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Fluorescence-Detected Circular Dichroism of Ethidium Bound to Poly(dG-dC) and Poly(dG-m^{5}dC) under B- and Z-Form Conditions†

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ABSTRACT: The equilibrium binding of ethidium to poly(dG-dC) and poly(dG-m^{5}dC) under conditions favoring B and Z forms was investigated with fluorescence-detected circular dichroism (FDCD) and optical titration methods. FDCD spectra indicate a similar geometry for the intercalated ethidium under both B- and Z-form conditions, even at low levels of bound ethidium. The magnitude of the 310–330-nm FDCD band as a function of the bound drug to base pair ratio (r) indicates ethidium binds to poly(dG-dC) in 4.4 M NaCl and to poly(dG-m^{5}dC) in 25 mM $MgCl_2$ by clustering. Under these conditions, circular dichroism spectra indicate the polymer is largely Z form. Thus, it appears ethidium clusters into regions it has induced into a right-handed form. For all conditions studied, the FDCD spectra provided no evidence for a left-handed binding site. Under B-form conditions, binding is random.

Pohl & Jovin (1972) discovered a salt-induced conformational transition in poly(dG-dC) that has been assigned to conversion to left-handed poly(dG-dC) (Wang et al., 1979, 1981; Patel et al., 1979; Drew & Dickerson, 1982; Wartell et al., 1983). Although ethidium binding to native and synthetic DNAs under B-form conditions has been well characterized (Lepecq & Paoletti, 1967; Dalgleish et al., 1971; Houssier et al., 1974; Aktipis et al., 1975; Krugh et al., 1975; Olmstead & Kearns, 1977; Bresloff & Crothers, 1981; Dahl et al., 1982), much less is known of the interaction of ethidium with left-handed DNA. Pohl et al. (1972) reported that ethidium binding to left-handed poly(dG-dC) in 4.4 M NaCl solution results in the reversal of the salt-induced conformational transition. Krugh & Walker (1984) and Walker et al. (1985) used circular dichroism and optical absorption spectroscopies to monitor the binding of ethidium to poly(dG-dC) and poly(dG-m^{5}dC) under both B- and Z-form conditions and concluded that ethidium intercalation results in the formation of a right-handed binding site. Conversely, Shafer et al. (1984) studied the binding of ethidium to left-handed poly(dG-dC) in 4.4 M NaCl and suggested that ethidium forms a left-handed intercalation site at low levels of bound drug. The plausibility of a left-handed intercalation site is supported by model building and theoretical studies (Gupta et al., 1983). Van de Sande & Jovin (1982) showed that Z* DNA [a condensed form of poly(dG-dC) in ethanol solution] supports the binding of several intercalating ligands, including ethidium.

A complicating factor in interpreting the CD[1] spectra of ethidium complexes with polymers is that the observed CD

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[1] Abbreviations: FDCD, fluorescence-detected circular dichroism; CD, circular dichroism; bp, base pair(s); EDTA, ethylenediaminetetraacetic acid.

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is a sum of the CD arising from intercalated ethidium and the base pairs both at the intercalation site and in the rest of the polymer. Thus, at low r values the majority of the 200–300-nm CD spectrum arises from uncomplexed polymer. The technique of fluorescence-detected circular dichroism (FDCD) spectroscopy (Turner et al., 1974; Turner, 1978) provides a technique to directly probe the conformation of bound ethidium. The FDCD data indicate a similar conformation at the intercalation site under both B- and Z-form conditions, from which we conclude that ethidium is intercalated into a right-handed region of the polymer.

Materials and Methods

FDCD. The FDCD instrument used in these studies has been described previously (Lobenstine et al., 1981; Lamos & Turner, 1985). It measures (Tinoco & Turner, 1976; Turner, 1978)

$$2\frac{F_L - F_R}{F_L + F_R} = \frac{2(g_F - 2R)}{2 - g_F R} \approx g_F - 2R \qquad (1)$$

where

$$R = \frac{A_L(1 - 10^{-A_R}) - A_R(1 - 10^{-A_L})}{A_L(1 - 10^{-A_R}) + A_R(1 - 10^{-A_L})} \qquad (2)$$

Here, F_L and F_R are fluorescence intensities excited by left and right circularly polarized light, respectively. A_L and A_R are the absorbances of the sample for left and right circularly polarized light, respectively, and are obtained from the CD and absorbance of the sample. If all fluorescence is due to absorption by one chromophore, then $g_F = \Delta\epsilon_F/\epsilon_F$, the Kuhn dissymmetry factor of the fluorophore. Otherwise, it is more complicated (Tinoco & Turner, 1976). When only the fluorophore absorbs, $g_F = (A_L - A_R)/A$, so the FDCD spectrum can be predicted from the CD and absorption spectra by using eq 1 and 2. Equation 1 can also be rearranged so that g_F can be calculated if the FDCD, CD, and absorbance spectra are known. For comparison with previous work, $\theta_F = -28.65(F_L - F_R)/(F_L + F_R)$.

