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Interaction of Copper(II) Ions with the Daunomycin–Calf Thymus Deoxyribonucleic Acid Complex†

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ABSTRACT: The interaction of Cu(II) with the native and heat-denatured DNA complexes of daunomycin (**1**) and *N*-(trifluoroacetyl)daunomycin (**3**) has been examined by using absorption and circular dichroism spectroscopies. At low r_t, where r_t is the input molar ratio of drug to DNA phosphate, Cu(II) interacts with the native and heat-denatured calf thymus DNA complex of daunomycin to form a ternary complex involving the aglycon portion of the antibiotic, Cu(II), and DNA. A Job plot of the titration involving Cu(II) and heat-denatured DNA shows that the Cu(II)–drug stoichiometry in the ternary complex is ≤1. Although the *N*-(trifluoroacetyl)daunomycin–native DNA complex does not form a ternary complex, the denatured DNA complex with the antibiotic does. Copper(II) titrations of the daunomycin–native DNA complex, at high r_t, where both strongly and weakly bound antibiotic molecules are very likely present in solution result in the formation of both the ternary species as well as a binary complex involving only the metal ion and the antibiotic. The spectroscopic results indicate that in the ternary complex, the Cu(II) ion is bound to the unintercalated aglycon portion of the antibiotic and very likely also to the heterocyclic bases of DNA.

The antitumor antibiotics daunomycin (**1**) (daunorubicin) and adriamycin (**2**) (deoxyrubicin) (Chart I) are in wide clinical use for the treatment of various neoplastic diseases (Carter, 1980). The mechanism by which the drugs exhibit their antineoplastic activity is not completely understood, but their strong DNA binding properties led the discoverers of the antibiotics to suggest that the drug receptor site is DNA (DiMarco et al., 1964; Arcamone et al., 1969). Most of the available evidence indicates that the antibiotics bind to DNA via an intercalative process employing the hydroxyquinone portion of the drug and also through an electrostatic interaction involving the protonated amine group on daunosamine (DiMarco & Arcamone, 1975; Pigram et al., 1972). Recent crystallographic studies on the hexanucleotide duplex d(CpGpTpApCpG), containing intercalated daunomycin molecules at both CG sites, show that the long axis of the aglycon portion of the drug is roughly perpendicular to the base pair axis at the intercalation site (Quigley et al., 1980).

In an effort to gain further information about the binding mechanism, several investigators have studied the binding of metal ions to the anthracycline–DNA complex. Fishman & Schwartz (1974) demonstrated that the addition of Cu(II) to the denatured calf thymus DNA–daunomycin complex affects the visible absorption and fluorescence spectra of the drug–DNA complex. In the presence of native DNA, however, the study reported that Cu(II) did not affect the spectral properties of the intercalated drug. These observations led Fishman and

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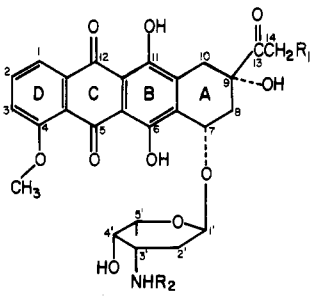
Chart I



$R_1, R_2 = H$ Daunomycin, 1
$R_1 = OH, R_2 = H$ Adriamycin, 2
$R_1 = H, R_2 = COCF_3$ N-Trifluoroacetyl Daunomycin, 3

Schwartz to the conclusion that daunomycin can form a ternary complex involving the metal ion, the drug, and denatured DNA. The observed lack of formation of a ternary complex with native DNA was presumably due to the fact that the drug chromophore could not interact with a Cu(II) ion while the antibiotic was intercalated into double-helical DNA. A later study showed that single-stranded homopolymers, e.g., poly(dA), are capable of forming a ternary complex with daunomycin and Cu(II) (Mikelens & Levinson, 1978).

In addition to reporting the DNA binding properties of daunomycin, Calendi et al. (1965) discussed the effects of various metal salts on the optical properties of the drug–native DNA complex. Contrary to Fishman & Schwartz (1974), the study reported that Cu(II) affected the spectrum of the drug–native DNA complex and that the cation caused the release of the antibiotic into solution as a simple Cu(II)–anthracycline complex. A similar conclusion was reached by Rusconi (1966), who studied the effects of addition of Ag(I) to the drug–native DNA complex. However, neither study presented evidence of the existence of simple metal–daunomycin complexes in solution.

The uncertainty in what factors influence the formation of a proposed ternary complex involving Cu(II), the anthracyclines, and DNA and the inconsistency in the spectroscopic data reported for the titration of the drug–DNA complex with Cu(II) prompted us to reexamine the system. Such an investigation assumed added importance in light of recent reports that the anthracyclines in the presence of reducing agents are capable of causing strand scission of DNA (Lown et al., 1977; Bachur, 1979). At least one study has shown that DNA degradation via the reductive mechanism is stimulated by the presence of Cu(II) (Someya & Tanaka, 1979).

In this report we show that Cu(II) interacts with the calf thymus DNA complexes of daunomycin (**1**) and *N*-(trifluoroacetyl)daunomycin (**3**) to produce a ternary complex involving the drug, the cation, and DNA, eq 1. Visible absorption and circular dichroism spectroscopies show that the formation of the ternary complex is sensitive to the drug–DNA ratio, the physical state of DNA (native or heat denatured), the antibiotic structure, and the salt concentration of the medium.

$$\text{drug} + \text{Cu(II)} + \text{DNA} \rightleftharpoons \text{drug–Cu(II)–DNA} \quad (1)$$

Experimental Procedures

Materials. Daunomycin hydrochloride (**1**) (Farmitalia) and *N*-(trifluoroacetyl)daunomycin (**3**) (National Cancer Institute) were stored as solids in the dark in a desiccator at 4 °C. Aqueous stock solutions of **1** were prepared immediately prior to use. Because of the limited solubility of **3** in water, the stock solution of this compound was prepared by dissolving the antibiotic in 95% ethanol. Addition of the appropriate amount of the ethanol solution of **3** to an aqueous DNA solution resulted in a drug–DNA solution containing less than 5% (v/v) ethanol. Concentrations of **1** and **3** in solution were determined optically at 480 nm with an extinction coefficient of 11 500 L mol^{-1} cm^{-1} (Gabbay et al., 1976). The source of Cu(II) as $Cu^{II}Cl_2 \cdot 4H_2O$ used in the experiment was obtained from Alfa Inorganics.

