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Binding of Daunomycin to Calf Thymus Nucleosomes†

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ABSTRACT: We report equilibrium, hydrodynamic, and electric dichroism studies of the complex of daunomycin with H1-depleted 175 base pair nucleosomes, along with some comparative data for ethidium. In contrast to ethidium, daunomycin binding to nucleosomes is strongly reduced relative to the affinity for free DNA. The salt concentration dependence of the binding constant indicates that approximately one Na^+ ion is released from both nucleosomes and free DNA upon daunomycin binding. The early melting transition of nucleosomes is preferentially stabilized by low levels of both drugs, but more markedly by ethidium. Ethidium also stabilizes the second nucleosome melting transition, but daunomycin barely does so. Dichroism and rotational relaxation time measurements indicate that daunomycin unfolds nucleosomes in a manner analogous to the influence of ethidium, although about twice as much daunomycin as ethidium is required to complete the unfolding process. The data support an unfolded structure in which the nucleosome elongates along the DNA superhelical axis. Levels of daunomycin greater than about 0.15 per DNA base pair promote nucleosome aggregation. To relate our results to the activity of daunomycin as an antitumor agent, we propose that the druug, because of its special intercalation geometry, strongly prefers free DNA regions over the bent helices found in nucleosomes and chromatin. The result of this preference should be an increased local concentration of the drug in the genetically active regions of nuclear DNA in which nucleosomal structure is less prevalent. Presumably the abundance of such regions in tumor cells makes them especially sensitive to daunomycin.

The anthracycline antibiotic daunomycin is widely used in the treatment of human cancers. It is proposed that the drug acts by direct interaction with nuclear DNA and subsequent inhibition of DNA replication and RNA transcription [for reviews, see Crooke & Reich (1980), Arcamone (1978), and Neidle (1978)].

Numerous studies of the interaction of daunomycin with DNA have appeared (Zunnio et al., 1972, 1980; Gabbay et al., 1976; Plumbridge & Brown, 1977; Barthalemy-Clavey et al., 1973; Huang & Phillips, 1977; Molinier-Jumel et al., 1978; Schütz et al., 1979). We have reported the details of our research on the self-association of daunomycin (Chaires et al., 1982a), equilibrium aspects of the daunomycin–DNA interaction (Chaires et al., 1982b), and the geometry of the daunomycin–DNA complex (Fritzsche et al., 1982).

In the cell, however, it is not naked DNA that serves as a binding site for daunomycin, but rather chromatin, consisting of DNA complexed with histones and other nuclear proteins. How the presence of proteins on the DNA might affect the binding of daunomycin is a question of importance in efforts to understand the action of the drug.

To explore this question, we have examined the interaction of daunomycin with H1-depleted nucleosomes containing lengths of DNA of 146 and 175 base pairs (bp). We find that the presence of core histones drastically reduces the affinity of daunomycin for DNA. Hydrodynamic studies show that

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daunomycin causes unfolding of nucleosomal particles and, at saturation, promotes their aggregation.

This work provides interesting similarities and contrasts with previous studies on the effects of ethidium bromide on the structure of nucleosomes (Wu et al., 1980). Both drugs are strong intercalators, and they show general similarity in their ability to cause nucleosome unfolding by elongation along the DNA superhelical axis. However, there are also some pronounced specific differences between the two drugs. For example, whereas the binding affinity of ethidium for nucleosomes is greater than for free DNA at small degrees of binding (Wu et al., 1980), binding of daunomycin is drastically reduced in nucleosomes, compared to free DNA. Our experiments also reveal other differences, such as the tendency of nucleosomes to aggregate in the presence of daunomycin and the greater stabilization of nucleosomes against thermal melting by ethidium, compared to daunomycin.

Materials and Methods

Preparation of 175-bp Nucleosomes. H1-depleted nucleosomes were prepared from frozen calf thymus glands (Pel-freeze) as follows. Minced tissue (100 g) was suspended in 600 mL of a buffer (buffer A) containing 50 mM triethanolamine, 25 mM NaCl, 0.25 M sucrose, 1 mM $PhCH_2SO_2F$ [phenylmethanesulfonyl fluoride (PMSF)], and 4 mM $MgCl_2$, pH 6.5. The suspended tissue was blended at 80 V for 1 min and then at 50 V for 3 min in a Waring blender and filtered through three layers of sterilized cheesecloth. The filtrate was then centrifuged at 5000 rpm in a Sorvall GSA rotor. The resultant supernatant was discarded; the nuclear pellet was retained and washed 2 times in buffer A. The nuclei were then suspended in digestion buffer (50 mM Tris, 25 mM NaCl, 0.25 mM sucrose, 3 mM $MgCl_2$, and 1 mM $CaCl_2$, pH 7.5) and adjusted to a concentration of 100–150 A_{260} units/mL. For estimation of the absorbance, 10 μL of the suspension of nuclei was diluted into 1 mL of 1 M NaOH, and the absorbance at 260 nm was measured. Micrococcal nuclease was then added to a concentration of 100 units/mL, and the suspension was digested for 15–20 min at 37 °C. The digestion solution was removed to ice, and the nuclei were pelleted by centrifugation in a Sorvall GSA rotor for 10 min at 5000 rpm. The pellet was resuspended, by using a Dounce homogenizer, and lysed in 0.25 mM ethylenediaminetetraacetic acid (EDTA) and 1 mM PMSF, pH 8.0. The solution was centrifuged at 10000 rpm for 10 min in a Sorvall SS-34 rotor to remove cell debris. The supernatant was retained and brought to 0.1 M KCl by addition of a 1 M KCl stock solution. After being incubated for 15 min at 0 °C, the solution was centrifuged for 20 min at 10000 rpm in a Sorvall SS-34 rotor. The supernatant, containing H1-depleted nucleosomes, was concentrated by using an Aminco concentrator, loaded onto a 5–20% sucrose gradient containing 10 mM tris(hydroxymethyl)aminomethane (Tris), 1 mM EDTA, and 0.4 M NaCl, and centrifuged for 27 h in an SW 27 rotor at 25000 rpm. The central portions of the "monomer" elution peak were pooled and dialyzed extensively against 10 mM Tris and 1 mM EDTA, pH 7.5 (TE buffer).

This preparation yielded 175-bp nucleosomes in sufficient purity and quantity for all of the experiments reported here.

146-bp Nucleosomes. Nucleosomal core particles containing 146-bp-long DNA were prepared as previously described (Wu et al., 1980) and were generously provided by H. M. Wu.

