(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whereas the 390-nm band is only sensitive to the base composition at the binding site (M. L. Lamos and D. H. Turner, unpublished experiments).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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adriamycin may be important in its biochemical activity. Sugioka and Nakamo (1982) found that an iron–ADP–adriamycin complex induced phospholipid peroxidation. Myers et al. (1982) reported that erythrocyte ghost membranes were oxidatively destructed by an adriamycin–iron complex. Muindi et al. (1984) have also shown that DNA damage can be caused by the iron complex.

Metal ion binding to anthracyclines has been the object of many investigations. Calendi and co-workers (1965) reported that large spectral changes took place in the visible absorption spectrum on complexation with metal ions. Stability constants of adriamycin–metal complexes have been reported (May, 1980; Kiraly & Martin, 1982). Mikelens and Levinson (1978) showed that metal–adriamycin complexes exhibit DNA binding ability. Knowledge of the molecular structure of the various forms of these drugs is essential to understand the mechanisms of their action. Such studies are the focus of many recent research efforts. Greenway and Dabrowiak (1982) used visible absorption spectroscopy and circular dichroism (CD) to determine that the $Cu^{2+}$ ion formed bis complexes with anthracyclines in the pH range of 6.0–8.5. Beraldo et al. (1983) came to similar conclusions on the basis of spectrophotometric and potentiometric titration studies. They also reported on the resonance Raman spectrum of the copper–drug complex and showed that the metal ion was coordinated to quinone and phenolate oxygen atoms. The proton NMR relaxation study of Yb(III)–adriamycin has confirmed that the metal ion is bound to the quinone, phenolic functionality that is distant from the sugar group ($C_{11}$, $C_{12}$) (McLennan & Lenkinski, 1984). Spinneli and Dabrowiak (1982), on the basis of visible absorption studies, reported on the formation of a ternary copper–drug DNA complex, in which the drug was not intercalated into DNA.

Resonance Raman spectroscopy has been a powerful probe for investigating chromophoric groups in biological systems (Carey, 1982). Such studies have been reported on adriamycin (Hillig & Morris, 1976; Manfait, 1981), adriamycin–DNA complexes (Angeloni, 1982; Manfait, 1982), and copper– and iron–adriamycin complexes (Beraldo, 1983, 1985). This paper presents a systematic investigation of the Raman spectra of (i) adriamycin and its deprotonated forms and their band assignments, (ii) copper–adriamycin complexes at various pHs, and (iii) adriamycin–$Cu^{2+}$–DNA complexes.

## Materials and Methods

The Raman spectra of the various samples were obtained by excitation with a Spectra-Physics Model 171 $Ar^+$ laser, and the scattered light was measured with a Spex 1403 double monochromator and RCA C31034 photomultiplier. The power at the sample was of the order of 25–50 mW. All samples were prepared anaerobically and sealed in NMR sample tubes and spun while recording the spectra to avoid decomposition. In the past, Raman studies of adriamycin at high pHs have failed because of the extreme photochemical instability in the presence of oxygen. It is only under strict anaerobic conditions that we were able to obtain reasonable quality Raman spectra. There have been several reports in the literature on the ultraviolet light induced decomposition of adriamycin in the presence of oxygen (Carmichael & Riesz, 1985). Slit widths were of the order of 6 $cm^{-1}$ and collection times varied from 3 to 6 s/wavenumber.

Adriamycin was obtained from Adria Laboratories and calf thymus DNA from Sigma and used as such. All of the samples were made up in unbuffered solutions. The concentration and laser excitation lines are described in the figure captions. The UV–visible spectra were recorded after the Raman experiments. $CuCl_2$ was used as the source for metal ions.