Calf thymus DNA (Sigma) was prepared by dissolving the DNA (~2 mg/mL) in 0.1 M NaCl and exhaustively dialyzing first against 0.1 M EDTA[1] and then against either 0.1 M or 1 mM NaCl at 4 °C. DNA concentrations are expressed as DNA phosphate (DNA P) with a molar extinction coefficient of 6600 at 260 nm. Since the spectroscopic results with phenol-extracted DNA and those with unextracted DNA were identical, the latter was used in the titrations. The DNA samples in 10 mM NaCl at pH 7.0 had an A_{260nm}/A_{280nm} ratio of ~1.9, indicating they were relatively protein free. DNA was denatured, immediately prior to the titration, by heating in a boiling water bath at 100 °C for 10 min (1 mM NaCl), followed by rapid quenching of the solution in ice.

Methods. The absorption spectra were obtained by using either a Cary 118C, 219, or 14 spectrophotometer. The CD spectra were collected on a Durrum Jasco J-20 spectropolarimeter while the IR spectrum of the violet precipitate was obtained on a Beckman 4220 spectrometer. The absorption and CD data were collected in 1-cm path-length cells. A Beckman pH meter having a combination electrode (Orion No. 91-15) was calibrated against a sodium phosphate buffer at pH 7.00. All of the spectroscopic work was carried out at ambient temperature.

Titration of the Drug–DNA Complex with Cu(II). The drug–DNA complexes were formed by adding an aqueous solution of the drugs to a solution of DNA at a specific ionic strength and adjusting the pH to 7.00 ± 0.05 with 0.1 N NaOH. Salt concentrations used for the native DNA titration were 1 mM, 10 mM, 0.1 M, 1 M, and 2 M while a single salt concentration of 1 mM was employed for the studies with heat-denatured DNA. The drug–DNA ratio was varied by altering the DNA concentration in solution while the drug concentration was maintained at $(6.7–8.0) \times 10^{-5}$ M. Separate titrations were carried out with various input drug–DNA P ratios, r_t, in the range $0.02 \leq r_t \leq 0.5$.

To the drug–DNA solutions (total volume 4 mL) were added microliter quantities of 10 mM or 0.1 M aqueous $CuCl_2$. Addition of the copper salt caused the pH of the unbuffered solution to decrease by 0.3–0.6 pH unit. The pH of the solution was readjusted to 7.00 ± 0.05 by the addition of microliter quantities of 0.1 N NaOH, and a portion of the solution was placed in a spectrophotometer cell for recording the optical data. The error introduced by dilution in the experiment was minor, and it did not significantly affect the spectroscopic results. The time required for recording each spectrum in the titration (5–20 spectra) was ~15 min. Titrations were also performed on drug–DNA solutions in Hepes (0.01 M) or Tris (0.01 M) buffer to maintain the pH (pH 7.00). The presence of the buffers affected the spectral changes that occurred only in the sense that additional amounts of Cu(II) were necessary to produce a spectral change equivalent to that observed in the absence of buffer. The

[1] Abbreviations: EDTA, ethylenediaminetetraacetic acid; CD, circular dichroism; Hepes, *N*-(2-hydroxyethyl)piperazine-*N*'-2-ethanesulfonic acid; Tris, tris(hydroxymethyl)aminomethane.

Table I: Spectroscopic Data for the Complexes[a]

complex[b]	r_t[c]	[Cu(II)]/[DNA P][d]	[Cu(II)]/[drug][d]	absorption (nm)	CD (nm)
1-nDNA	0.02			540 (sh)	
				507	506 (+)
				490 (sh)	382 (+)
				390	
1-Cu(II)-nDNA	0.02	0.18	9	510 (sh)	
				545	590 (+)
				581	552 (+)
				390 (sh)	355 (+)
1-nDNA	0.5			540 (sh)	520 (+)
				490 (br)	375 (+)
				390 (sh)	
1-Cu(II)-nDNA	0.5	1	2	585 (sh)	600 (+)
				545	570 (+)
				515 (sh)	500 (−)
				400	430 (sh) (+)
					390 (+)
3-nDNA	0.02			540 (sh)	
				508	520 (+)
				490 (sh)	365 (+)
				400 (sh)	
3-Cu(II)-nDNA	0.02	0.12	6	580 (sh)	
				540	566 (+)
				515	500 (−)
				400 (sh)	435 (sh) (+)
					390 (+)
1-dDNA	0.02			540 (sh)	510 (+)
				506	366 (+)
				485 (sh)	
				390 (sh)	
1-Cu(II)-dDNA	0.02	0.12	6	587	595 (+)
				557	525 (+)
				390 (sh)	364 (+)
3-dDNA	0.02			540 (sh)	460 (+)
				495 (br)	
				390 (sh)	360 (+)
3-Cu(II)-dDNA	0.02	0.16	8	589	600 (+)
				555	562 (+)
				390 (sh)	460 (sh) (+)
					415 (+)
Cu(II)-1 or 3[e]				572	580 (+)
				532	540 (+)
				499 (sh)	400 (sh) (+)
				390	350 (+)
Cu(II)-1 or 3[f]				572	580 (+)
				532	540 (+)
				499 (sh)	400 (sh) (+)
				390	350 (+)

[a] The salt concentration of the solution containing DNA was 1 mM. [b] nDNA is native calf thymus DNA. dDNA is heat-denatured calf thymus DNA. [c] r_t is the input drug-DNA ratio. [d] Values given in these columns define the end point of the titration. [e] In water solution. [f] In ethanol solution.

spectral changes that occurred during the titration did not change with time after the time of mixing of the reagents (up to 2 h), and they were independent of the order of addition of the reagents used [drug, Cu(II), DNA] to form the complex.

At high concentrations of DNA (5.8×10^{-3} M, P) and after significant amounts of Cu(II) had been added to solution ([Cu(II)]/[drug] 9), titrations produced a small amount of violet precipitate. The color of the precipitate indicated the presence of a copper-bond hydroxyquinone function (Greenaway & Dabrowiak, 1982), while an infrared spectrum of the solid recovered by centrifugation showed the presence of DNA: Nujol mull (cm^{-1}) ~1650 (m, br), 1300 (w), 1235, 1090, 970. The formation of the precipitate did not significantly interfere with the optical studies on the complex.

