Contribution from LA 71, Département de Recherches Physiques, Université Pierre et Marie Curie, 75230 Paris, France, and Laboratoire de Chimie Bioinorganique, UER de Santé, Médecine et Biologie Humaine, Université Paris XIII, 93000 Bobigny, France

Copper(II)–Adriamycin Complexes. A Circular Dichroism and Resonance Raman Study

HELOISA BERALDO,[†] ARLETTE GARNIER-SUILLEROT,*[‡] and LUCIA TOSI*[§]

Received March 16, 1983

Depending on pH and molar ratio, adriamycin forms two well-defined species with Cu(II), which can be formulated $Cu(HAd)_2$ and Cu(Ad). In these formulas, HAd and Ad stand for adriamycin in which the 1,4-dihydroxyanthraquinone moiety is respectively half or totally deprotonated. Both complexes are six-membered chelates. $Cu(HAd)_2$ is a bis chelate of the stacked type that is fully defined at pH 5.8. Cu(Ad) is a long-chain polymer that forms at pH 7.2. Stability constants were obtained by two methods: spectrometric titrations and competing equilibria. They give for the first complex $\beta_I = (4.6 \pm 1.1) \times 10^{16}$ and for the second $\beta_{II} = (1.8 \pm 0.7) \times 10^{12}$. Resonance Raman spectra indicate coordination through quinone and phenolate oxygens forming nondelocalized chelate rings. In addition, CD data suggest intermolecular hydrogen bonding at high concentrations of adriamycin (>100 μM) when self-association takes place. Although formation of $Cu(HAd)_2$ brings about self-association at lower concentration of adriamycin (50 μM), resonance Raman data show disruption of intermolecular hydrogen bonding.

Introduction

Adriamycin (Figure 1) is an anthracycline antibiotic widely used in the treatment of various human cancers. Its chemistry and physical properties and its effects in biological systems both in vivo and in vitro have recently been reviewed.[1,2] Once in the cell, adriamycin localizes in the nucleus and is believed to act by inhibiting both DNA replication and RNA transcription.[3]

Several recent observations have focused attention on the interaction of adriamycin with metal ions. An early report noted the effect of metal ions on its visible absorption spectrum,[4] Mikelens and Levinson[5] having lately shown that adriamycin is a chelating agent that forms metal ion complexes exhibiting DNA binding ability in a filter retention assay.

Metal ions are present in all biological processes involving the nucleic acids. As far as DNA seems to be the target of adriamycin action, the occurrence of metal chelates of the drug inside the cell may be an important step in the course of its pharmacological activity. If this is so, a better understanding of their formation and structure should enable clear insight in the way of action of anthracyclines. Several recent reports deal with the metal ion binding to adriamycin and other related anthracycline antibiotics. With use of spectroscopy and potentiometry, the stability constants of some species have been measured.[6–8] In addition, some data on the Cu(II)–adriamycin interaction have already been published.[9] However, many of the results obtained are not in agreement.

These considerations led us to examine the behavior of metal–adriamycin interaction in more detail. In this paper we present the results of an investigation aimed to accurately characterize the formation of two Cu(II)–adriamycin complexes, $Cu(HAd)_2$ and Cu(Ad), their spectral pattern, and their stability constants.

Experimental Section

Purified adriamycin was provided by Laboratoire Roger Bellon. Concentrations were determined by diluting stock solutions to approximately 10 μM and using $\epsilon_{480} = 11\,500$ M^{-1}.[10] As adriamycin solution is sensitive to light and oxygen, stock solutions were prepared just before use. Standard Cu(II) solutions were prepared from reagent grade material ($Cu(ClO_4)_2 \cdot 6H_2O$). All other reagents were of the highest quality available, and deionized bidistilled water was used throughout the experiments.