FDCD cells had 1-cm path lengths. Filters used to block excitation light while transmitting ethidium fluorescence were Corning CS3-66. Separate Pockels cells were used for the 240–350- and 300–420-nm regions in order to increase the lifetime of the Pockels cells.

Other Spectra. CD and absorption spectra were recorded on a Jasco J-40 spectropolarimeter and a Perkin-Elmer 330 spectrophotometer, respectively. Both were interfaced to a PDP-11/34 computer. Molar ellipticity, $[\theta]$, was calculated from

$$[\theta] = \frac{\theta}{Cl}100 \qquad (3)$$

where θ is the ellipticity (in degrees), C is the molar base pair concentration of polynucleotide, and l is the path length in centimeters. Cell path lengths were 2 cm unless otherwise noted.

Sample Preparation. Ethidium bromide was purchased from Sigma and recrystallized from methanol. Poly(dG-dC) and poly(dG-m^{5}dC) were purchased from Pharmacia P-L Biochemicals and used without further purification.

A buffer consisting of 50 mM NaCl and 5 mM tris(hydroxymethyl)aminomethane (Tris), pH 8, will be referred to as 50 mM sodium buffer. This buffer was also used with the addition of varying amounts of $MgCl_2$; these buffers will be referred to respectively as 0.2, 2, and 25 mM magnesium buffer. A buffer of 4.4 M NaCl, 10 mM Na_2PO_4, and 10 mM Na_2EDTA, pH 7, will be referred to as 4.4 M sodium buffer.

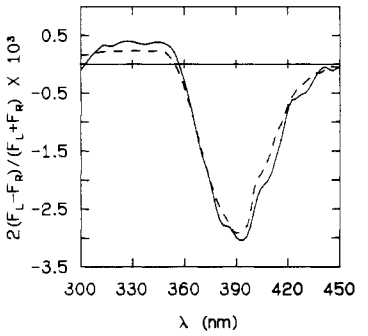

FIGURE 1: Experimental (—) and calculated (--) FDCD spectra of 5×10^{-6} M ethidium with 5×10^{-5} M poly(dG-dC) in 50 mM sodium buffer. CD and absorbance data were recorded in a 10-cm cell.

Independent samples were prepared, and the bound drug to base pair ratio, r, was calculated from the absorbance at 480 nm as previously described (Walker et al., 1985). All polymer concentrations are expressed in terms of base pairs. For titrations, the polymer concentrations were approximately 50 μM in base pairs.

Results

Spectra of Ethidium Complexes with Poly(dG-dC). Figure 1 shows measured and calculated FDCD spectra from 310 to 420 nm for ethidium bound to B-form poly(dG-dC) at $r = 0.1$ in 50 mM sodium buffer. Only ethidium absorbs above 310 nm so the FDCD spectrum may be calculated from absorbance and CD data by using eq 1. The calculated and experimental spectra are in excellent agreement, indicating the absence of artifacts in the FDCD spectra. The spectra show a large negative band centered at 390 nm and a small positive band in the 310–340-nm region. The 390-nm band is small in absorption CD (Aktipis & Martz, 1970) but large in FDCD because it arises from an ethidium transition with a small extinction coefficient, ϵ, but large $\Delta\epsilon/\epsilon$ ($=g_F$; see eq 1) (Hudson & Jacobs, 1975; Giacomoni & LeBret, 1973; Houssier et al., 1974).