Results

Daunomycin at $r_t < \sim 0.1$. Addition of Cu(II) to the native DNA complex of daunomycin at $r_t < \sim 0.1$ causes the absorption band associated with the hydroxyquinone chromophore of the drug to sharpen and shift to lower energy, Table I and Figure 1. The absorbance data for this and other titrations at six different wavelengths are collected in Table II. The titration is characterized by a sharp isosbestic point at 520 nm. At the end point of the titration, as defined by $\Delta Abs/\Delta[Cu(II)] < \sim 5 \times 10^{-2}$ M^{-1}, the solution containing the drug, the Cu(II) ion, and DNA exhibits two well-defined absorption maxima at 545 and 581 nm, Table I. The amount of Cu(II) necessary to reach an end point as measured by the ratio, [Cu(II)]/[drug], is dependent on both r_t and the salt concentration of the medium. For r_t values of 0.11 and 0.02 and a salt concentration of 1 mM, the values of [Cu(II)]/[**1**] necessary to produce an end point are ~4 and ~9, respectively. If, on the other hand, r_t and [Cu(II)]/[**1**] are held constant and the salt concentration of the medium is varied, an *increase* in the salt concentration results in a *decrease* in the amount of the new optical species present in solution. For $r_t = 0.02$ and [Cu(II)]/[**1**] of 9, the relative amounts of the new optical species present in solutions having salt concen-

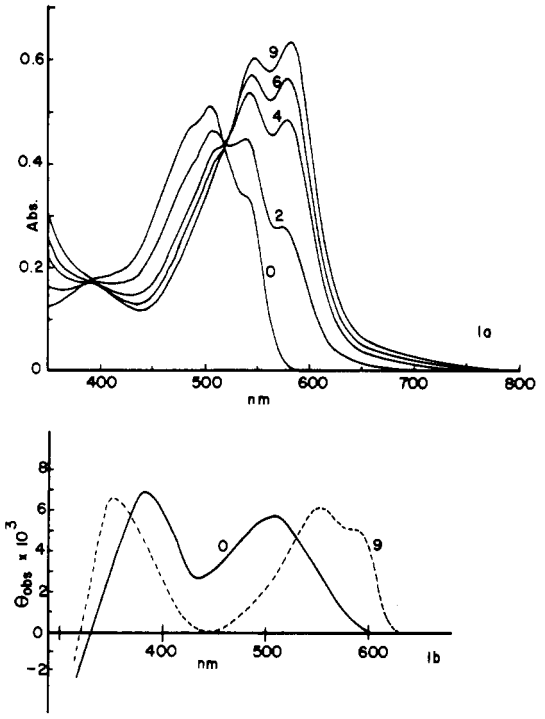


FIGURE 1: (a) Absorbance changes that occur when the daunomycin–native calf thymus DNA complex, $r_t = 0.02$, is titrated with Cu(II): [1] 7.0×10^{-5} M, [DNAP] 3.5×10^{-3} M, and [NaCl] 1 mM. The values of [Cu(II)]/[1] are given on the individual spectra. (b) The CD spectra of the above titration at a [Cu(II)]/[1] of 0 and 9 are shown.

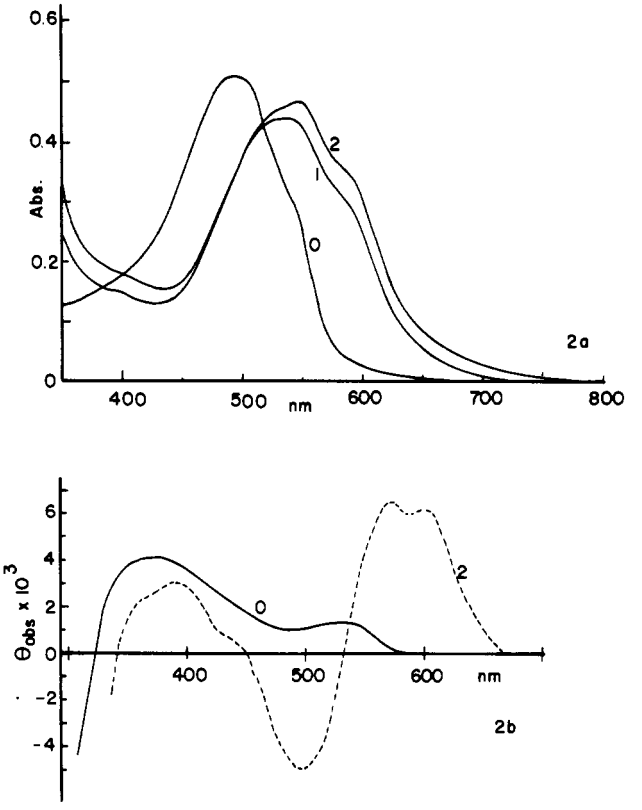


FIGURE 2: (a) Absorbance changes that occur when the daunomycin–native calf thymus DNA complex, $r_t = 0.5$, is titrated with Cu(II): [1] 7.0×10^{-5} M, [DNAP] 1.4×10^{-4} M, and [NaCl] 1 mM. The values of [Cu(II)]/[1] are given on the individual spectra. (b) The CD spectra of the above titration at a [Cu(II)]/[1] of 0 and 2 are shown.

trations of 1 mM, 10 mM, 0.1 M, 1 M, and 2 M were 1.0, 0.93, 0.86, 0.74, and 0.58, respectively. The relative amounts were determined by measurement of the absorbance at 581 nm. Titrations with 1 and 2 M NaCl, at $r_t = 0.02$, did not exhibit isosbestic behavior, and the end point spectra were less well-defined than titrations carried out at low salt.

The Cu(II) titration of the drug–native DNA complex was also followed by circular dichroism. Addition of Cu(II) caused the positive CD band of the daunomycin–DNA complex at 506 nm to split into two bands at 552 and 590 nm. The positive CD band of the drug–DNA complex at 382 nm retains the same sign but shifts to 355 nm in the presence of Cu(II), Figure 1b, Table I. Spectrometer noise, the relatively small magnitude of the effect, and the concentration at which the experiments were carried out obscured observation of sharp isoeliptic points, but they appear to be present at ~520 and ~370 nm. Strong overlap of the transitions from DNA with those of the antibiotics below ~350 nm prevented interpretation of absorption and circular dichroism data, in the high-energy region of the spectrum.

Daunomycin at $r_t > \sim 0.1$. Addition of Cu(II) to the drug–native DNA complex at r_t of 0.5 to ~0.1 results in spectral changes that are different from those observed at $r_t < \sim 0.1$. The titration did not exhibit an isosbestic point, and the absorption spectrum at the end of the titration is broad and relatively featureless, Figure 2a. Circular dichroism clearly shows that the species that is produced at the end of the titration with r_t of 0.5 is not the same as that observed with $r_t < \sim 0.1$, Figure 2b. The CD spectrum of the solution at the end of the titration has positive CD bands at 600, 570, 430, and 390 nm and a negative CD absorption at 500 nm. The titration does not exhibit an isoeliptic point.