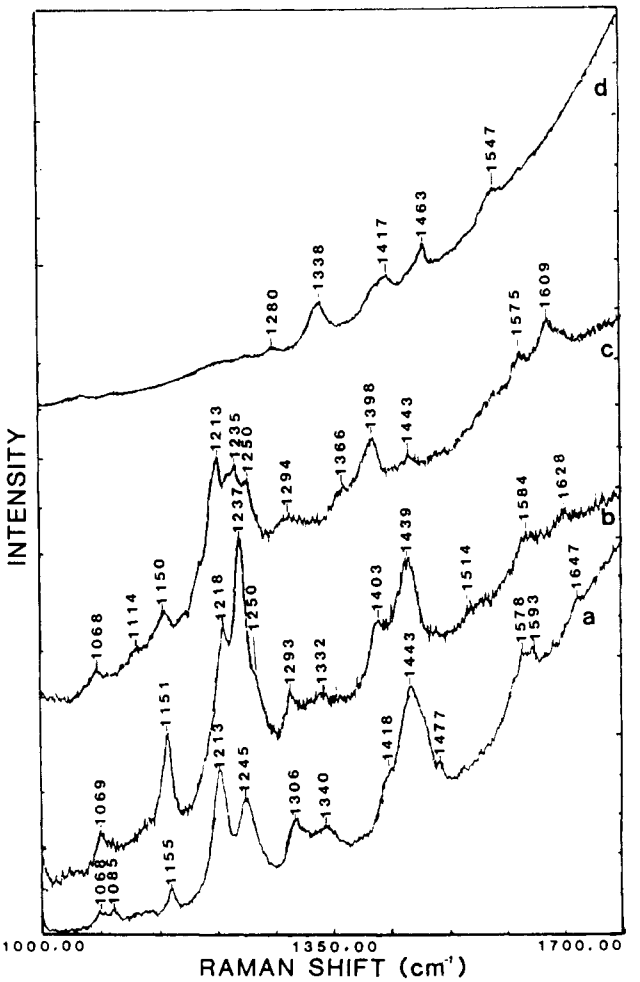


FIGURE 1: Resonance Raman spectra of (a) adriamycin ($1.1 \times 10^{-4}$ M) at pH ~5.4, excitation wavelngth 457.9 nm, (b) adriamycin ($7 \times 10^{-4}$ M) at pH ~12, excitation wavelength 457.9 nm, (c) adriamycin ($7 \times 10^{-4}$ M) at pH ~13.5, excitation wavelength 457.9 nm, and (d) adriamycin ($1.1 \times 10^{-4}$ M) in $D_2O$ at pD ~5, excitation wavelength 457.9 nm.

## Results

*Adriamycin.* Figure 1a–c shows the resonance Raman spectra of adriamycin at pH ~5.4, pH ~12, and pH ~13.5 and Figure 1d is the spectrum of adriamycin in $D_2O$ at pD ~5. When spectra a and b of Figure 1 are compared, it is seen that at pH ~12 the bands at 1085, 1213, 1443, 1578, and 1593 $cm^{-1}$ have disappeared or decreased considerably in intensity. New bands are observed at 1218, 1250, 1514, and 1628 $cm^{-1}$. Bands at 1155, 1245, 1306, 1418, and 1443 $cm^{-1}$ have decreased in frequencies by 3–10 $cm^{-1}$. In the lower frequency region, major changes are the replacement of the bands at 444 and 467 $cm^{-1}$ by a broad band (half-width ~75 $cm^{-1}$ centered at 470 $cm^{-1}$).

On increasing the pH to 13.5, further changes are observed in the Raman spectrum. New bands appear at 1114 and 1609 $cm^{-1}$. The bands at 1213, 1235, and 1250 $cm^{-1}$ are clearly seen. The band at 1443 $cm^{-1}$ has almost disappeared, and the major band in this region is at 1398 $cm^{-1}$. On raising the pH to 12, the major change in the electronic spectrum is the disappearance of the band at ~480 nm and the appearance of a band at 552 nm (Beraldo, 1983). At pH ~13.5, a shoulder is observed at 607 nm, in addition to the 552-nm band.