CD and FDCD spectra for an ethidium titration with poly(dG-dC) in 50 mM sodium buffer are shown in Figure 2A,B. The initial CD spectrum (Figure 2A, curve a) is characteristic of B-form poly(dG-dC). The binding of ethidium produces positive increases in the CD at 270 and 320 nm, while isoelliptical points are observed at ~250 and ~295 nm as previously reported (Walker et al., 1985). The FDCD spectra have positive bands at 270 and 320 nm, a negative band at 390 nm, and zero crossing points at about 255, 290, and 300 nm. The general shape of the FDCD spectra agree with those obtained with ethidium bound to the dinucleotide dC-dG (Dahl et al., 1982). As shown in Figure 3, as the ethidium to base pair ratio, r, increases, the FDCD magnitude at 320 nm increases while the magnitude at 390 nm remains fairly constant.

CD and FDCD spectra for an ethidium titration of poly(dG-dC) in 4.4 M sodium buffer are shown in panels C and D of Figure 2, respectively. The initial CD spectrum (Figure 2C, curve a) is characteristic of Z-form poly(dG-dC). Upon addition of ethidium, the CD spectrum of the Z form is converted toward that characteristic of ethidium-saturated right-handed poly(dG-dC) as previously reported (Pohl et al., 1972; Walker et al., 1985). The FDCD spectra remain essentially constant for all r values greater than 0.05 (Figures 2D and 3) and closely resemble the FDCD spectrum observed in a 50 mM sodium buffer at an r of 0.3. This characteristic FDCD spectrum is observed even when most of the poly(dG-dC) is left-handed, as judged by the corresponding CD spectra

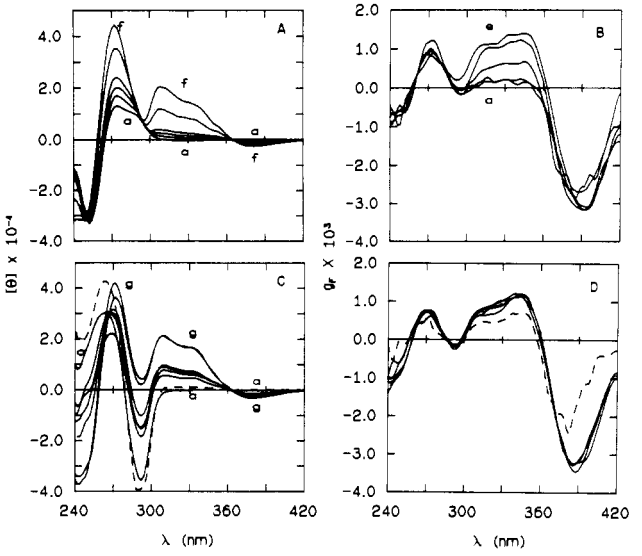

FIGURE 2: CD and FDCD spectra of ethidium with poly(dG-dC) in 50 mM sodium buffer (A and B) and 4.4 M sodium buffer (C and D). Values of r for spectra a–f in (A) are respectively 0.0, 0.05, 0.1, 0.15, 0.3, and 0.439. Values of r for spectra a–e in (B) are respectively 0.04, 0.1, 0.15, 0.3, and 0.439. Values of r for spectra a–g in (C) are respectively 0.0, 0.04, 0.05, 0.1, 0.15, 0.3, and 0.4. Values of r for the spectra in (D) are 0.04, 0.05, 0.1, 0.15, 0.3, and 0.4. In (C) and (D) the dashed line (– –) is the spectrum recorded from a sample containing 10.2 μM ethidium and 22.1 μM poly(dG-dC) in 4.4 M sodium buffer; the r value determined by absorbance spectrscopy is $r = 0.0083$.

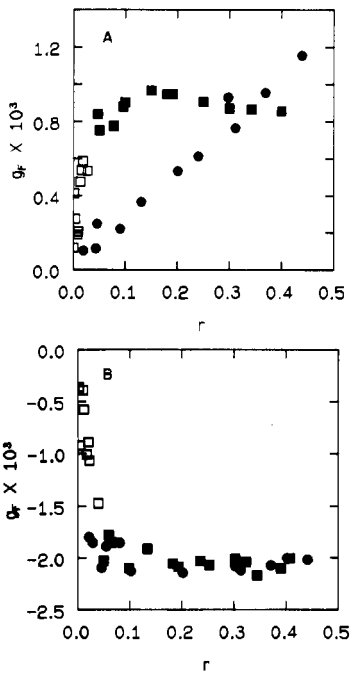

FIGURE 3: g_F vs. r for ethidium binding to poly(dG-dC): (●) 50 mM sodium buffer; (■) 4.4 M sodium buffer; (□) 4.4 M sodium buffer. [Ethidium] < 20 μM. (A) g_F averaged from 310 to 330 nm. (B) g_F averaged from 360 to 420 nm.