Antibiotic 3 at r_t of 0.02. Addition of Cu(II) to the *N*-(trifluoroacetyl)daunomycin (3) complex of native DNA (1 mM NaCl) causes the drug chromophore to broaden and shift

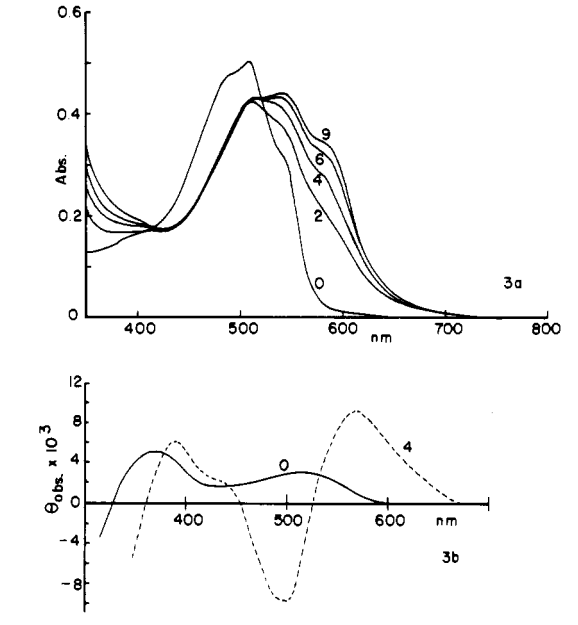


FIGURE 3: (a) Absorbance changes that occur when the *N*-(trifluoroacetyl)daunomycin–native calf thymus DNA complex, $r_t = 0.02$, is titrated with Cu(II): [3] 6.7×10^{-5} M, [DNAP] 3.35×10^{-3} M, and [NaCl] 1 mM. The values of [Cu(II)]/[3] are given on the individual spectra. (b) The CD spectra of the above titration at a [Cu(II)]/[3] of 0 and 4 are shown.

to lower energy, Figure 3a. The titration does not exhibit isosbestic behavior, and at the end point, the absorption spectrum possesses three broad maxima at 580, 540, and 515 nm. The CD spectrum at the end point, Figure 3b, exhibits positive maxima at 566, 435, and 390 nm, while a negative

Table II: Absorbance Data[a]

conditions			absorbance at a wavelength (nm) of					
[drug] × 10^5 (M)	[DNAP] × 10^3 (M)	[Cu(II)] × 10^4 (M)	600	580	550	500	475	450
7.0[b]	3.5	0	0	0.010	0.286	0.526	0.457	0.308
		1.4	0.080	0.201	0.373	0.488	0.392	0.258
		2.8	0.260	0.455	0.502	0.435	0.314	0.200
		4.2	0.401	0.601	0.582	0.391	0.255	0.156
		6.3	0.481	0.675	0.625	0.362	0.218	0.129
8.0[c]	4.0	0	0.006	0.034	0.344	0.656	0.580	0.409
		0.7	0.275	0.468	0.578	0.550	0.412	0.275
		1.4	0.510	0.700	0.711	0.465	0.312	0.207
		2.1	0.690	0.845	0.788	0.411	0.252	0.166
		2.8	0.725	0.904	0.824	0.386	0.225	0.149
		3.5	0.780	0.930	0.845	0.367	0.215	0.137
		4.2	0.825	0.950	0.848	0.350	0.204	0.130
6.7[d]	3.35	0	0.035	0.052	0.254	0.578	0.547	0.391
		1.34	0.396	0.493	0.505	0.350	0.236	0.162
		2.68	0.510	0.569	0.555	0.334	0.219	0.155
		4.02	0.560	0.620	0.591	0.328	0.214	0.151
		5.36	0.593	0.652	0.608	0.315	0.205	0.146
		6.7	0.608	0.658	0.611	0.310	0.198	0.139

[a] All data were collected in 1 mM NaCl. [b] Daunomycin with native DNA. [c] Daunomycin with denatured DNA. [d] *N*-(Trifluoroacetyl)-daunomycin with denatured DNA.

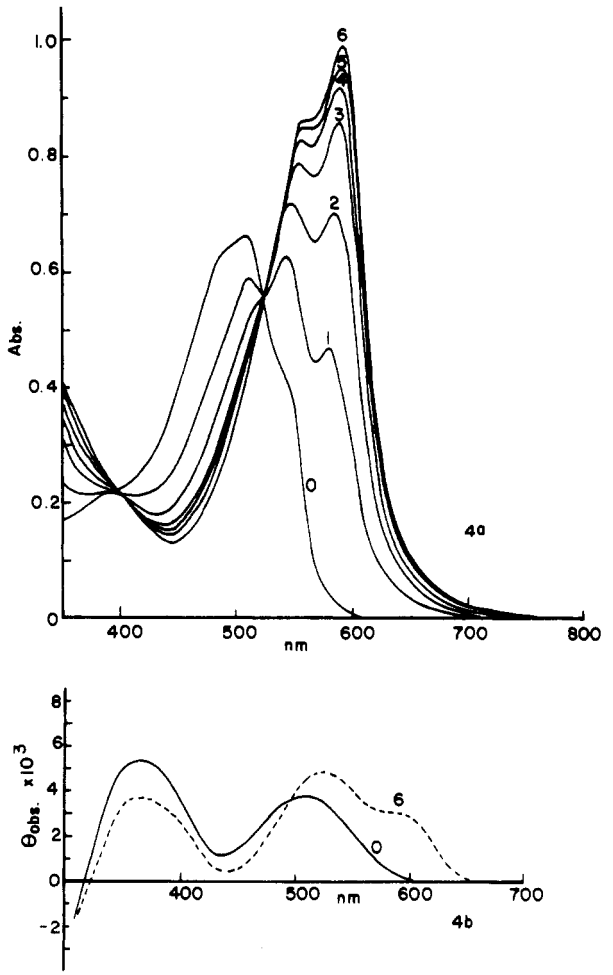


FIGURE 4: (a) Absorbance changes that occur when the daunomycin–heat-denatured calf thymus DNA complex, $r_t = 0.02$, is titrated with Cu(II): [1] 8.0×10^{-5} M, [DNAP] 4.0×10^{-3} M, and [NaCl] 1 mM. The values of [Cu(II)]/[1] are given on the individual spectra. (b) The CD spectra of the above titration at a [Cu(II)]/[1] of 0 and 6 are shown.