Absorption spectra were recorded on a Cary 219 spectrophotometer; CD spectra on a Jobin Yvon dicrograph, Model Mark V. Results are expressed in terms of ϵ (molar absorption coefficient) and $\Delta\epsilon = \epsilon_L - \epsilon_R$ (molar CD coefficients). The values of ϵ and $\Delta\epsilon$ are expressed in terms of [Adr], molar concentrations of adriamycin. Raman spectra were measured on a Coderg D 800 spectrometer using the exciting lines of Ar^+ and Kr^+ Spectra Physics lasers; 90° scattering was used throughout. Frequencies are reported with ± 2 cm^{-1} accuracy. Raman intensity measurements were made relative to the intensity of the ν_1 mode of the SO_4^{2-} (or ClO_4^-) band used as internal standard. The excitation profiles are reported as the ratio of the peak heights of the bands. The ratios were corrected for instrumental spectral response and ν dependence (ν = scattered radiation frequency).

Potentiometric measurements were obtained with a Tacussel pH meter, Model Isis 20000, at 25 °C under nitrogen atmosphere, using a Metrohm EA 147 combined-glass electrode.

Results

Potentiometric and Spectroscopic Titration of Adriamycin. Before studying the Cu(II)–adriamycin system we performed a potentiometric and spectroscopic reinvestigation of the behavior of adriamycin as a function of pH. Potentiometric titrations of adriamycin have already been done by May et al.,[6] and spectroscopic and potentiometric titrations of the parent drug daunorubicin (see Figure 1) by Kiraly and Martin.[7] We repeated the potentiometric and spectroscopic titrations of adriamycin for two reasons: (i) The results obtained by both teams are not in agreement. (ii) In order to facilitate comparisons, all our measurements were made in the same conditions as those used in the study of the Cu(II)–adriamycin system, namely 50 μM in adriamycin and $I = 0.05$ M KCl. It must be recalled that the adriamycin concentrations used by the authors above cited for potentiometric titrations were higher than 1 mM.

The adriamycin concentration was chosen low enough to avoid self-association. It is well documented that adriamycin and other anthracyclines tend to form aggregates even at moderately low concentrations. The problem to know whether the associated form is a dimer[11] or if aggregation beyond the dimer occurs[10] is still a matter of discussion. On the other hand, our standard procedures enabled us to readily obtain

[†] On leave from the Department of Chemistry, Federal University of Minas Gerais, Belo Horizonte, Brazil.
[‡] Université Paris XIII.
[§] Université Pierre et Marie Curie.

(1) Arcamone, F. *Top. Antibiotic Chem.* **1978**, *2*, 102.
(2) Neidle, S. *Top. Antibiotic Chem.* **1978**, *2*, 242.
(3) DiMarco, A.; Zunnio, F.; Silverstrini, R.; Gambarucci, C.; Gambetta, R. A. *Biochem. Pharmacol.* **1971**, *20*, 1323.
(4) Calendi, E.; DiMarco, A.; Reggiani, M.; Scarpinato, B. M.; Valentini, L. *Biochem. Biophys. Acta* **1965**, *103*, 25.
(5) Mikelens, P.; Levinson, W. *Bioinorg. Chem.* **1978**, *9*, 441.
(6) May, P. M.; Williams, G. K.; Williams, D. R. *Inorg. Chim. Acta* **1980**, *46*, 221.
(7) Kiraly, R.; Martin, R. B. *Inorg. Chim. Acta* **1982**, *67*, 13.
(8) Greenaway, F. T.; Dabrowiak, J. C. *J. Inorg. Biochem.* **1982**, *16*, 91.
(9) Spinelli, M.; Dabrowiak, J. C. *Biochemistry* **1982**, *21*, 5862.
(10) Chaires, J. B.; Dattagupta, N.; Crothers, D. M. *Biochemistry* **1982**, *21*, 3927.
(11) Martin, S. R. *Biopolymers* **1980**, *19*, 713.

0020-1669/83/1322-4117$01.50/0

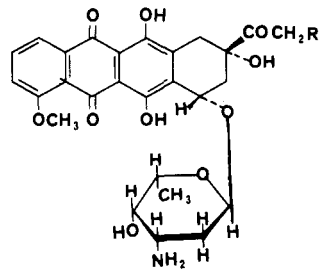

Figure 1. Structures of adriamycin (R = OH) and daunorubicin (R = H).

accurate results at concentrations not lower than 50 μM. We can estimate, however, from Chaires et al.[10] and from our own data that, at this concentration and at pH 6 (i.e., when adriamycin is fully protonated), 85%, at least, is in the monomeric form. As the titration proceeds, the monomer/dimer equilibrium changes and the concentration of the monomeric form increases. The small amount of the associated form does not impair our results. This was confirmed by the data of spectrophotometric titrations. Owing to the high sensitivity of the apparatus, we were able to work at much lower concentrations (10 μM) when aggregation is negligible.