in Figure 2C. The only exceptions to this generalization are observed when the total ethidium concentration is less than 20 μM. As shown in Figures 2D and 3, for total ethidium concentrations below 20 μM, the magnitudes of the FDCD peaks decrease, although the signs remain the same. An important aspect of ethidium binding to poly(dG-dC) in 4.4 M sodium buffer is that very little ethidium binds until the free ethidium concentration reaches 20 μM (Pohl et al., 1972; Walker et al., 1985). Absorbance measurements at these low

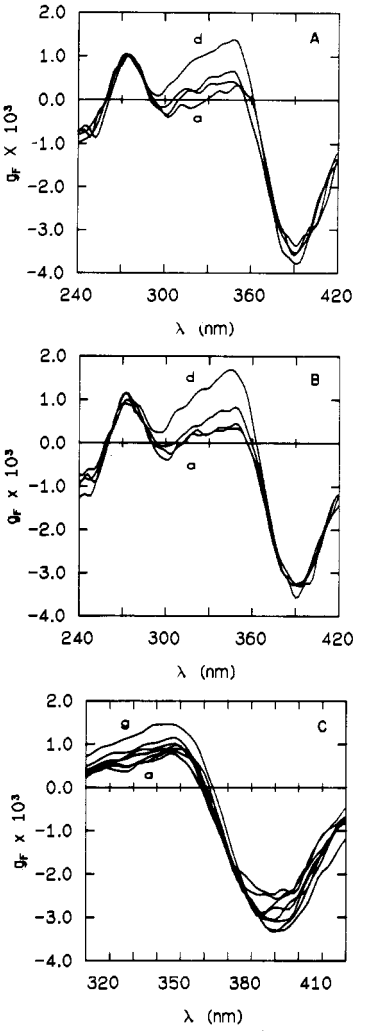

FIGURE 4: FDCD spectra of ethidium with poly(dG-m^{5}dC) in 0.2 (A), 2.0 (B), and 25 (C) mM magnesium buffer. Values of r for spectra a–d in (A) are respectively 0.05, 0.1, 0.2, and 0.4. Values of r for spectra a–d in (B) are respectively 0.05, 0.1, 0.2, and 0.4. Values of r for spectra a–g in (C) are respectively 0.0142, 0.0567, 0.067, 0.104, 0.15, 0.204, 0.286, and 0.367.

concentrations indicate that less than 10% of the ethidium is bound. This can account for the decreased FDCD magnitudes since the contributions of bound and unbound ethidium to the FDCD spectrum are weighted by their CD, concentration, and quantum yield (Tinoco & Turner, 1976).

Spectra of Ethidium Complexes with Poly(dG-m^{5}dC). CD spectra show that poly(dG-m^{5}dC) undergoes a B–Z conformational transition as the concentration of magnesium ion increases from 0.2 to 2.0 mM (Behe & Felsenfeld, 1981) and is also in a left-handed form in 25 mM magnesium buffer. As ethidium is added, the CD spectra at 2 and 25 mM magnesium progressively approach the spectra observed at 0.2 mM magnesium (Walker et al., 1985; G. T. Walker, J. M. Castle, and T. R. Krugh, unpublished data), in a manner much like that shown for poly(dG-dC) in Figure 2C.

FDCD spectra for ethidium bound to poly(dG-m^{5}dC) under B-form (0.2 mM magnesium) and Z-form (2 and 25 mM magnesium) conditions are shown in Figure 4. The FDCD spectra in 0.2 and 2 mM magnesium buffer are similar to one another at each r value studied and resemble the spectra observed with B-form poly(dG-dC) (Figure 2B). The magnitude of the 320-nm band increases in a similar way as a function of r (Figure 5). Raising the magnesium concentration to 25 mM increases the stability of Z-form poly(dG-m^{5}dC) (G. T.