*$Cu^{2+}$–Adriamycin Complexes.* Figure 2 shows the resonance Raman spectra of the $Cu^{2+}$–adriamycin at pH ~5.5,

Table I: Resonance Raman Frequencies ($cm^{-1}$) of Various Forms of Adriamycin

| adriamycin | | | Cu–adriamycin | | | DNA–adriamycin at pH 5.2 | Cu–DNA–adriamycin at pH 5.2 | band assignments |
|---|---|---|---|---|---|---|---|---|
| pH 5.4 | pH 12 | pH 13.5 | pH 5.5 | pD 5 | pH 13 | | | |
| 1068 | 1069 | 1068 | | | | 1068 | | $C-O-CH_3$ |
| 1085 | | | | | | 1089 | | $^{+}C-O-H$ |
| | | 1114 | | | 1110 | | | phenolate |
| 1155 | 1151 | 1150 | 1160 | 1159 | 1154 | 1155 | 1159 | $C-O-CH_3$ |
| 1213 | | | | | | 1214 | | $^{+}C-O-H$ |
| | 1218 | 1213 | 1226 | 1222 | | | 1217 | phenolate |
| 1245 | 1237 | 1235 | 1246 | 1243 | | 1244 | 1245 | ring |
| | 1250 | 1250 | 1264 | 1264 | 1257 | | 1264 | phenolate |
| 1306 | 1293 | 1294 | 1304 | 1302 | 1304 | 1302 | 1303 | ring |
| | | | | 1333 | | | | $^{+}C-O-D$ |
| 1340 | 1332 | | | | 1337 | 1340 | | ring |
| | | 1366 | | | | | | phenolate |
| | | | 1376 | 1374 | | | 1380 | phenolate |
| 1418 | 1403 | 1398 | 1412 | 1413 | 1409 | 1435 | | ring |
| 1443 | 1439 | 1443 | 1445 | 1444 | 1444 | 1450 | 1447 | $^{+}C-O-H$ |
| | 1514 | | 1524 | 1545 | | | | phenolate |
| 1578 | | | | | | 1576 | | $^{+}C-O-H$ |
| | 1584 | 1575 | | | | | | ring |
| | 1584 | 1575 | | | | | | ring |
| 1593 | | | | | | 1591 | | $^{+}C-O-H$ |
| | 1628 | 1609 | 1635 | 1631 | 1607 | | 1634 | phenolate |
| 1647 | | | | | | | 1641 | $^{+}C-O-H$ |

in $D_2O$ at pD ~5, and at pH ~13. The spectra shown were at a $Cu^{2+}$:adriamycin ratio of 10:1. We have confirmed that the spectrum remains unchanged at metal:drug ratios of 5:1, and Figure 2a resembles the Raman spectrum reported by Beraldo et al. (1983) at ratios corresponding to 1:1.

At pH ~5.5 (Figure 2a), new bands are observed at 1226, 1264, 1376, 1524, and 1635 $cm^{-1}$. In the low frequency, a broad band centered at 450 $cm^{-1}$ is observed. The band at 1412 $cm^{-1}$ is clearly observable. Bands at 1000, 1160, 1246, 1304, and 1445 $cm^{-1}$ remain unshifted as compared to those of free adriamycin (Figure 1a). A weak band is observed at 1214 $cm^{-1}$. Upon formation of the complex in $D_2O$ (Figure 2b), most of the bands remain unshifted in frequency except for the 1524-$cm^{-1}$ band, which appears at 1545 $cm^{-1}$. New bands are observed at 1333 and 1427 $cm^{-1}$. Also, a band at 846 $cm^{-1}$ is observed in the deuterated spectrum. On raising the pH to 13 (Figure 2c), new bands are observed at 1110 and 1607 $cm^{-1}$, and the 1444-$cm^{-1}$ band has lost most of its intensity. A broad band at 1257 $cm^{-1}$ is also observed. At pH ~6, the visible absorption spectrum showed a red shift of the 480-nm band to 524 nm, followed by a further lowering to 554 and 596 nm at pH ~13.

