batch Calorimetric Data for Daunomycin Aggregation

We used the drug dissociation enthalpy calculated from our proton chemical shift data to correct published daunomycin–DNA binding enthalpies measured by batch calorimetry (Remeta et al., 1984; Marky et al., 1983b; Breslauer et al., 1987; Remeta, 1990). Table III compares the previously reported binding enthalpies corrected via spectrophotometric determination of the van't Hoff dissociation enthalpy, $\Delta H_{reported}$, and the daunomycin–DNA binding enthalpies calculated in accordance with the NMR-derived van't Hoff dissociation enthalpy, $\Delta H°_{corrected}$. Comparison of the binding enthalpies listed in columns 4 and 5 of Table III reveals remarkable agreement between the $\Delta H°_{corrected}$ batch data and the $\Delta H°_{observed}$ stopped-flow data. This agreement supports our analysis of the NMR data associated with the daunomycin aggregation equilibrium. Thus, for daunomycin, NMR analysis of chemical shift data provides a reliable estimate of intrinsic association constants from which drug dissociation enthalpies may be accurately determined and applied to the correction of batch microcalorimetric data.

Some DNA Duplexes Exhibit Significant Enthalpies of Dilution

We have noted that the enhanced sensitivity of the stopped-flow instrument enabled us to make the first determinations of daunomycin–DNA binding enthalpies at monomeric drug concentrations and over a range of binding densities. This enhanced sensitivity also has allowed us to detect significant dilution enthalpies for several of the DNA duplexes we have studied. This observation has broader implications for drug–DNA binding studies since previous investigators frequently have assumed the duplex dilution enthalpies to be negligible. Our stopped-flow measurements reveal that, depending on the nature of the DNA sequence being studied, such an assumption may not always be correct. For example, we measure significant exothermic enthalpies for the 1:1 dilutions of both the A-T and A-U homopolymers at concentrations above 300 μM. To be specific, in the absence of drug, a 1:1 dilution of the poly[d(A)]·poly[d(T)] homopolymeric duplex results in a measurable exotherm of −4.9 μJ (Figure 2, panel A). This dilution enthalpy was measured by placing an 8.0×10^{-4} M standard solution of poly[d(A)]·poly[d(T)] in one of the sample channel syringes and the sodium phosphate buffer in the remaining three syringes. Thus, the net endotherm characterizing formation of the daunomycin–poly[d(A)]·poly[d(T)] complex (i.e., +30.4 μJ) was obtained by subtracting the exothermic heat of dilution (i.e., −4.9 μJ) from the total observed reaction heat (i.e., +25.5 μJ) for daunomycin binding to the homopolymer (Figure 2, panel B). The solid line in panel C of Figure 2 represents the net endotherm determined in this manner. The differential design of the stopped-flow microcalorimeter also enabled us to measure this heat directly in a single mixing experiment by conducting the daunomycin–poly[d(A)]·poly[d(T)] binding reaction in the sample chamber and the poly[d(A)]·poly[d(T)] buffer dilution "reaction" in the reference chamber. The net endotherm of +30.5 μJ appears as the dashed thermogram in panel C of Figure 2. Comparison of the drug–DNA binding enthalpy measured directly with that determined by subtracting individual reaction heats reveals remarkable agreement (the integrated heats are within 24 cal/mol, which represents a coefficient of variation of less than 0.5%). Despite this agreement, the resultant thermograms are not directly superimposable (see panel C of Figure 2), due to differences in the time constants for the binding and the dilution reactions (compare panels A and B of Figure 2). Nevertheless, the comparability of the two binding enthalpies demonstrates that the differential design of the instrument permits one to determine directly the net reaction enthalpy from a single mixing experiment.