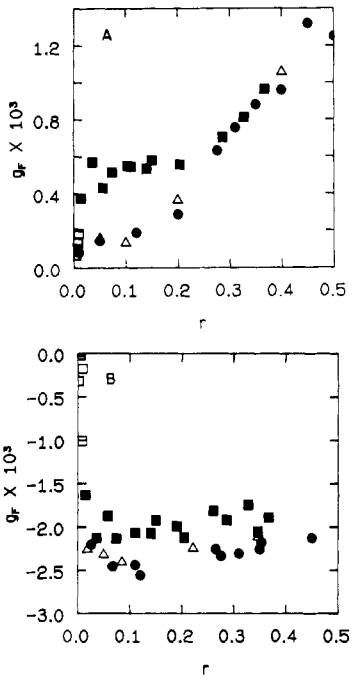

FIGURE 5: g_F vs. r for ethidium binding to poly(dG-m^{5}dC): (●) 0.2 mM magnesium buffer; (△) 2 mM magnesium buffer; (■) 25 mM magnesium buffer; (□) 25 mM magnesium buffer, [ethidium] < 3 μM. (A) g_F averaged from 310 to 330 nm. (B) g_F averaged from 360 to 420 nm.

Walker, J. M. Castle, and T. R. Krugh, unpublished data). In 25 mM magnesium buffer and >3 μM ethidium, the FDCD spectra are constant when $r \leq 0.25$ and closely resemble spectra at 0.2 and 2 mM magnesium when $r = 0.25$. When $r > 0.25$, the FDCD spectra at all three magnesium concentrations change in the same way as r increases (see Figure 5). FDCD spectra for 25 mM magnesium depend on r when $r <$ 0.25 only if the total ethidium concentration is less than 3 μM (see Figure 5). This corresponds to the threshold concentration for ethidium binding in this buffer (G. T. Walker, J. M. Castle, and T. R. Krugh, unpublished data).

Discussion

Previous absorption, CD, and binding studies show that binding of ethidium to poly(dG-dC) or poly(dG-m^{5}dC) under Z-form conditions induces a "sequential" conversion of the polymer from a left-handed to a right-handed conformation (Walker et al., 1985). For poly(dG-dC) in 4.4 M sodium buffer, the CD at 320 nm per mole of DNA-bound ethidium remained constant as ethidium was added, and was equal to the molar CD for ethidium bound to poly(dG-dC) in 50 mM sodium buffer when $r \sim 0.4$. This suggested the conversion under these conditions is associated with a clustering of ethidium in the B-form regions of poly(dG-dC). For poly(dG-m^{5}dC) induced into Z form by $MgCl_2$, the CD at 320 nm indicates clustering with a spacing dependent on $MgCl_2$ concentration (G. T. Walker, J. M. Castle, and T. R. Krugh, unpublished data). These conclusions appear to differ from those of Shafer et al. (1984), who suggest that ethidium forms a complex "directly with the left-handed polynucleotide".

FDCD makes it possible to measure a spectrum that is only sensitive to the environment around ethidium. FDCD spectra of ethidium bound to macromolecules nonintercalatively do not exhibit bands at 320 and 390 nm (Lamos & Turner, 1985). The magnitudes of FDCD bands for ethidium bound by intercalation depend on the base composition of the binding site (Dahl et al., 1982; M. L. Lamos and D. H. Turner, unpublished results). The most striking observation in this paper is that above 20 μM ethidium the entire FDCD spectrum for poly(dG-dC) in 4.4 M sodium buffer remains constant as ethidium is added and corresponds to the spectrum observed in 50 mM sodium buffer when $r = 0.3$. Since FDCD spectra are very sensitive to conformation (Lobenstine et al., 1981; Lamos & Turner, 1985), this implies that throughout the titration in 4.4 M sodium buffer the binding sites are equivalent to those in 50 mM sodium buffer when ethidiums are spaced by about three base pairs. This confirms previous CD data that indicated two to four base pairs of left-handed poly(dG-dC) in 4.4 M sodium buffer are converted to right-handed form per bound ethidium (Walker et al., 1985). Thus, the results are consistent with the suggestion of Walker et al. (1985) that the left- to right-handed conversion is accomplished by a "sequential" conversion of the polymer. Even at low overall levels of saturation, the regions of the poly(dG-dC) in a right-handed conformation have one ethidium bound every two to four base pairs.

Below 20 μM ethidium for poly(dG-dC) in 4.4 M sodium buffer and below 3 μM ethidium in 25 mM magnesium buffer, FDCD spectra depend on the r value. The shapes of the spectra are similar above and below these ethidium concentrations, but the magnitudes of the bands are lower (see Figures 2, 3, and 5). At these low ethidium concentrations, less than 10% of the ethidium is bound to polymer. When more than one species contributes to the FDCD spectrum, the relative contributions are weighted by the CD, concentration, and quantum yields of each species (Tinoco & Turner, 1976). Thus, the presence of free ethidium, which has no CD, decreases the magnitude of the FDCD spectrum without affecting its shape. Therefore, the FDCD spectra at low ethidium concentrations are also consistent with binding to a right-handed helix. Under no conditions is the shape of the FDCD spectrum drastically altered as might be expected for binding to a left-handed helix.