*Cu–Adriamycin–DNA.* Figure 3a shows the resonance Raman spectrum of adriamycin bound to calf thymus DNA, and Figure 3b is that of the drug–DNA complex mixed with $Cu^{2+}$ in the adriamycin:$Cu^{2+}$ ratio of 1:20; both spectra were taken at pH ~5.2. At this ratio, the absorption band of adriamycin has shifted from 480 nm to 588 nm, 550 nm, and a shoulder at 510 nm, and beyond this ratio no further changes in the absorption spectrum were observed, in agreement with the data reported by Spinnelli and Dabrowiak (1982). On binding to DNA, the major change in the Raman spectrum is the shift of the 1418-$cm^{-1}$ shoulder to a distinct band at 1435 $cm^{-1}$ along with the shift of the 1443-$cm^{-1}$ band to 1450 $cm^{-1}$. On addition of $Cu^{2+}$ ion, new bands are observed at 1217, 1264, 1380, and 1634 $cm^{-1}$ along with disappearance of the band at ~1435 $cm^{-1}$. The low-frequency region showed a broad band at 450 $cm^{-1}$.

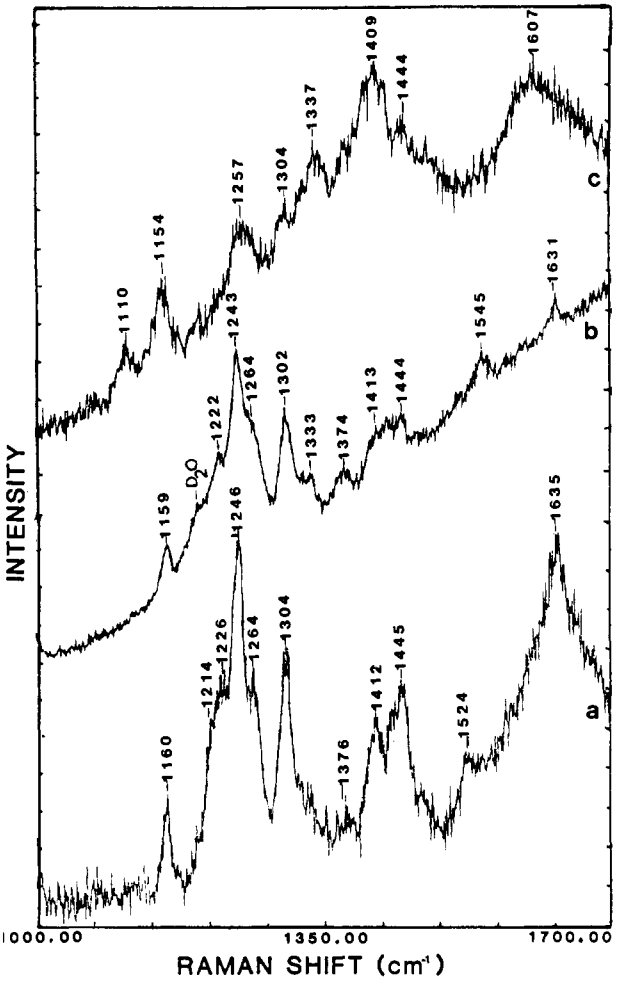


FIGURE 2: Resonance Raman spectra of $Cu^{2+}$–adriamycin complexes ([$Cu^{2+}$] = 1.1 × $10^{-3}$ M, [adriamycin] = 1.1 × $10^{-4}$ M (a) at pH ~5.5, excitation wavelength 476.5 nm, (b) in $D_2O$ at pD ~5, excitation wavelength 496.5 nm, and (c) at pH ~13, excitation wavelength 472.7 nm.