The measured duplex dilution enthalpies of ≈−15 cal/mol may appear negligible when compared with the reaction heats of ≈2–4 kcal/mol determined for daunomycin complexation with the A-T and A-U homopolymers. However, it should be noted that this dilution enthalpy is expressed on a per mole basis of DNA, which is the excess reagent in our stopped-flow microcalorimetric measurements. The importance of measuring these dilution enthalpies ultimately resides in the cor-

rection of our drug–DNA binding enthalpies. Consequently, a more realistic or direct evaluation of the overall significance of DNA dilution enthalpies rests in expressing these heats on a per mole basis of bound drug. In this regard, the heats measured for the dilution of poly[d(A)]·poly[d(T)] at polymer concentrations equivalent to D:P ratios of 1:20, 1:40, and 1:80 in the drug binding experiments result in respective correction terms of 0.33, 0.69, and 1.23 kcal/mol for the daunomycin–poly[d(A)]·poly[d(T)] reaction. This is clearly evident in the thermograms comprising Figure 2, where nearly one-sixth of the net endothermic heat may be attributed to correction for dilution of the A-T homopolymer. The drug–DNA binding enthalpies listed in Table II for both homopolymer host duplexes at D:P ratios of 1:20, 1:40, and 1:80 are corrected to account for these dilution heats.

The detection and quantification of these DNA dilution enthalpies at moderately dilute reagent concentrations was made possible by the enhanced sensitivity of the stopped-flow microcalorimeter employed in this investigation. Such a heat would not be detectable or distinguishable from baseline noise in conventional batch calorimeters, which are inherently less sensitive and have slower response times, thereby effectively broadening the thermogram. Nevertheless, a correction term of such magnitude is especially critical in batch calorimetry, since the concentrations of both the limiting and excess reagents are at least one order of magnitude greater than in stopped-flow microcalorimetry. The 3-fold order increase in sensitivity and 5-fold faster response afforded by the stopped-flow instrument permit accurate quantitation of such duplex dilution enthalpies.

Speculation on the origin of these dilution enthalpies may be premature due to the limited basis set of deoxypolynucleotides studied. However, it is interesting to note that the poly[d(A)]·poly[d(T)] duplex assumes a modified B-DNA structure in solution with unique hydration properties relative to classic B-form DNA helices (Pilet et al., 1975). Previous studies also reveal that poly[d(A)]·poly[d(T)] solutions are more viscous than other DNA polymers (Wilson et al., 1985), that the homopolymer undergoes a premelting transition (Herrera & Chaires, 1989; Chan et al., 1990; Breslauer, 1991), and that it exhibits aberrant drug binding properties (Breslauer et al., 1987). Hence, it is tempting to suggest that the *exothermic dilution enthalpies* measured for the A-T and A-U homopolymers may arise from specific hydrodynamic and/or solvation properties peculiar to this class of altered B-form DNA helices. Clearly, additional microcalorimetric dilution data must be acquired for other DNA duplexes before a more complete understanding of the origins of this effect can be defined.

Concluding Remarks

The calorimetric measurements reported in this paper represent the most direct model-independent determination of daunomycin–DNA binding enthalpies performed to date. These measurements were made possible by the microjoule detection capabilities of a newly developed stopped-flow microcalorimeter, which allowed us to determine daunomycin–DNA binding enthalpies at extremely dilute reagent concentrations, thereby precluding the need to correct for drug aggregation effects. For the 10 DNA host duplexes studied, we observed a broad range of daunomycin binding enthalpies, including both exothermic and endothermic reaction heats. Such a range of values may reflect sequence-dependent differences in the enthalpic contributions and even the nature of the specific daunomycin–DNA interactions that drive the overall binding event. Possible molecular interpretations of our thermodynamic data will be presented in a subsequent paper (Remeta et al., manuscript in preparation) in which we report *complete* thermodynamic profiles for daunomycin binding to the 10 host duplexes studied here.

The enhanced sensitivity of the stopped-flow instrument also enabled us to discover that the binding enthalpies exhibit an r dependence. This intriguing observation may be related to specific anticooperative ligand–ligand interactions at higher drug–DNA binding densities. Our calorimetric measurements also reveal that at moderately dilute DNA concentrations (i.e., 300 μM), the dilution enthalpies of certain duplexes are not negligible, as commonly has been assumed in previous studies. In the aggregate, these results demonstrate the need to employ highly sensitive calorimeters to measure directly the enthalpy changes accompanying drug–DNA interactions, particularly for drugs that tend to self-associate and/or for drugs that exhibit binding properties that depend on binding density. The binding enthalpies obtained from such measurements represent an essential component of the complete thermodynamic profile required to define the overall nature of the forces that control the binding event. Repetition of these stopped-flow calorimetric measurements at additional temperatures will allow us to calculate ΔC_p (the heat capacity change), a parameter that may provide insight into the role of solvent in the binding process.

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