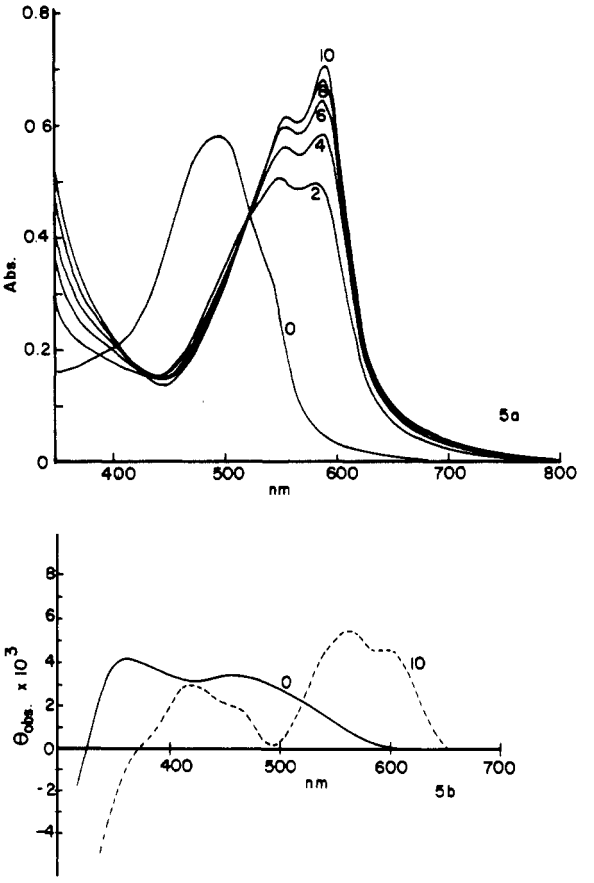


FIGURE 5: (a) Absorbance changes that occur when the *N*-(trifluoroacetyl)daunomycin (**3**)–heat-denatured DNA complex, $r_t = 0.02$, is titrated with Cu(II): [**3**] 6.7×10^{-5} M, [DNAP] 3.35×10^{-3} M, and [NaCl] 1 mM. The values of [Cu(II)]/[**3**] are given on the individual spectra. (b) The CD spectra for the above titration at a [Cu(II)]/[**3**] of 0 and 10 are shown.

CD envelope appears at 500 nm.

Heat-Denatured DNA. Antibiotics **1** *and* **3**. Titration of the *denatured* DNA complexes of daunomycin or its N-trifluoroacylated derivative with Cu(II) at $r_t < \sim 0.1$ leads to a species that is optically very similar to that produced by addition of Cu(II) to the *native* DNA complex of **1** at $r_t < \sim 0.1$. The absorption and CD spectra associated with the titrations involving the two drugs are shown in Figures 4 and 5, and the spectral data for the complexes are collected in

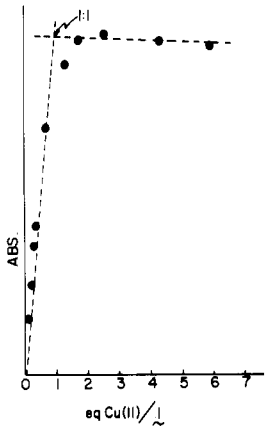


FIGURE 6: A Job plot of the titration of **1**–dDNA at $r_t = 0.11$ is shown. The absorbance of the solution at 587 nm is plotted against the equivalents of Cu(II) added per equivalent of antibiotic.

Tables I and II. The titration of the denatured DNA complex with either **1** or **3** exhibits an isosbestic point at 522 nm. The absorption spectra at the end of the titration possesses two well-defined absorption maxima in the region 555–590 nm. The spectra are qualitatively similar to the end point spectrum involving native DNA and **1** at $r_t < \sim 0.1$, Figure 1. The CD spectra of the species present at the end point exhibit two positive CD maxima in the region 520–600 nm and an additional positive band at 360–415 nm, Table I. For daunomycin, the amount of Cu(II) necessary to reach the end point in the titration involving *denatured* DNA was significantly less than that necessary to reach the end point with *native* DNA, Table I. The increased sensitivity of the spectral changes to added Cu(II) in the denatured system allowed the determination of the Cu(II)–drug stoichiometry of the new species. As is shown in Figure 6, a Job plot of the absorbance at 585 nm (the low-energy maximum of the new optical species formed in the titration) vs. the equivalents of Cu(II) added to the daunomycin–*denatured* DNA complex at $r_t = 0.11$ shows that the Cu(II)–drug stoichiometry is ≤ 1.

At $r_t > \sim 0.1$, the titration of the denatured DNA complex of the drugs with Cu(II) results in spectral changes that do not exhibit isosbestic behavior. The absorption and CD spectra at the end point of the titration indicate the presence of both Cu(II)–**1** [Cu(II)–**3**] and a new optical species.

Discussion

Binary Equilibria. Crucial to understanding the factors that control the formation of a ternary complex between Cu(II), the antibiotic, and DNA is knowledge of the binary equilibria, eq 2–4.

$$\text{drug} + \text{DNA} \rightleftharpoons \text{drug–DNA} \quad (2)$$

$$\text{DNA} + \text{Cu(II)} \rightleftharpoons \text{DNA–Cu(II)} \quad (3)$$

$$\text{drug} + \text{Cu(II)} \rightleftharpoons \text{drug–Cu(II)} \quad (4)$$

It is well-known that daunomycin interacts with calf thymus DNA to form a drug–DNA complex, eq 2 (DiMarco & Arcamone, 1975; Barthelemy-Clavey et al., 1973). The type of binding that occurs between the drug and DNA, and its strength, appears to be sensitive to antibiotic and DNA structure, salt concentration, and the drug–DNA ratio in solution. At $r_b < \sim 0.2$, where r_b is the molar ratio of bound drug to DNA P, daunomycin strongly binds to native calf thymus DNA to form (**1**–nDNA)$_s$. The association constant of the complex is 1.2×10^7 M^{-1} (10 mM NaCl). The binding constant is salt dependent, and at a salt concentration of 1 M it is reduced to 1.3×10^6 M^{-1} (Zunino et al., 1980). The strong binding interaction is characterized by an intercalation of the aglycon portion of the antibiotic into the base pairs of DNA, accompanied by an electrostatic interaction between the protonated amine group of daunosamine and a nearby phosphate group on DNA. Optical measurements further show that at $r_b > \sim 0.2$ additional binding can occur but that it is weaker than the primary binding and it exhibits a significant salt dependence. It has been suggested that the weaker binding is associated with daunomycin molecules that are only electrostatically bound to (not intercalated into) the DNA helix (**1**–nDNA)$_w$. Daunomycin is also capable of binding to heat-denatured DNA, and both strong and weak binding (**1**–dDNA)$_{s,w}$, as a function of r_b, have been observed (Barthelemy-Clavey et al., 1973). The structure of the drug–denatured DNA complex is unknown.

Reduction of the basic properties of the amine function on C3′ of daunosamine significantly weakens the binding affinity of the antibiotic for DNA. Thus, *N*-(trifluoroacetyl)daunomycin (**3**) has a native DNA association constant of $\sim 10^3$ M^{-1} and exhibits only a modest effect on the melting point of DNA (ΔT_m 1 °C). Although the structure of the drug–DNA complex involving **3** (**3**–nDNA) is unknown, the melting point and binding data suggest that the antibiotic does not intercalate into DNA.