## Discussion

*Band Assignments.* A listing of the Raman frequencies of the various forms of adriamycin along with the band assignments is shown in Table I. Adriamycin contains three acidic groups, the amino group with a $pK_a$ of 3.0 and the two phenolic groups with $pK_a$ of 10.0 and 13.0 (Kiraly & Martin, 1982). At pH ~1, the Raman spectrum of the drug is identical with that at pH ~5, suggesting that the state of protonation of the

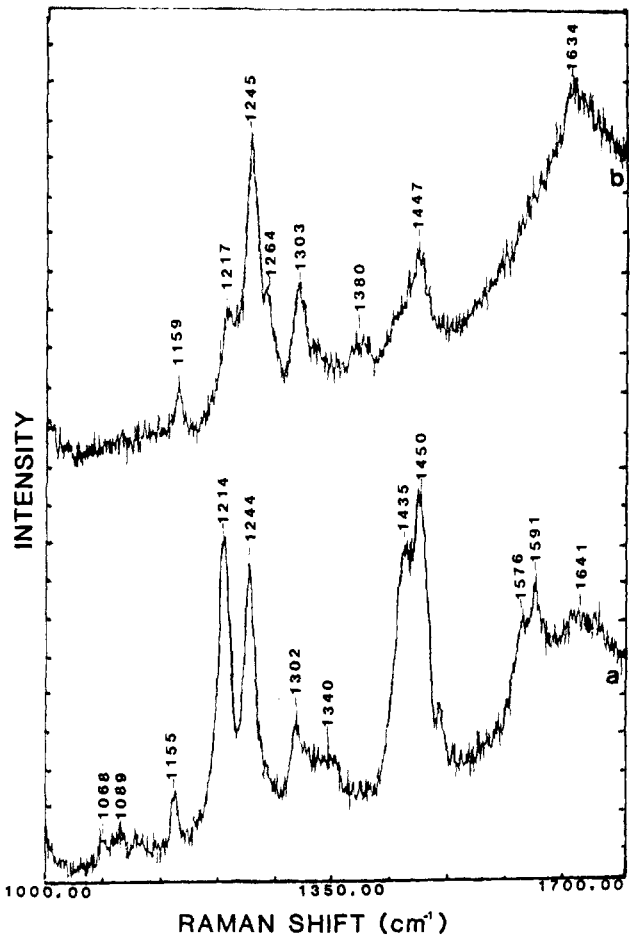


FIGURE 3: Resonance Raman spectra of (a) adriamycin–DNA complex ([adriamycin] = $1.1 \times 10^{-4}$ M, [DNA] = 0.76 mg/mL) at pH ~5.2, excitation wavelength 457.9 nm, and (b) adriamycin–DNA–$Cu^{2+}$ complex ([adriamycin] = $1.1 \times 10^{-4}$ M, [$Cu^{2+}$] = $2.2 \times 10^{-3}$ M, [DNA] = 0.76 mg/mL) at pH ~5.2, excitation wavelength 476.5 nm.

amino group does not affect the resonance Raman spectrum of the anthraquinone chromophore. This is not surprising, since the amino group is part of the sugar moiety and does not in any way influence the electronic structure of the anthraquinone ring.

However, at pH ~12, there are major changes in the Raman spectrum as one of the phenolic groups on the ring gets deprotonated. Three distinct patterns can be discerned. First, there is the disappearance or weakening in intensity of bands that are coupled with $\delta$(C–O–H) motion. These bands occur at frequencies of 1085, 1213, 1443, 1578, 1593, and 1647 $cm^{-1}$. Second, the ionization of the phenolic group leads to electron delocalization over the anthraquinone ring. This results in a shift of bands at 1245, 1306, and 1418 $cm^{-1}$ to lower frequencies by about 12–15 $cm^{-1}$. So, it appears that these ring vibrations are sensitive to the electron density of the anthraquinone ring.