Electrophoretic Characterization of Nucleosome Preparations. Nucleosomes and nucleosomal DNA were characterized electrophoretically by using 7.5% acrylamide slab gels and a Tris–borate buffer system [90 mM Tris–borate and 2.5 mM EDTA, pH 8.2 (Maniatis et al., 1975)]. Runs were performed at a constant voltage of 120 V for 2–3 h at ambient temperature. Nucleosomal DNA for gels was prepared by an overnight incubation of nucleosomes with Proteinase K in 0.1% sodium dodecyl sulfate ($NaDodSO_4$), extraction with water-saturated phenol, and ethanol precipitation. Gels for the characterization of histones contained a 5% stacking slab gel and a 15% acrylamide gel, with 0.1% $NaDodSO_4$, and were run for 14–15 h at 50 mA (Wu et al., 1980).

Binding Isotherms. Binding isotherms were obtained from equilibrium dialysis or fluorescence titration experiments as previously described (Chaires et al., 1982b; Wu et al., 1980).

Data were cast in the form of a Scatchard plot (Scatchard, 1949) and analyzed in terms of the neighbor exclusion binding model (Crothers, 1968; McGhee & von Hippel, 1974):

$$r/C_f = K(1 - nr)\left[\frac{1 - nr}{1 - (n - 1)r}\right]^{n-1} \quad (1)$$

where r is the ratio of bound drug to total base pair concentration, C_f is the amount of free drug, K is the intrinsic binding constant to an isolated site, and n is the exclusion parameter in base pairs. Values of K and n were estimated by the least-squares fitting of experimental data to eq 1 by using the procedure "FIT FUNCTION" provided by the Prophet computer resource system.

Sedimentation Studies. Sedimentation studies were performed on a model E analytical ultracentrifuge (Beckman) equipped with an electronic speed control, UV optics, and a photoelectric scanner. Sedimentation velocity runs were typically made by using 12-mm double-sector centerpieces and an AnF rotor at 30000 rpm. Sedimentation coefficients were obtained from the slopes of plots of ln X vs. time, where the radial position X was taken to be the distance from the center of rotation to the half-height of the UV scanner trace. Sedimentation values were corrected to 20 °C for viscosity effects, neglecting density corrections.

Sedimentation equilibrium experiments were performed by using 12-mm double-sector centerpieces and 12000 rpm, 20.4 °C. Nucleosomes in TE buffer at an initial concentration of 1.25 A_{260} units/mL were sedimented for 72 h. Scans at 60 and 72 h were superposable. Column heights were 7–8 mm. Plots of ln absorbance (λ = 300 nm) vs. X^2 were used to obtain the quantity $M(1 - \bar{v}\rho)$, from which M was calculated by assuming $\bar{v}$ = 0.661 (Olins et al., 1976).

Melting Experiments. For thermal denaturation experiments, nucleosome samples were dialyzed extensively against a buffer containing 1 mM Tris and 0.1 mM Na_2EDTA, pH 7.5. Thermal denaturation experiments were conducted as previously described (Chaires et al., 1982b). Derivative melting curves were calculated at each temperature T_i by measuring the slope of the hypochromism between adjacent temperatures:

$$\frac{dH}{dT_i} = \frac{H_{i+1} - H_{i-1}}{T_{i+1} - T_{i-1}}$$

using 1 °C intervals between temperature points.

Translational Diffusion Coefficients. Translational diffusion coefficients were obtained from quasi-elastic light scattering measurements by using an argon ion laser, λ = 488 nm, with the autocorrelation function calculated by a Malvern K7025 correlator and fit by a least-squares routine to a single exponential decay. The decay time constant (τ) is related to the translational diffusion coefficient (D_T) by

$$1/\tau = 2q^2D_T$$

where $q = 4\pi n/\lambda \sin(\theta/2)$, θ being the scattering angle and

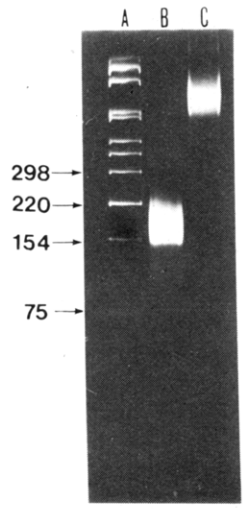

FIGURE 1: Gel electrophoresis of nucleosomes and nucleosomal DNA preparation. Samples were run in 7.5% acrylamide for 3 h at 120 V in TBE buffer (Maniatis et al., 1975). (A) *Hae*III digest of PBR 322; (B) nucleosomal DNA; (C) nucleosomes. The gel was stained with ethidium bromide.

n the refractive index (Berne & Pecora, 1976). An angle of 90° was used at a temperature of 25 °C.

Nucleosome samples (9.6 A_{260} units/mL in TE buffer) were filtered 3 times through a 0.22-μm Millipore filter prior to light scattering measurements. Daunomycin was added incrementally in volumes of 10 μL to the 1-mL sample volume to obtain the apparent diffusion coefficient as a function of added drug. Since daunomycin absorbs at the wavelength of the laser used, we found it necessary to take the following precautions. For each addition of daunomycin, the scattering intensity was adjusted to the value of the nucleosome solution in the absence of drug by adjusting the intensity of the incident beam. Exposure to the light was minimized by removal of the sample immediately upon data collection. No divergence from exponential autocorrelation decay due to sample heating could be discerned. Upon completion of the experiment, we found that all of the added daunomycin could be removed by dialysis against a solution containing excess DNA, indicating that exposure of the drug–DNA complex to laser light did not result in formation of any covalent complexes.

Electric Dichroism. Electric dichroism measurements were done on a modified temperature jump device. For low-field measurements ($E < 2$ kV/cm), square pulses were applied from a Cober 605P pulse generator. The dichroism signal from the photomultiplier tube was stored in a LeCroy 2256 waveform digitizer coupled to a LeCroy 8501 programmable clock and interfaced with a PDP 11/40 digital computer. Several hundred to a few thousand pulses were used for signal averaging. The dichroism rise and decay were fitted to single exponential curves. At higher voltages ($E > 5$ kV/cm), the device was similar to that described earlier (Hogan et al., 1978), modified to allow the use of the computer for data analysis and signal averaging.

Results

Characterization of Preparation. Figure 1 shows an electrophoretic characterization of the 175-bp nucleosomes used for most of these studies. DNA extracted from the nucleosomal preparation ran as a broad band centered at around 170 bp in length. As we have in the past (Schlessinger et al., 1982), we call this preparation "175-bp nucleosomes". The nucleosomes were found to contain all of the core histones and no detectable amounts of histones H1 or H5. The hydrodynamic properties of the preparation were as follows: $s_{20,w} = 10.8$ S; $D_{app} = 3.4 \times 10^{-7}$ cm^2 s^{-1}; $M_w = 2.26 \times 10^5$ (assuming $\bar{v} = 0.661$); TE buffer. In TE buffer, these particles show monophasic melting profiles with $T_m = 79.1$ °C.