Copper(II) interactions with DNA have been extensively studied (Eichhorn, 1973; Förster et al., 1979; Rifkind et al., 1976; Zimmer et al., 1971; Eichhorn & Clark, 1965). At low Cu(II)–DNA ratios, [Cu(II)]/[DNA P] $< \sim 0.3$, and at low salt concentration, Cu(II) binds in a nondenaturing fashion to native DNA. The binding interaction is characterized by an increase in the melting point of DNA and by significant changes in the absorption and CD spectra of DNA. Although the structure of the complex is unknown, the binding interaction most likely involves the phosphate groups of DNA (Zimmer et al., 1971; Eichhorn & Clark, 1965; Förster et al., 1979). At Cu(II)–DNA P ratios $> \sim 0.3$, Cu(II) produces further changes in the physical properties of native DNA. Kinetically, the rate of formation of the second type of interaction is slower than that of the first type, and it appears to involve direct binding of Cu(II), in a denaturing fashion, to the heterocyclic bases of DNA. Studies with various homopolymers and with DNAs of varying GC content have suggested that guanine and cytosine are involved in the second type of binding. Native, duplex DNA can be regenerated from the Cu(II)–DNA adduct, by increasing the salt concentration of the medium or by addition of EDTA. Structurally, the second type of binding is thought to involve G-C base pairs on opposite strands of DNA that are linked by a copper ion. Heat denaturation of DNA followed by the addition of Cu(II), or denaturation of DNA in the presence of Cu(II), produces complexes that are spectroscopically very similar to that produced by addition of Cu(II) to native DNA at Cu(II)–DNA ratios of $> \sim 0.3$ (Förster et al., 1979).

Copper(II) binding studies with the anthracyclines, eq 4, have recently been reported by Greenaway & Dabrowiak (1982). In unbuffered aqueous media in the pH range 4–8, **1**–**3** react with Cu(II) to form two water-soluble complexes having Cu(II)–drug stoichiometries of 1:1 and 1:2. The compounds exhibit similar absorption spectra, but their CD spectra are dramatically different from one another. Copper(II) also reacts with **1**–**3** in ethanol solution to produce a 1:1 complex having optical properties different than those of the 1:1 complex that forms in water, Figures 7 and 8. The tendency of the antibiotic to self-associate in aqueous media

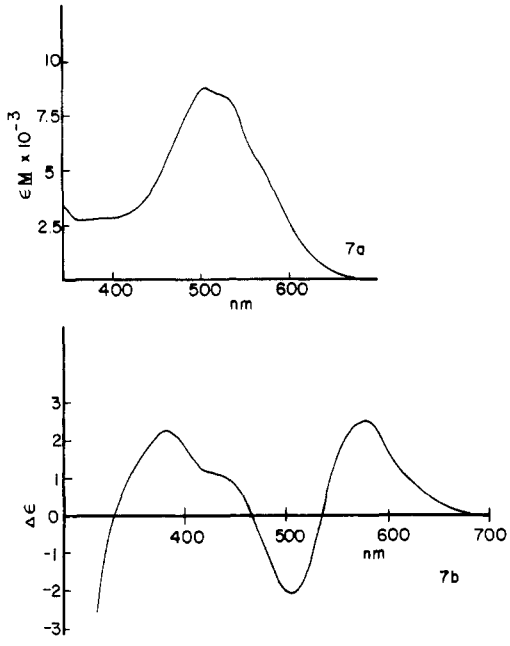


FIGURE 7: Absorption (a) and CD (b) spectra of Cu(II)–**1** or **3** in water are shown. Although these complexes have pK_as of formation of 5.6 in water, the formation is sensitive to other ions in solution. They readily form at pH 7.0 in aqueous solution containing NaCl. The values of ϵ_M and $\Delta\epsilon$ were determined by using daunomycin and assuming that the total amount of antibiotic present in solution exists as Cu(II)–**1**. The positions of the absorption maxima and the CD maxima and minima for both Cu(II)–**1** and Cu(II)–**3** were identical (Greenaway & Dabrowiak, 1982).

(Martin, 1980) suggests that the copper–daunomycin complexes that form in water are polynuclear while the one that forms in ethanol is mononuclear. In either solvent, metal binding has been interpreted in terms of Cu(II) interacting with one of the deprotonated quinone functions at C5,6 or C11,12 of the aglycon portion of the antibiotic. The amine group on C3′ of daunosamine does not appear to be important in binding Cu(II) (Greenaway & Dabrowiak, 1982).

Ternary System. Metal Ion–Drug–DNA. At low r_t, $0.02 \leq r_t < \sim 0.1$, where a high population of antibiotic molecules are bound to DNA, titration of the native DNA complex of daunomycin and the heat-denatured DNA complexes of **1** and **3** with Cu(II) results in the formation of a ternary complex involving the drug, Cu(II), and DNA, eq 5–7. Although, the

$$\text{Cu(II)} + (\textbf{1}\text{–nDNA})_s \rightleftharpoons \text{Cu(II)}\text{–}\textbf{1}\text{–nDNA} \quad (5)$$

$$\text{Cu(II)} + (\textbf{1}\text{–dDNA})_s \rightleftharpoons \text{Cu(II)}\text{–}\textbf{1}\text{–dDNA} \quad (6)$$

$$\text{Cu(II)} + (\textbf{3}\text{–dDNA}) \rightleftharpoons \text{Cu(II)}\text{–}\textbf{3}\text{–dDNA} \quad (7)$$

absorption and CD spectra at the end point of the titration are qualitatively similar to those of Cu(II)–**1** [Cu(II)–**3**] in ethanol, Figures 1, 4, 5, and 8 and Table I, they are significantly different than any of those of the known Cu(II)–daunomycin complexes. The isosbestic nature of the titration indicates that the bound drug chromophore is converted to a single new species or to a mixture of species each having identical or nearly identical formation constants.

The optical properties of the ternary complex indicate that the Cu(II) ion is bound directly to the aglycon of the antibiotic. The π–π^* transitions of the chromophore in the ternary complexes are shifted $\sim$60 nm to lower energy relative to either the metal-free antibiotics or their DNA complexes. Spectroscopic studies on complexes of hydroxyquinones (Underwood et al., 1950) and optical, magnetic resonance, and proton release studies on Cu(II) complexes of daunomycin and its analogues (Greenaway & Dabrowiak, 1982) show that metal

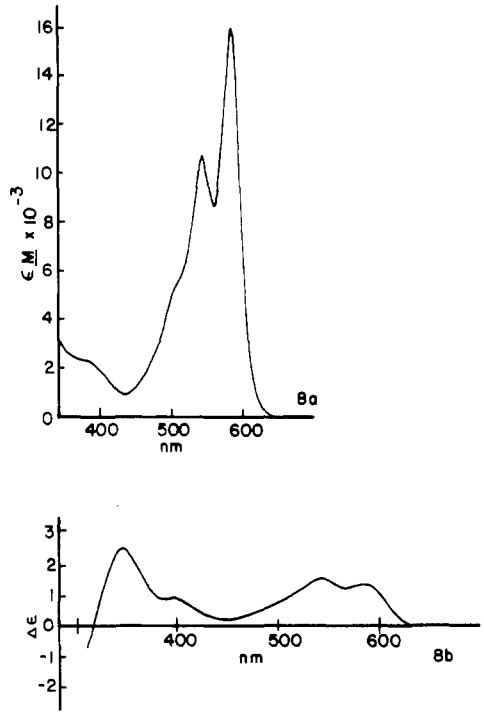


FIGURE 8: Absorption (a) and CD (b) spectra of Cu(II)–**1** in ethanol are shown. The values of ϵ_M and $\Delta\epsilon$ were calculated as stated in the caption to Figure 7. The positions of the absorption and CD bands for Cu(II)–**1** and Cu(II)–**3** were identical.

binding to the deprotonated quinone functions of either **1** or **3** causes the π–π^* transitions in the visible region of the spectrum to shift to lower energy, Figures 1, 4, 5, and 8.