For poly(dG-m^{5}dC) in 25 mM magnesium buffer in the presence of more than 3 μM ethidium at $r \leq 0.25$, the FDCD spectrum corresponds to the spectrum observed in 0.2 mM magnesium buffer when $r = 0.25$. Above $r = 0.25$, the spectra for both buffers are essentially the same. This again correlates with transmission CD and binding studies that indicate each bound ethidium converts four to five base pairs from left- to right-handed form under these conditions and that very little ethidium binds until the free concentration reaches 3 μM (G. T. Walker, J. M. Castle, and T. R. Krugh, unpublished data). The FDCD data strongly suggest that until the left- to right-handed conversion is complete the average distance between bound ethidiums is about four base pairs. Moreover, the conformation of the binding sites are similar to those in right-handed poly(dG-m^{5}dC), as evidenced by the similar shape of the FDCD bands. At r values greater than 0.25, the poly(dG-m^{5}dC) is completely reversed to a right-handed form, and ethidium binds as under B-form conditions.

For poly(dG-m^{5}dC) in 2 mM magnesium buffer, the FDCD spectra continuously change as ethidium is added, and they are similar to the spectra for corresponding r values in 0.2 mM magnesium buffer. Transmission CD and binding studies indicate each bound ethidium converts seven base pairs from left to right-handed form under these conditions (Walker et al., 1985). Evidently, an average spacing of seven base pairs is too large for adjacent ethidiums to affect the CD spectra of each other in poly(dG-m^{5}dC). Interestingly, under B-form conditions, the FDCD spectra are more constant at $r < 0.2$ for ethidium bound to poly(dG-m^{5}dC) than bound to poly-

(dG-dC). This may indicate the methyl group stabilizes the initial structure, since the dependence of CD on the r value has been suggested to arise from conformational effects (Dahl et al., 1982).

This work also demonstrates the advantages of using FDCD spectroscopy for studying ethidium binding to nucleic acids [e.g., see Lamos & Turner (1985)]. The increased sensitivity permitted measurements below the threshold concentration for ethidium binding. Since the magnitude of FDCD bands depends on $g_F = \Delta\epsilon_F/\epsilon_F$ (see eq 1), the sensitivity is particularly enhanced for transitions with small extinction coefficients, but large rotatory strengths. This permits measurements on the 390-nm band of ethidium. Different spectral bands are apparently sensitive to different features of the binding. Thus, the 320-nm band is sensitive to the distance between ethidiums on dG-dC polymers (see Figures 3 and 5), whereas the 390-nm band is only sensitive to the base composition at the binding site (M. L. Lamos and D. H. Turner, unpublished experiments). Presumably, these advantages will also be useful for studies of other fluorescent drugs.

Registry No. Poly(dG-dC), 36786-90-0; poly(dG-m^{5}dC), 51853-63-5; ethidium bromide, 1239-45-8.

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Resonance Raman Spectroscopic Studies of Adriamycin and Copper(II)–Adriamycin and Copper(II)–Adriamycin–DNA Complexes

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ABSTRACT: Characteristic resonance Raman spectra are observed on ionization of the phenolic groups in adriamycin. On the basis of these results, vibrational assignments for the Raman bands of adriamycin are reported. Distinct Raman spectra are observed for Cu(II)–adriamycin complexes at pH ~5 and pH ~13. The data indicate that at lower pH a bis complex of Cu(II) is formed, which transforms to a polymeric Cu(II) chelate at higher pH. Upon interaction of the metal–drug complex with calf thymus DNA at pH ~5, a ternary complex is formed in which the Cu(II)-complexed adriamycin is intercalated into DNA.

Adriamycin, a glycosidic anthracycline antibiotic, has been in wide clinical use for the treatment of various types of cancers (Remers, 1979). Various pathways have been suggested for the mechanism of action and cardiac toxicity of these drugs. These include intercalation into DNA (Pigram, 1972), binding to membranes (Goormaghtigh, 1983), and free radical reactions of the reduced forms of these drugs (Bachur, 1982). Recent studies have indicated that metal-chelated forms of

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