Third, new bands appear at 1218, 1250, 1514, and 1628 $cm^{-1}$ and are characteristic of the deprotonated form ($HAd^{-1}$) of adriamycin. These bands, by comparison with other phenolates, can be assigned to phenolate ring deformations; e.g., tyrosinates exhibit bands at 1250, 1501, and 1602 $cm^{-1}$ (Que, 1983). Bands that remain unaffected in frequency at 1068 and 1155 $cm^{-1}$ could arise from $\nu$(C–O) of the methoxy group.

Upon further deprotonation (Figure 1c), the anthraquinone ring modes are observed at 1235, 1294, and 1398 $cm^{-1}$. The characteristic phenolate ring deformations of the completely deprotonated drug ($Ad^{2-}$) occur at 1114, 1213, 1250, 1366, and 1609 $cm^{-1}$. The band at 1443 $cm^{-1}$ is very weak in intensity, confirming our earlier suggestion that this mode is coupled with $\delta$(C–O–H) motion.

An exact assignment of the various bands can only be done from vibrational studies of isotopic forms of the adriamycin molecule. What can be concluded at this point is that three distinct types of vibrational bands are resonance-enhanced in adriamycin. These are (1) methoxy group vibrations at 1068 and 1155 $cm^{-1}$, which appear insensitive to chemical changes, (2) anthraquinone ring modes at 1245, 1306, and 1418 $cm^{-1}$, which decrease in frequency as electron density of the ring increases, and (3) various ring and carbonyl modes that are coupled to $\delta$(C–O–H) motion and appear at 1213, 1443, 1578, 1597, and 1647 $cm^{-1}$. These bands will be sensitive to the electronic distribution in the rings as well as to H-bonding interactions through the phenolic groups.

*Cu–Adriamycin Complex.* Beraldo et al. (1983) reported the appearance of phenolate ring deformations on complexation of adriamycin with $Cu^{2+}$ ion and concluded that chelation is taking place through the phenolic and quinone functionalities. There is some ambiguity about the exact state of protonation of the adriamycin ligand in these complexes. Upon comparison of the spectra of the $HAd^{-}$ ion (Figure 1b) with the $Cu^{2+}$ complex (Figure 2a), it is quite evident that the characteristic phenolate ring deformations of the $HAd^{-}$ ion at 1218, 1250, 1514, and 1628 $cm^{-1}$ are appearing in the $Cu^{2+}$–drug complex at 1226, 1264, 1524, and 1635 $cm^{-1}$. The increase in frequencies of these modes indicates increased electron localization on the phenolate C–O bond, thus raising its double-bond character. In agreement with this observation, the anthraquinone ring modes, most notably the 1237- and 1403-$cm^{-1}$ bands, increase in frequency to 1246 and 1412 $cm^{-1}$, as electrons from the ring get localized on the C–O band. That the adriamycin is in the form $HAd^{-}$ is also supported by studies in $D_2O$. Two bands characteristic of the presence of O–D groups are observed at 896 and 1333 $cm^{-1}$. So, the ligand at pH ~5.8 is most definitely $HAd^{-}$. The resonance Raman spectrum remains unchanged until the pH reaches 12, suggesting that $Cu(HAd)_2$ is stable until pH ~12.

At pH 13, further changes are observed in the Raman spectrum (Figure 2c). Bands that are characteristic of the $Ad^{2-}$ ion are observed at 1110 and 1607 $cm^{-1}$. The anthraquinone ring modes at 1235 and 1398 $cm^{-1}$ appear at 1257 and 1409 $cm^{-1}$, indicating that $Ad^{2-}$ is complexed with the $Cu^{2+}$ ion. It is difficult from the raman spectroscopic data to determine the exact structure of these complexes, but the data are well explained by a polymeric ...Cu–Ad–Cu... complex, in agreement with potentiometric studies (Beraldo, 1983).