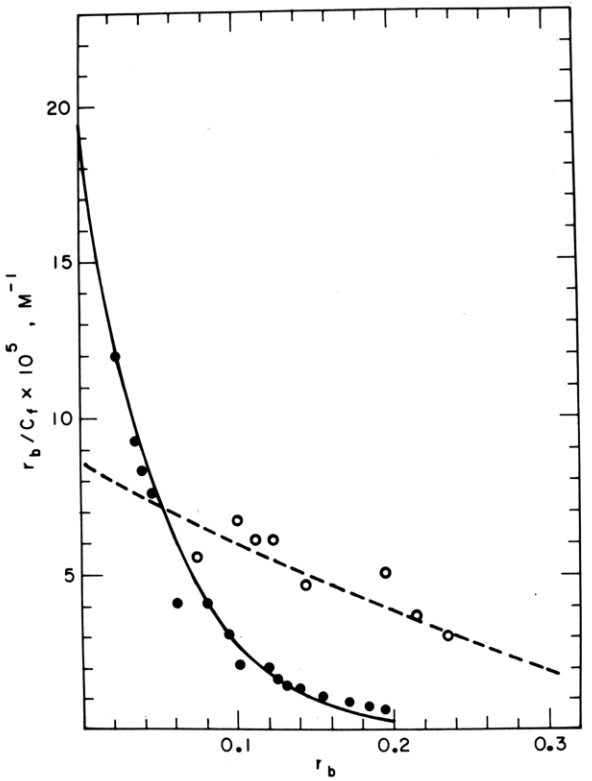

FIGURE 2: Binding of ethidium bromide to nucleosomal DNA (○) and 175-bp nucleosomes (●). Binding was measured by fluorescence titration as described in the text. Data were fit to eq 1 for DNA, with $K = 8.5 \times 10^5$ M^{-1} and $n = 2$ (---), or eq 2 for nucleosomes, with $K = 6 \times 10^5$ M^{-1}, $f = 0.7$, $n = 2$, $r_c = 0.11$, and $a_1 = 14$. Binding experiments were performed in TE buffer, 20 ± 2 °C.

Binding of Ethidium Bromide to 175-bp Nucleosomes. Figure 2 shows the binding isotherm obtained from fluorescence titration experiments for the binding of ethidium bromide to 175-bp nucleosomes. At low values of the ratio r of bound drug per base pair, the nucleosome and DNA isotherms are seen to cross, as was reported earlier for 146-bp core particles (Wu et al., 1980). This indicates that the binding of ethidium to nucleosomes is tighter than to free DNA at low r. We confirmed stronger binding of ethidium to nucleosomal core particles than to free DNA by competition dialysis. In a dialysis cell, solutions of DNA and nucleosomes of known concentrations were separated by the membrane, and a few microliters of ethidium bromide was added to one side (final drug concentration 0.5–1 μM). The cell was shaken on a water bath shaker for 48 h. The fluorescence intensities of the solution in both chambers were measured. The experiment was done in duplicate with addition of ethidium to the other side of the cell, allowing us to be sure that equilibrium is achieved. The relative fluorescence intensities at $r \sim 0.013$ indicate that ethidium shows 10 times higher affinity for nucleosomal core particles than for free DNA.

A model to account for this behavior has been proposed in an earlier study from this laboratory (Wu et al., 1980), which we use to fit the data of Figure 2. The expression previously derived for the binding of ethidium to 146-bp nucleosomes is

$$r/C_f = fK(1 - n^*r/f)\left[\frac{1 - n^*r/f}{1 - (n^* - 1)r/f}\right]^{n^*-1} \exp[-a_1(r - r_c)] \quad (2)$$

where r and C_f have been defined in connection with eq 1, f

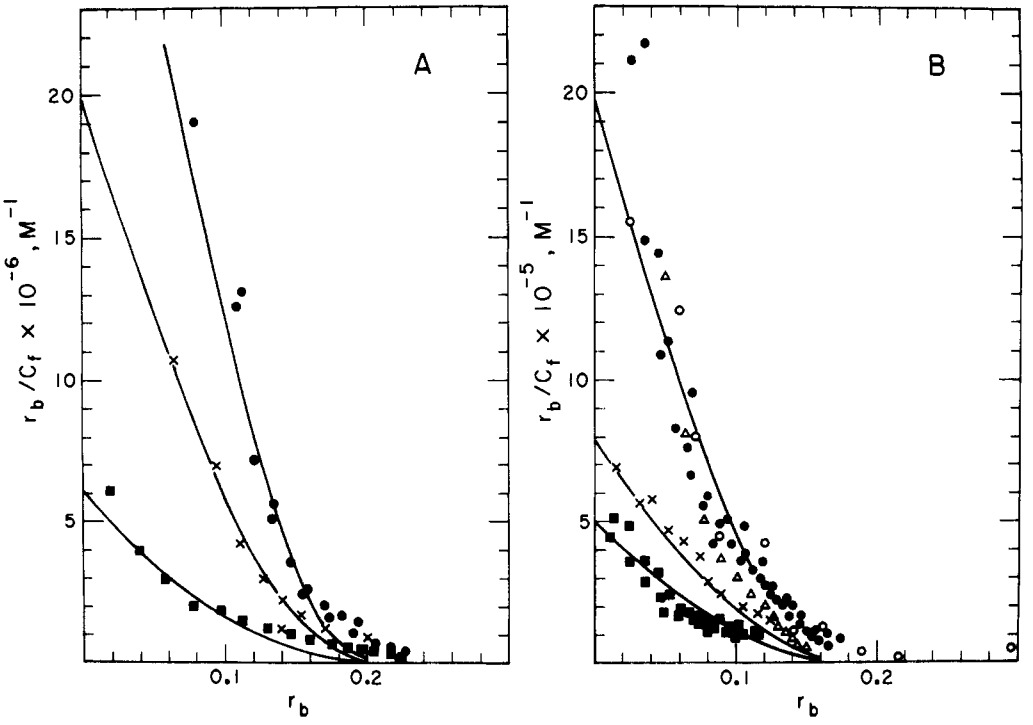

FIGURE 3: Binding of daunomycin to DNA (A) or 175-bp nucleosomes (B), shown as Scatchard plots, where r_b is the bound drug per DNA base pair and C_f is the free drug concentration. Symbols indicated the ionic conditions: (●, ○) TE buffer; (+) TE + 10 mM NaCl; (■) TE + 50 mM NaCl. For nucleosomes (B), (○) was obtained from equilibrium dialysis experiments. All other results were obtained from fluorescence titration. (△) refers to data obtained on 146-bp nucleosomes in TE buffer. Data were fit to the neighbor exclusion model, eq 1, yielding the parameters shown in Table I.

is the fraction of sites (relative to free DNA) available for binding, K is the intrinsic binding constant of the drug to free DNA, n^* is the exclusion parameter in the nucleosomal particle, a_1 is a force constant, and r_c is that r value at which all the superhelical turns in the nucleosomal DNA are relaxed (Wu et al., 1980). The values used to fit the data of Figure 2 are identical with those found earlier for the binding of ethidium to 146-bp nucleosomes, with the exception of K, which is 25% higher. Thus, the model previously proposed for 146-bp nucleosomes can account both qualitatively and quantitatively for the interaction of ethidium with 175-bp nucleosomes.