The CD spectra of the ternary complex are also consistent with a direct Cu(II)–drug interaction. The low-energy positive CD bands, associated with electronic transitions polarized along the short axis of the aglycon (Gabby et al., 1976), are shifted to low energy relative to either the metal-free antibiotics or their DNA complexes, Table I. In addition, the positive CD bands in the region 350–415 nm (long axis polarized) for Cu(II)–**1**–nDNA (dDNA) and Cu(II)–**3**–dDNA have the same sign as do the Cu(II) complexes of daunomycin and its *N*-trifluoroacetyl analogue in either ethanol or water, Table I.

The dichroic strengths, $\Delta\epsilon_i/\epsilon_i$, of the low-energy bands of the ternary species are sensitive to complex structure. The uncertainty in the binding constant for eq 1 and the complicated equilibria for all of the species involved preclude accurate determination of $\Delta\epsilon_i$ and ϵ_i. However, reference to Figures 1 and 4 and the spectrum of Cu(II)–**1** in ethanol, Figure 8, clearly shows that the dichroic strengths of the short axis polarized transitions of the drug chromophore are $\sim 10^{-4}$ and that $\Delta\epsilon_i/\epsilon_i$ increases in the order Cu(I)–**1** < Cu(II)–**1**–DNA < **1**–DNA. On the other hand, the dichroic strength for the long axis polarized band (at 380 nm) is $\sim 10^{-3}$, and it is essentially constant for all of the aforementioned complexes. Thus, the CD data suggest that in the ternary complex, the asymemtry of DNA exerts itself along the short axis of the aglycon of the antibiotic.

Although the Cu(II)–antibiotic stoichiometry in the ternary complex cannot be unequivocally assigned, it must be ≤ 1. Job plots of the absorbance at 587 nm vs. the equivalents of Cu(II) added for daunomycin and *denatured* DNA show that the apparent stoichiometry of the interaction is sensitive to r_t. At an r_t of 0.02 where the DNA is in large excess and a high population of antibiotic molecules are bound to DNA, a Job plot indicates that the Cu(II)–drug ratio for the ternary complex is ~ 4. However, as r_t is increased and r_b approaches

the transition region between strong and weak binding of daunomycin to DNA (Barthelemy-Clavey et al., 1973), the apparent Cu(II)–drug stoichiometry for Cu(II)–**1**–dDNA, as determined from a Job plot, approaches 1:1. A Job plot of the absorbance vs. added Cu(II) at r_t of 0.11 for Cu(II)–**1**–dDNA is shown in Figure 6. The variation in the apparent stoichiometry as a function of r_t within the strong binding region appears to be due to the fact that Cu(II) ions can bind competitively to DNA sites that do not have bound antibiotic (eq 3) as well as interact with the drug chromophore. Cu(II) binding to sites other than the drug chromophore reduces the amount of available $[Cu^{II}\cdot H_2O)_6]^{2+}$ in solution. Since only spectroscopic changes associated with the drug chromophore are being measured during the titration, competitive binding of Cu(II) to denatured DNA has the effect of overestimating the true Cu(II)–drug stoichiometry as determined from a Job plot. Although increasing r_t should result in a more accurate determination of the Cu(II)–drug stoichiometry, at $r_t > \sim 0.1$, the titration loses isosbestic behavior, and both the ternary complex and Cu(II)–**1** form, eq 8. Thus, it appears that, while

$$\text{Cu(II)} + (\textbf{1}\text{–dDNA})_{s,w} \rightleftharpoons \text{Cu(II)}\text{–}\textbf{1}\text{–dDNA} + \text{Cu(II)}\text{–}\textbf{1} \quad (8)$$

the strongly bound antibiotic molecules react with Cu(II) to produce the ternary species, $r_t < \sim 0.1$, those that are only weakly bound or are free in solution, $r_t > \sim 0.1$, yield the binary complex Cu(II)–**1**. Since the spectroscopic changes for the system involving **1** and native DNA were less sensitive to added Cu(II) than those of the analogous system with denatured DNA, Cu(II)–drug stoichiometries determined from Job plots for the former system were $\gg 1$. As with denatured DNA, titration of $(\textbf{1}\text{–nDNA})_{s,w}$, $r_t > \sim 0.1$, resulted in the formation of both Cu(II)–**1** and the ternary complex Cu(II)–**1**–nDNA, eq 9. The spectral similarities between all of

$$\text{Cu(II)} + (\textbf{1}\text{–nDNA})_{s,w} \rightleftharpoons \text{Cu(II)}\text{–}\textbf{1}\text{–nDNA} + \text{Cu(II)}\text{–}\textbf{1} \quad (9)$$

the ternary complexes and Cu(II)–**1** [Cu(II)–**3**] in ethanol suggest that all complexes have a common stoichiometry.

Comparison of the titration results of **3**–nDNA with those of **3**–dDNA indicates that the DNA bases are important to the formation of the ternary complex. Titration of the native DNA complex of **3** with Cu(II), under conditions that produce a high population of bound but very likely unintercalated antibiotic (r_t of 0.02), does not result in the formation of a ternary complex. Absorption and CD spectra show that the species present in solution at the end point of the titration is Cu(II)–**3**, Table I, eq 10. However, if the DNA is first heat

$$\text{Cu(II)} + \textbf{3}\text{–nDNA} \rightleftharpoons \text{nDNA} + \text{Cu(II)}\text{–}\textbf{3} \quad (10)$$

denatured and the denatured DNA complex of **3** titrated with Cu(II), the ternary species forms, eq 7 and Figure 5. Since denaturation exposes binding sites on the heterocyclic bases of DNA, it appears that the bases are important in ternary complex formation. If binding to only the phosphate groups of DNA were important to the formation of the ternary complex, **3**–nDNA would be expected to produce the ternary complex. However, the titration results show that this is not the case.