Figure 2 shows the visible absorption spectra of adriamycin at different pH values. As has already been noticed by Kiraly and Martin,[7] at basic pH adriamycin is very unstable. This is attested to by the decrease of absorbance as time elapses (at pH 10.5, for instance, the absorbance decreases 50% in 1 h). So, to obtain reliable results, the pH of the solution was quickly raised from 5.8 (pH of the stock solution) to the desired value and the spectrum recorded in less than 2 min. A new solution was used for each of the curves of Figure 2.

At pH lower than 6 the absorption spectrum exhibits a broad band centered at 480 nm (ϵ = 11 500) with fine structure at 448, 478, 498, and 535 nm. Adriamycin is then fully protonated. When the pH is raised from 6 to about 7.6, a slight decrease in molar absorptivity is observed at 480 nm (from 11 500 to 10 700). Concomitantly, the potentiometric titration indicates one proton released per adriamycin as illustrated in Figure 3, with a pK_a = 6.8. Such deprotonation occurring without shift of the absorption band can be ascribed to the ammonium group of the amino sugar. Yet the small decrease of absorbance at 480 nm suggests some interaction between this group and the 1,4-dihydroxyanthraquinone moiety.

When the pH is raised from 8 to 11, the absorption at 480 nm decreases even more and the band shifts to higher wavelengths. At pH 11 the band is centered at 555 nm (ϵ = 12 800) with fine structure at 480, 516, 550, and 590 nm. This large shift may be attributed to deprotonation of the phenolic groups. We have used absorptivity at 590 nm, where the fully protonated form does not absorb, to monitor the deprotonation of the phenolic groups, and Figure 4 shows the plot of ϵ_{590} as a function of pH. As can be noticed, the absorptivity levels off at pH higher than 11. When the pH is increased from 11 to 13, no further modification is noticeable, strongly suggesting that both phenolic groups deprotonate in the pH range 8–11.

Owing to the increasing instability of adriamycin as pH increases (see above) potentiometric titrations could not be performed above pH 8. Hence the plot of absorptivity at 590 nm against pH has been used to determine the deprotonation constants, k_1 and k_2, of each one of the phenolic groups, defined as

$$k_1 = [HAd][H^+]/[H_2Ad] \qquad k_2 = [Ad][H^+]/[HAd]$$

where H_2Ad, HAd, and Ad stand for adriamycin with the 1,4-hydroxyanthraquinone moiety fully protonated, half-deprotonated, and fully deprotonated, respectively. In this formulation the state of protonation of the amino group is not taken into consideration although it is implicit, as we have shown above, that at pH higher than 8 the ammonium group