Daunomycin Binds Less Tightly to Nucleosomes Than to DNA. Figure 3 shows the binding isotherms obtained for the interaction of daunomycin with DNA (Figure 3A) and nucleosomes (Figure 3B) at several salt concentrations. In marked contrast to the behavior seen for ethidium bromide, the DNA and nucleosome isotherms do not cross. The binding of daunomycin to nucleosomes is weaker than to free DNA over the entire range of r. The model used to fit the ethidium binding data does not account for such behavior; attempts to fit the data of Figure 3B to eq 2 were not successful when realistic values of the parameters were used. Lacking a basis for postulating a more detailed model, we have simply fit the binding data of Figure 3 to the standard neighbor exclusion model, yielding the empirical parameters shown in Table I.

The values presented in Table I were obtained from a nonlinear least-squares fit of the data of Figure 3 to eq 1. The confidence limits on K and n are obtained from the fitting routine. Relative to free DNA, the binding of daunomycin to nucleosomes is characterized by a much lower binding constant, K, and a small increase in the exclusion parameter, n. For both DNA and nucleosomes, there are systematic deviations from the fitted curve at high r values as may be seen in Figure 3. Plausible explanations for this include weaker contribution from an ionic binding mode or incomplete

Table I: Summary of Binding Parameters for the Interaction of Daunomycin with DNA and 175-bp Nucleosomes[a]

	DNA		nucleosomes	
salt	$10^{-7}K$ (M^{-1})	n (bp)	$10^{-6}K$ (M^{-1})	n (bp)
TE	4.7 ± 0.4	4.6 ± 0.1	2.3 ± 0.9	5.1 ± 0.1
TE + 10 mM NaCl	2.1 ± 0.2	4.6 ± 0.2	0.85 ± 0.03	5.0 ± 0.2
TE + 50 mM NaCl	0.6 ± 0.05	4.4 ± 0.3	0.47 ± 0.03	5.0 ± 0.3

[a] The values shown are obtained from a least-squares fit of eq 1 to the experimental data of Figure 3.

neighbor exclusion. Control experiments have shown that addition of daunomycin to purified core histones does not alter the absorbance or fluorescence spectra of the drug, from which we infer that drug–histone interactions are unlikely and are not reasonable explanations for the deviations seen in Figure 3B. Other control experiments included pelleting of nucleosomes in the presence of daunomycin, with $NaDodSO_4$ gel electrophoretic analysis of the pellet and supernatant for histone proteins. Added daunomycin produced no change in the relative amounts of the four core histones in these fractions, from which we concluded that daunomycin does not cause dissociation of histones from nucleosomes up to $r = 0.2$.

As can be seen in Figure 3B, the affinity of 146-bp nucleosomes for daunomycin at higher r values is smaller than that of 175-bp nucleosomes. This difference is reproducible and will be reported in more detail in a later paper (F. Schlessinger, N. Dattagupta, and D. Crothers, unpublished experiments).

Salt Dependence. Table I shows that the interaction of daunomycin with both DNA and nucleosomes is dependent upon ionic strength. This results from the linkage of salt and drug binding and may be characterized by a plot according to the equation (Record et al., 1978)

$$\frac{d \ln K}{d \ln [Na^+]} = -\Delta r \qquad (3)$$

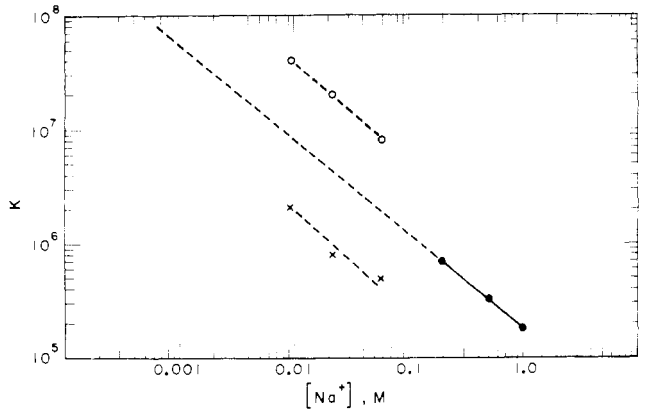

FIGURE 4: Apparent binding constant K as a function of ionic strength. Data of Table I were plotted according to eq 3. (○) DNA; (●) DNA in phosphate buffer [data taken from Chaires et al. (1982b)]; (×) 175-bp nucleosomes.

where Δr is the number of Na^+ ions released per drug bound. (More precisely, with the assumption of no change in anion binding, Δr is the difference in thermodynamic cation binding by free DNA or nucleosomes and their drug complex.) From the plots in Figure 4, we find $\Delta r = 1.04$ for binding daunomycin to DNA and $\Delta r = 0.73$ for binding daunomycin to nucleosomes. These values are both reasonably close to $\Delta r = 0.88$ expected for a singly charged ligand such as daunomycin, binding to free double-helical DNA (Record et al., 1978). We note that the K values obtained for the drug–DNA interaction in this study are somewhat higher than those expected from extrapolation of the earlier data (Chaires et al., 1982b) to the salt conditions used here. Differences in buffer cations, anions, and/or ionic strength account for this, as we verified directly by comparing the binding constant in BPES buffer (200 mM Na^+), $K = 9.8 \times 10^5$ M^{-1}, with the value $K = 1.7 \times 10^6$ M^{-1} in TE buffer + 200 mM NaCl.

The ion release value of $\Delta r = 0.73$ for daunomycin binding to nucleosomes, roughly the same as that for free DNA, implies that the drug binds primarily to DNA regions whose charge is not fully neutralized by histone protein side chains. As a consequence, Na^+ displacement occurs, rather than disruption of ionic protein–DNA interactions by the charged drug.