*Interaction with DNA.* Previous Raman studies on adriamycin–DNA complexes have shown noticeable hyper- and hypochromism of many of the resonance-enhanced bands of adriamycin (Manfait, 1982; Angeloni, 1982). Besides these intensity changes, there are also frequency shifts, the most noteworthy being the shifts of the 1418- and 1443-$cm^{-1}$ bands to 1435 and 1450 $cm^{-1}$ (Figure 3a). The intensity changes are taken as evidence that the adriamycin intercalates with the DNA, i.e., slides in between the DNA base pairs. Intensity changes are commonly encountered in UV–visible absorption spectroscopy, if electronic intercalations between vertically stacked groups can occur. The origin of the Raman effect is similar and arises from the dipole–dipole interactions between the parallel groups (Tomlinson & Peticolas, 1970; Painter & Koenig, 1976). An excellent discussion of the Raman intensity changes and intercalation of adriamycin with DNA is given

by Manfait et al. (1982). The frequency shifts that we report indicate that bonding changes are also taking place in the ground state of the molecule upon interaction with DNA. As we have noted before, the 1418-$cm^{-1}$ bands arises from an anthraquinone ring mode, and its increase in frequency to 1435 $cm^{-1}$ upon intercalation suggests that electron density is decreasing on the ring. The fact that the other ring modes are not affected could indicate a localized interaction between the DNA base pairs and one of the aromatic rings on the anthraquinone moiety. Specific assignment of the vibrational mode is necessary to understand the exact origin of this interaction. The origin of the shift of the 1443-$cm^{-1}$ band to 1450 $cm^{-1}$ could also arise from such an interaction. Ring-localized vibrational modes have been observed for similar delocalized systems, such as flavins (Dutta, 1980).

Figure 3b shows the resonance Raman spectrum of the adriamycin–DNA–$Cu^{2+}$ mixture at pH ~5.2. The appearance of the characteristic phenolate modes at 1217, 1264, and 1634 $cm^{-1}$ clearly indicates that the adriamycin is in the form $HAd^-$ and is complexed to the $Cu^{2+}$ ion. However, there are some changes in the adriamycin spectrum as compared to that of the free $Cu^{2+}$–drug complex (Figure 2a), most notable being the enhancement of the 1380-$cm^{-1}$ band and the disappearance of the 1412-$cm^{-1}$ band. Again, such hyper- and hypochromic effects indicate that the adriamycin ring is stacking with other $\pi$-systems. Many possibilities exist. One of these is the association of drug complexes with each other. Spinelli and Dabrowiak (1982) indicated that such association effects are predominant in ethanol. Even though there were pronounced changes in the UV–visible absorption spectrum (Spinelli & Dabrowiak, 1982), there were no alterations in the Raman spectrum in ethanol vs. water. Among the other possibilities are positioning of adriamycin in the groove without intercalation and the association of the drug with the external part of DNA. These associations will probably involve interactions between the alicyclic groups of the adriamycin and host and should not perturb the Raman spectrum. To observe the hyper- and hypochromic effects seen in Figure 3b, it is essential that strong electronic interactions between the anthraquinone ring and another $\pi$-system take place. Clearly, even the self-association of adriamycin molecules in ethanol is not strong enough to produce these Raman intensity changes. We therefore suggest that these changes can only be brought about if the metal-complexed adriamycin is stacked between DNA bases leading to specific $\pi$–$\pi$ interactions between the anthraquinone ring and DNA bases.

In conclusion, we have shown the following: (i) Adriamycin and its two phenolic deprotonated forms have characteristic Raman spectra. On the basis of these results, we have been able to make general band assignments for the various bands in adriamycin. (ii) The $Cu^{2+}$–drug complexes formed depend on the pH. (iii) a ternary DNA–drug–$Cu^{2+}$ complex is formed in which the intercalated adriamycin is bound to the metal ion.

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