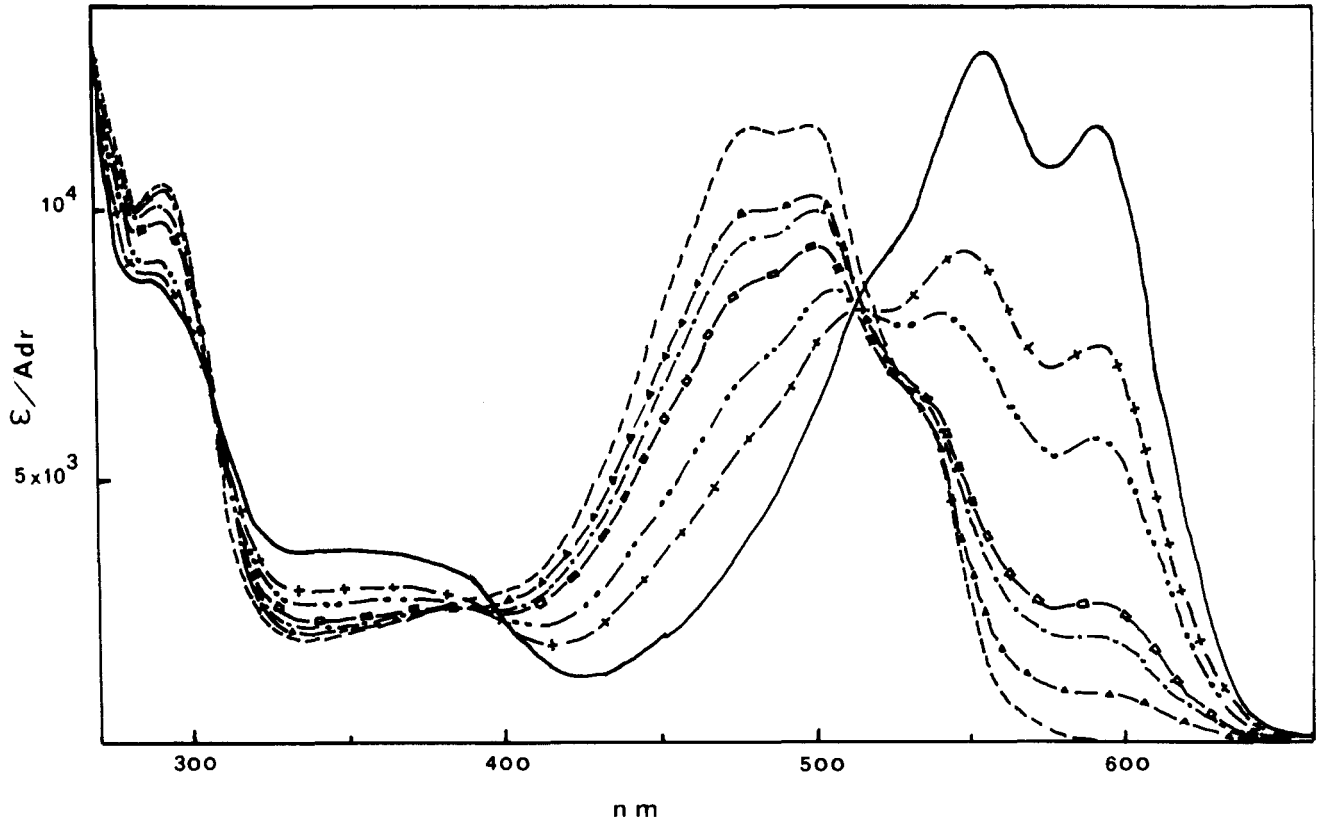

Figure 2. Absorption spectrum of adriamycin in aqueous solution at different pH values ([Adr] = 6.91 μM, [KCl] = 0.05 M): pH 5.80 (---), 8.30 (-▲-), 8.80 (-·-), 9.00 (-□-), 9.60 (-··-), 9.95 (-+-), 11.20 (—). For more detailed experimental conditions, see the text.

Table I. CD and Absorption Spectral Data (λ in nm) of Adriamycin at Different pH Values and Different Concentrations and of $Cu(HAd)_2$ and Cu(Ad) Complexes

Adr (pH 7)	10 μM	λ_M	232	252	290	380			480		
		ϵ_M	32 000	20 000	8000	1700			11 500		
	10 μM	λ_M	235	250	290	350		465			
		$\Delta\epsilon_M$	+12.0	+5.0	−2.6	+1.7		+1.0			
	10^{-3} M	λ_M			290	340		445	520		
		$\Delta\epsilon_M$			−2.6	+1.2		+2.0	−1.1		
Adr (pH 11.2);	10 μM	λ_M	232	252	290	370				555	
Δt = 2 min		ϵ_M	15 000	13 000	3700	1700				12 800	
	50 μM	λ_M				340		450 (sh)	530 (sh)	550	600 (sh)
		$\Delta\epsilon_M$				+1.2		+0.6	+1.8	+2.6	+1.0
	10^{-3} M	λ_M				340		445	530 (sh)	555	600 (sh)
		$\Delta\epsilon_M$				0.75		+0.65	+3.2	+5	+1.5
$Cu(HAd)_2$		λ_M	230		304		400 (sh)		512		
		ϵ_M	25 000		8400		2400		6700		
		λ_M	230	260	300	350 (sh)	380	430 (sh)	490	550	
		$\Delta\epsilon_M$	−26.5	+9.0	−18.0	+4.0	+5.5	+1.7	−6.0	+8.5	
Cu(Ad)		λ_M	230		316			420 (sh)		540	
		ϵ_M	20 600		8400			1700		5700	
		λ_M		260	305					570	605
		$\Delta\epsilon_M$		+16.4	+2.0					+4.5	+6.2