Thermal Denaturation of Nucleosomes in the Presence of Ethidium and Daunomycin. Figure 5 shows the effect of ethidium bromide and daunomycin on the thermal denaturation of nucleosomes. In figure 5A, the denaturation of 146-bp particles is seen to be biphasic. With the addition of ethidium, the low-temperature transition is abolished, and the T_m of the main transition steadily increases with added drug.

Addition of daunomycin to 146-bp particles (Figure 5B) likewise stabilizes the low-temperature transition, although less dramatically. In contrast to ethidium, the T_m of the main transition is unaltered until the nucleosome is completely saturated with drug. The effect of daunomycin on 175-bp particles (Figure 5C) is qualitatively similar to that seen in Figure 5B for 146-bp particles.

In Figure 5B,C, a new transition is seen to arise at 80 °C upon addition of daunomycin. This may represent the stabilized low-temperature transition seen in the absence of daunomycin. We have previously shown (Chaires et al., 1982b) that daunomycin raises the T_m of free DNA by some 30–40 °C saturation, which is close to the difference between the highest and lowest transitions seen in panels B and C of Figure 5.

In interpreting thermal denaturation profiles of nucleosomes,

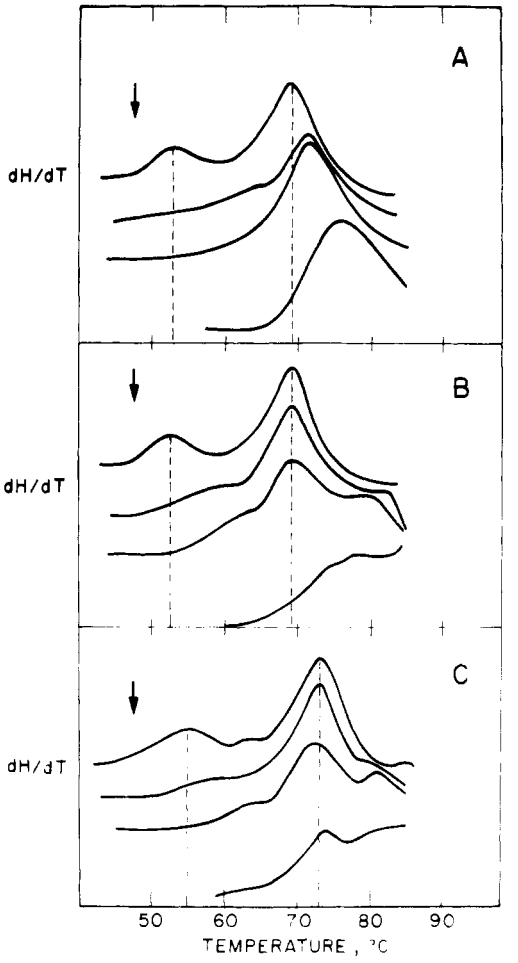

FIGURE 5: Differential melting curves for nucleosomes in TE/10. (A) 146-bp nucleosomes ± ethidium bromide. From top to bottom, $r = 0$, $r = 0.036$, $r = 0.108$, and $r = 0.27$. (B) 146-bp nucleosomes ± daunomycin. From top to bottom, $r = 0$, $r = 0.033$, $r = 0.1$, and $r = 0.2$. (C) 175-bp nucleosomes ± daunomycin. From top to bottom, $r = 0$, $r = 0.023$, $r = 0.08$, and $r = 0.208$. The arrows indicated the T_m of free calf thymus DNA under these conditions.

it should be recognized that the low salt buffer used (~1.3 mM ionic strength) causes partial unfolding of 175-bp nucleosomes, even at low temperature (Schlessinger et al., 1982). Hence, it is difficult to relate the melting characteristics observed to structural properties of the nucleosome. The main feature of Figure 5 is a substantially greater stabilization of nucleosomal DNA against thermal denaturation by ethidium, compared to daunomycin. Both drugs preferentially stabilize the first melting transition. The stronger stabilizing effects of ethidium may be due to tighter binding at elevated temperature, which would require a larger binding enthalpy for daunomycin, since the two drugs are nearly equal in affinity for nucleosomes at lower temperature (compare Figures 2 and 3).

Daunomycin Unfolds the Nucleosome and Promotes Nucleosome Aggregation. Addition of daunomycin to 175-bp nucleosome solutions alters the apparent sedimentation coefficient in the rather unusual manner shown in Figure 6A. First, the sedimentation coefficient is seen to droop to a minimum value of 9.45 at $r = 0.174$ and then to increase to a value exceeding that found for the nucleosome in the absence of any drug. We tentatively interpret this behavior as indicating unfolding of nucleosomes followed by their aggregation. In the experiment shown in Figure 6B, designed to test the second part of this hypothesis, the concentration dependence of the sedimentation coefficient of a nucleosome–daunomycin