Because of the large number of competing equilibria, calculation of the equilibrium constant for the ternary complex was not attempted. However, from the total amount of added Cu(II) and the Cu(II)–drug ratio in solution at the end point of the titration, the equilibrium constant for the ternary complex is about 1 order of magnitude greater than the binding affinity of a Cu(II) ion for a single nucleotide unit of DNA. The binding constant of Cu(II) for DNA at Cu(II)–DNA P ratios $< \sim 0.3$, the levels of Cu(II) used in the Cu(II)–drug–DNA titrations, is unknown. However, the binding constant for Cu(II) interacting with the heterocyclic bases of DNA ($[Cu(II)]/[DNA\ P] > \sim 0.3$) has been reported to be $116 \pm 36\ M^{-1}$ (Förster et al., 1979). The observation that weakly coordinating buffers, e.g., Hepes and Tris, inhibit the formation of the ternary complex (see Experimental Procedures) indicates that the stability constant for the complex is relatively small.

The equilibrium involving the ternary complex is sensitive to the salt concentration of the medium. Increasing the salt concentration is known to decrease the affinity of both the drug (Zunino et al., 1980) and Cu(II) (Förster et al., 1979) for DNA. Thus, high salt would decrease the population of bound drug thus inhibiting the formation of the ternary complex. Titrations of **1**–nDNA at r_t of 0.02 in 1 and 2 M NaCl solutions produced broadened absorption spectra indicative of a small amount of Cu(II)–**1** present in solution with the ternary complex.

At least three observations suggest that the ternary complex possesses an *unintercalated* aglycon.

(i) A ternary complex involving the *intercalated* aglycon would be expected to exhibit an optical spectrum that reflects the presence of both the metal interaction and the π interaction from the heterocyclic bases of DNA. The anthraquinone moiety of daunomycin possesses an absorption maximum at 477 nm (Gabbay et al., 1976) that shifts $\sim 1000\ cm^{-1}$ to lower energy when the drug is intercalated into DNA. On the other hand, Cu(II) binding to the aglycon results in a shift to lower energy of $\sim 3000\ cm^{-1}$ (Greenaway & Dabrowiak, 1982). Thus, a ternary species involving *both* the intercalated and Cu(II)-bound aglycon would be expected to exhibit a band pattern centered at ~ 590 nm ($4000\text{-}cm^{-1}$ red shifted from 477 nm) rather than at ~ 560 nm, which is observed for the ternary species. The observed spectral shifts can be accounted for solely on the basis of Cu(II) interacting with the aglycon of daunomycin, suggesting that the π interaction that accompanies intercalation is absent.

(ii) The spectral changes that occur with daunomycin and denatured DNA occur at lower levels of added Cu(II) than the changes observed with native DNA. Furthermore, the ternary complex with *N*-(trifluoroacetyl)daunomycin requires denaturation of DNA for its formation. Since thermodenaturation of the hydrogen-bonded base pairs of DNA facilitates the formation of the ternary complexes, donor sites on the heterocyclic bases appear to be important to complex formation. Coordination of the base and the drug to the Cu(II) ion would not appear to be possible if the antibiotic remained in the intercalation site.

(iii) X-ray crystallographic and solution studies of the daunomycin-DNA complex (Quigley et al., 1980; Patel & Canuel, 1978; Gabbay et al., 1976) show that the metal binding sites on the aglycon, C5,6 and C11,12, lie within the intercalation site and are not readily available for binding Cu(II). The earlier reported difficulty in deprotonating the intercalated aglycon is consistent with the sterically inhibited nature of the quinone oxygen atoms (Calendi et al., 1965).

In summary, addition of Cu(II) to the native calf thymus DNA complex of intercalated daunomycin results in the formation of a ternary complex involving Cu(II), the unintercalated but metal-bound aglycon, and most likely the heterocyclic basis of DNA. On the other hand, reducing the binding constant of daunomycin toward native DNA by saturation of the strong DNA binding sites, $r_t > \sim 0.1$ (1 mM NaCl), increasing the salt concentration of the medium, or

acylation of the amino group of daunosamine (3) results in the formation of a binary complex involving only the drug and Cu(II).

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Synthesis and Properties of ApU Analogues Containing 2′-Halo-2′-deoxyadenosines. Effects of 2′ Substituents on Oligonucleotide Conformation†

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ABSTRACT: Five A-U analogues containing deoxyadenosine or 2′-halo-2′-deoxyadenosines, which are known to have widely different C3′-endo conformer populations according to their electronegativities of the halogen substituents, dAfl-U, dAcl-U, dAbr-U, dAio-U, and dA-U, were synthesized chemically. Characterization of these dimers has been performed by UV absorption, circular dichroism, and proton nuclear magnetic resonance spectroscopy. The results show that the dimers containing 2′-halo-2′-deoxyadenosines have stacked conformations with a geometry similar to that of A-U and the degree of stacking decreases in the order dAfl-U > dAcl-U > dAbr-U > dAio-U. dAcl-U is assumed to have the same degree of stacking as A-U. dA-U takes a more stacked conformation than does dAio-U, but the mode of stacking is different from those of the other dimers. The effects of the 2′ substituents on dimer conformation are discussed in terms of electronegativity, molecular size, and hydrophobicity.

DNA and RNA have different structures as well as different functions. The difference in chemical structures resides in α substituents at C2′ (H for DNA and OH for RNA). It is known that DNA usually takes a B-form structure and that RNA only takes an A-form structure from X-ray diffraction analysis of nucleic acid fibers (Arnott, 1970). Recently, these conclusions were confirmed by X-ray analysis of crystals of a double-helical dodecadeoxyribonucleotide (Drew et al., 1981) and a tRNA [e.g., Quigley et al. (1975)]. One of the major conformational differences in the monomer units of DNA and RNA, which produces differences in polymer conformation as a whole, is the difference in sugar puckering (C2′ endo for B-form DNA and C3′ endo for RNA). It is known that the furanose ring conformation of nucleoside derivatives in solution can be described as an equilibrium between C3′-endo and C2′-endo forms (Altona & Sundaralingam, 1973; Evans & Sarma, 1974). Recently, it was shown from ^{1}H NMR[1] studies

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[1] Abbreviations: dAfl, 2′-fluoro-2′-deoxyadenosine; dAcl, 2′-chloro-2′-deoxyadenosine; dAbr, 2′-bromo-2′-deoxyadenosine; dAio, 2′-iodo-2′-deoxyadenosine; NMR, nuclear magnetic resonance; CD, circular dichroism; UV, ultraviolet; TLC, thin-layer chromatography; DSS, sodium 3-(trimethylsilyl)propane-1-sulfonate; MS, mass spectrum; MeOTr, monomethoxytrityl; Bz, benzoyl; DMF, dimethylformamide; TEAB, triethylammonium bicarbonate; DCC, dicyclohexylcarbodiimide; DEAE, diethylaminoethyl; other abbreviations principally follow the recommendation of the IUPAC–IUB Commission on Biochemical Nomenclature (1970, 1977).

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