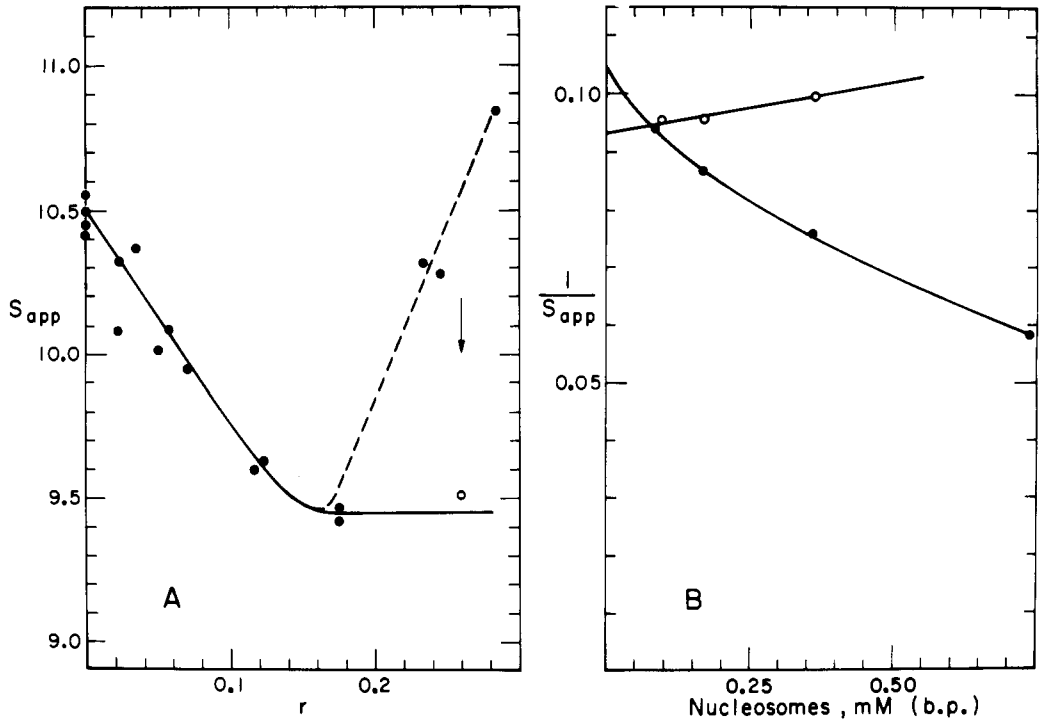

FIGURE 6: Sedimentation velocity experiments on 175-bp nucleosomes in TE buffer ± daunomycin. (A) Nucleosomes at 1 A_{260} unit/mL + drug as indicated. s has been corrected to 20 °C for viscosity but not density. (B) Concentration dependence of nucleosomes (○) and a nucleosome–daunomycin complex (●). The data in (B) were used to correct the data of (A) to construct the curve indicated by the open circle and the solid line.

complex at $r = 0.26$ was examined. The results are shown as a reciprocal plot. In the absence of the drug, the dependence of $1/s$ on concentration is slight and may be fit by a linear least-squares fit to the equation $1/s = 0.093 + 19.63$ (base pair concentration in millimolar). In the presence of drug, $1/s$ decreases strongly with increasing complex concentration, consistent with the expected behavior for an aggregating system. Extrapolating to zero complex concentration, we estimate $1/s^0 = 0.104\ S^{-1}$ or $s^0 = 9.5$ S. Plotting this corrected value in Figure 6A (open circle), we see that that data are consistent with a value of $s = 9.4$–9.5 S for the drug-saturated nucleosome.

Quasi-elastic laser light scattering experiments provide further evidence for nucleosomal aggregation in the presence of daunomycin. In the absence of daunomycin, we measured an apparent diffusion coefficient of 3.4×10^{-7} cm^2 s^{-1} at a nucleosome concentration of 0.175 mM base pairs in TE buffer, 25 °C. Using this value, and $s = 10.75$, we estimate an apparent molecular weight of 2.27×10^5 (assuming $\bar{v} = 0.661$). This is in good agreement with the independently measured value of 2.26×10^5 using sedimentation equilibrium, obtained at a 10-fold lower total nucleosome concentration. In the presence of daunomycin ($r = 0.26$), we observe $D_{app} = 1.64 \times 10^{-7}$ cm^2 s^{-1}. Combining this value with $s_{app} = 16.9$ S (Figure 6B), we calculate an apparent molecular weight of 7.4×10^5 ($\bar{v} = 0.661$), a 4-fold increase over that observed in the absence of drug, and a clear indication of nucleosomal aggregation. Figure 7 shows the effect of daunomycin on the apparent diffusion coefficient over a wide range of r values. From the sedimentation data of Figure 6A, and the known molecular weight of the nucleosome, we calculated the expected behavior as shown by the dashed line. The discrepancy at higher r values between this calculated curve and the experimental points is attributed to nucleosome aggregation, which is even more pronounced in this experiment because of the high nucleosome concentrations used.

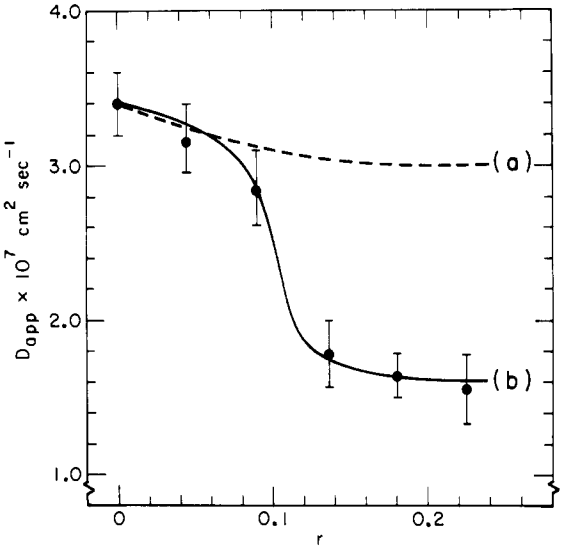

FIGURE 7: Apparent diffusion coefficient of nucleosomes in the presence of daunomycin (b). The diffusion coefficient of nucleosomes (9.6 A_{260} units, in TE buffer, 25 °C) was determined by using quasi-elastic light scattering as described under Materials and Methods. The error bars represent the range of values obtained for separate accumulations on a single sample. The dashed line is the expected diffusion coefficient calculated from the sedimentation data in Figure 6A (corrected for the difference in nucleosome concentration) and the known nucleosome molecular weight (corrected at each r for the added mass due to bound daunomycin).

Dichroism and Rotational Relaxation Times Indicate That Daunomycin Unfolds Nucleosomes. As we previously observed for ethidium (Wu et al., 1980), daunomycin increases the UV dichroism of nucleosomes. Figure 8 shows the variation with drug concentration of the measured reduced dichroism at 15 kV/cm. A sigmoidal lag in the dichroism increase occurs at low r values with both drugs, but in the case

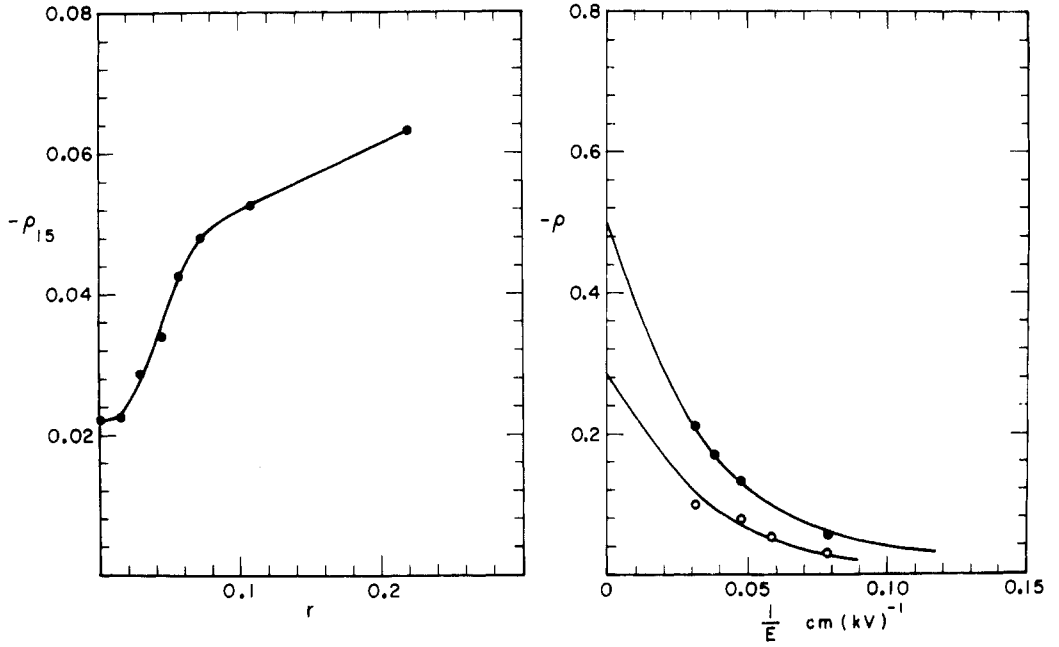

FIGURE 8: Electric dichroism of 146-bp nucleosomes in TE buffer. (Left panel) Variation of the dichroism amplitude (ρ_{15}) at 15 kV/cm. (Right panel) Reciprocal electric field dependence of $-\rho$ at 265 nm for nucleosomes (○) and the daunomycin–nucleosome complex at $r = 0.11$ (●).

Table II: Orientation Rise and Decay Times (μs) of 175-bp Nucleosomes[a]

r	τ_{decay}	τ_{rise}
0	1.0 ± 0.5	2.0 ± 0.5
0.096	2.5 ± 0.5	3.0 ± 1
0.18	2.6 ± 0.2	3.0 ± 0.3

[a] $T = 7$ °C, TE buffer.

of daunomycin, the dichroism amplitude continues to rise for r values above 0.08. Nucleosome aggregation may possibly contribute to this phenomenon.

Orientation rise and decay times are collected in Table II. The field-free decay time increases by about a factor 3 at $r = 0.1$, compared to $r = 0$, and remains constant thereafter, except for erratic effects probably due to aggregation at $r \geq 0.2$. When $0.1 \leq r \leq 0.2$, the field-free decay and orientation rise times are nearly equal, although the two are different at $r = 0$. For a particle which orients primarily by a permanent dipole moment mechanism, as deduced for drug-free nucleosomes (Crothers et al., 1978), the orientation rise time is expected to be about 3 times slower than the decay time. Equality of the rise and decay times at $r \geq 0.1$ strongly supports the idea that the orientation mechanism changes to the induced dipole type. We reached the same conclusion concerning the orientation mechanism for the ethidium–nucleosome complex from the salt concentration dependence of the apparent dipole moment (Wu et al., 1980). The increase of the field-free decay time and the change of the orientation mechanism to the induced dipole moment strongly suggest substantial elongation of the nucleosomal particle along the local superhelical axis.

Dichroism values at infinite field are useful in setting limits on structural models for the drug–nucleosome complex. Extrapolating as described previously (Hogan et al., 1978; Wu et al., 1980), we obtained for $r \simeq 0.1$, $\rho = -0.50$ at 262 nm, −0.52 at 313 nm and −1.00 at 486 nm.

Discussion

Our main findings may be summarized as follows: daunomycin binds to H1-depleted nucleosomes with considerably less affinity than it does to free DNA. Once bound to the nucleosome, the drug alters the hydrodynamic properties of the particle. At saturating values of bound drug, daunomycin promotes nucleosome aggregation. The drug preferentially stabilizes the low-temperature melting transition seen in thermal denaturation experiments.

The effect of daunomycin on the hydrodynamic properties of 175-bp nucleosomes is summarized in Table III. The hydrodynamic and equilibrium data are self-consistent: calculation of $M(1 - \bar{v}\rho)$ from s and D measurements yields a value in close agreement with that obtained from sedimentation equilibrium. Translational frictional coefficients, f (Tanford, 1960), calculated from s and D are also shown, along with the ratio of f to f_0, the latter being the frictional coefficient of an equivalent sphere of volume $M\bar{v}$.

With the exception of the limiting high-field dichroism (ρ_∞), the properties of daunomycin-saturated nucleosomes resemble those of the "expanded" form of 175-bp nucleosomes formed as an intermediate in low-salt unfolding (Schlessinger et al., 1982). However, the large dichroism amplitude of the drug-bound particle and its orientation by an induced dipole mechanism are not consistent with the expanded disklike structure proposed for the low salt unfolding intermediate.

The properties of the daunomycin-saturated 175-bp nucleosome complex strongly resemble those of the ethidium-saturated 146-bp nucleosome [$f = 1.44 \times 10^{-7}$ g s^{-1}; $\tau_{rise} = 3.0$ μs; $\rho_\infty(265) = -0.45$]. Hence, we propose the same model as we did in that case, an elongated superhelical winding of the DNA, yielding an equivalent cylinder about 335 Å long and 67 Å in diameter. The model for the low-salt "extended" structure is similar (Schlessinger et al., 1982), except that the estimated length is 470 Å, accounting for the slower rotational and translational motion, and the larger negative dichroism amplitude of the low-salt extended form.

We conclude that ethidium and daunomycin produce similar changes in nucleosomal structure when binding reaches saturation. The unfolding produced is analogous to, but slightly less extensive than, the elongation which occurs as the second unfolding step of 175-bp nucleosomes in low-salt solution.

Table III: Summary of Hydrodynamic and Dichroism Properties of 175-bp Nucleosomes[a]

	175-bp nucleosome	daunomycin, saturated	"expanded"[b]	"elongated"[b]
$s_{20,w}$ (S)	10.75	9.45[c]		
$10^{-7}D_T$ ($cm^2\ s^{-1}$) (20 °C)	3.4	2.72 (calcd)	2.85	1.94
$M(1-\bar{v}\rho)$	7.69×10^4 (S and D) 7.67×10^4 (SE)	8.3×10^4 (calcd)	7.67×10^4	7.67×10^4
$10^{-7}f$ ($g\ s^{-1}$)	1.19 (S) 1.19 (D)	1.47 (S)	1.42 (D)	2.08 (D)
f/f_0	1.6	1.9	1.84	2.8
τ_{rise} (μs) (70 °C)	1.16-2.0	3.0	2.0	5.4
$\rho_\infty(265)$	−0.49	−0.5	−0.33	−0.65

[a] Abbreviations: S and D, from combined sedimentation and diffusion data; SE, from sedimentation equilibrium experiments; S, from sedimentation velocity data; D, from translational diffusion data. [b] Data from Schlessinger et al. (1982). [c] Corrected for aggregation.

Comparison with Other Work. A significant disagreement between our work and the results of Erard et al. (1979) is their observation of cooperation binding of ethidium by core particles, whereas we have never been able to detect cooperativity in the binding of drugs by any nucleosome preparation. We repeated our titration under the buffer conditions used by Erard et al. (1979) and found (data not shown) that our results at degrees of binding r greater than about 0.13 were in reasonable agreement with theirs, but we never observed the drop in r/C_f at low r which is characteristic of cooperative binding. Since Scatchard's isotherms are notoriously inaccurate at low r values, especially when determined by spectroscopic methods, we checked our procedure by direct partition dialysis of ethidium between solutions of core particles and DNA. Independent of which side of the membrane the drug was added to at the start of the experiment, the core particle solution bound significantly (~10-fold) more drug than the free DNA at $r = 0.01$–0.015. This result confirms directly our conclusion that the binding isotherms of ethidium for nucleosomes and core particles do indeed cross as shown in Figure 2.

It is impossible for us to assess the possible role of optical artifacts or lack of dialysis equilibrium in the experiments of Erard et al. (1979). If it is assumed that no such factors intervene, the only possible explanation for the disagreement is that our nucleosome preparations are different. As judged from the gels, their core particles may be more highly trimmed than ours, but otherwise the preparative methods and general properties appear quite similar. Our preparations are electrophoretically homogeneous, as judged by ethidium staining. Each group has chosen different specialized methods for more elaborate characterization of the samples (Crothers et al., 1978; Wu et al., 1980; Schlessinger et al., 1982). In any event, larger nucleosomes of the type reported in this paper are the primary products of nuclease digestion of chromatin and therefore are probably closer to the native conformation in intact chromatin. We are confident that neither ethidium nor daunomycin binds cooperatively to such nucleosomes.

Origins of the Difference between Ethidium and Daunomycin. These two drugs differ in their nucleosome binding in three major ways: (1) Completion of unfolding requires about twice as much bound daunomycin as ethidium. (2) In contrast to ethidium, daunomycin binding to nucleosomes is strongly reduced compared to free DNA. (3) Daunomycin causes appreciable nucleosome aggregation.

The first of these differences may be related to the smaller DNA unwinding produced by daunomycin compared to ethidium (15° in low-salt buffer vs. 26° for ethidium; Fritzsche et al., 1982). In our analysis of the unfolding of nucleosomes by ethidium, we proposed that the DNA unwinding produced by ethidium intercalation is matched by a corresponding change in superhelical writhe, with no change in the sum of twist and writhe (Wu et al., 1980). (The sum of twist and writhe was proposed to be constant to explain the retention of the 10.4-bp DNase I cleavage repeat in the ethidium–nucleosome complex.) Since each daunomycin produces roughly half as much DNA unwinding as ethidium, it is expected that about twice as many daunomycin molecules will be necessary to complete the conversion to the elongated superhelix of altered writhe.

The sharply lessened relative binding of daunomycin to nucleosomes compared to free DNA reflects the specific intercalation geometry of daunomycin. Ethidium, which intercalates with its long axis parallel to the base pair long axis (Tsai et al., 1975), may be able to adapt well to a bent DNA structure by adjusting slightly the depth to which the drug enters a wedge-shaped intercalation cavity in curved DNA. Daunomycin, on the other hand, intercalates with its long axis perpendicular to the base pair long axis and extends through the helix (Quigley et al., 1980). Protrusion of the bound drug through to the side of the curved double helix which has a smaller radius of curvature would produce unfavorable interactions, and hence reduced binding. Thus, we propose that the anthracyclines such as daunomycin discriminate against bent DNA, whereas ethidium intercalation is able to accommodate helix bending.

Effects on Drug Activity. According to the relative daunomycin binding strengths of nucleosomes and free DNA, the drug in cell nuclei should partition strongly into free DNA regions. Hence, it is possible that genetically active regions which have less nucleosomal structure may be special targets for the drug. It is also possible that the contrasting properties of ethidium, which does not prefer free DNA, are related to the lesser activity of ethidium as an antitumor drug. By this hypothesis, the anthracyclines owe their exceptional antitumor activity to their unusual intercalation geometry, which strongly favors binding to linear over bent DNA, and hence focuses the drug in active DNA regions.

Aggregations of nucleosomes by daunomycin at $r > 0.1$ is clearly indicated by our experiments. Since saturation of the hydrodynamic changes induced in nucleosomes by ethidium is reached at about 2 times smaller r values than for daunomycin, we did not extend our r values to as high a range in the earlier ethidium experiments (Wu et al., 1980). Hence, it is difficult to be certain that the two drugs are fundamentally different in inducing aggregation. Possibly the drastic cytological effects of daunomycin reported for HeLa cells (Dorigotti, 1964) might be due to drug-induced condensation of chromatin, to which the aggregation reported here would contribute.

Registry No. Daunomycin, 20830-81-3.

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Thermodynamic Binding Constants for Gallium Transferrin†

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ABSTRACT: Gallium-67 is widely used as an imaging agent for tumors and inflammatory abscesses. It is well established that Ga^{3+} travels through the circulatory system bound to the serum iron transport protein transferrin and that this protein binding is an essential step in tumor localization. However, there have been conflicting reports on the magnitude of the gallium–transferrin binding constants. Therefore, thermodynamic binding constants for gallium complexation at the two specific metal binding sites of human serum transferrin at pH 7.4 and 5 mM $NaHCO_3$ have been determined by UV difference spectroscopy. The conditional constants calculated for 27 mM $NaHCO_3$ are log K_1^* = 20.3 and log K_2^* = 19.3. These results are discussed in relation to the thermodynamics of transferrin binding of Fe^{3+} and to previous reports on gallium binding. The strength of transferrin complexation is also compared to that of a series of low molecular weight ligands by using calculated pM values (pM = -log [$Ga(H_2O)_6$]) to express the effective binding strength at pH 7.4.

Gallium-67 is widely used as an imaging agent for a variety of soft tissue tumors and inflammatory abscesses (Hayes, 1978; Welch & Moerlein, 1980), although the mechanism of tumor localization has not been firmly established (Larson, 1978). Even though this radioisotope is commonly administered as a citrate complex, once in the blood the gallium rapidly binds to the iron transport protein transferrin (Clausen et al., 1974; Gunasekera et al., 1972; Vallabhajosula et al., 1980). Since serum transferrin is normally only about 30% saturated with iron (Larson et al., 1979a), it retains a relatively high capacity for binding other metal ions. In addition to this role in serum transport of gallium, it appears that transferrin facilitates gallium movement across tumor cell membranes (Larson et al., 1979a,b; Harris & Sephton, 1977). In some cell culture

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