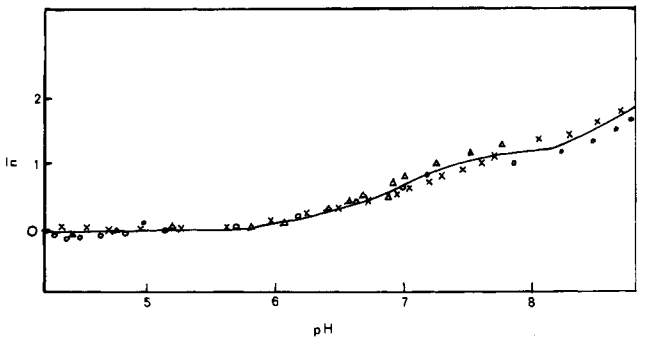

Figure 3. Potentiometric titration of adriamycin in the pH range 5–9, where $\bar{n}$ represents the number of protons released per adriamycin ([Adr] = 50 μM, [KCl] = 0.05 M, t = 25 °C). Three independent measurements have been performed.

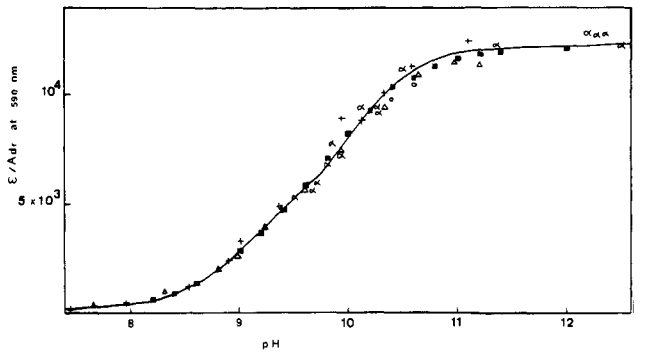

Figure 4. Spectrophotometric titration of adriamycin, as shown by the variation of the molar absorption, at 590 nm, of adriamycin in aqueous solution ([KCl] = 0.05 M): [Adr] = 6.91 μM (Δ); [Adr] = 8.10 μM (+); [Adr] = 12.52 μM (α). ■ represents the points calculated from ϵ_1 = 4700, pk_1 = 8.94, and pk_2 = 9.95 (see the text).

is fully deprotonated. Adr will stand hereafter for anyone of these forms.

At 590 nm the observed molar absorptivity $\langle\epsilon\rangle$ may be expressed by

$$\langle\epsilon\rangle = \frac{k_1[H^+]\epsilon_1 + k_1k_2\epsilon_2}{[H^+]^2 + k_1[H^+] + k_1k_2}$$

where ϵ_1 and ϵ_2 refer to the species HAd and Ad, respectively. At 590 nm ϵ_2 is equal to 12 200 (Figure 4) and ϵ_1 is unknown. The above equation was fitted by a nonlinear least-square analysis to 16 experimental intensities, at 590 nm, in the pH range 8.2–10.8 with ≃10 μM adriamycin. The best fit yields pk_1 = 8.94 and pk_2 = 9.95 with ϵ_1 = 4700.

At this stage it is interesting to compare our results with those of the authors referred to above. The values of pk_1 and pk_2 differ approximately in 1 order of magnitude from those obtained by May et al.[6] (8.1, 11). This difference is quite acceptable if one considers that these authors have worked at much higher concentrations (1–10 mM) at which the drug is completely in the aggregated form and have used potentiometric titrations at pH where decomposition is appreciable. The third pk value obtained by these authors, on the other hand, is too low (2.1) to be assigned to the ammonium group.[7]

A greater discrepancy is observed between our results and those of Kiraly and Martin[7] in the case of daunorubicin. They obtained pk values 2 orders of magnitude higher than ours (8.6, 10.0, 13.7) by using the same spectrophotometric method at the same concentration. This difference cannot be ascribed to the presence of H at R instead of the OH of adriamycin (see Figure 1) since they obtained similar values in the case of quinizarin. It might be accounted for by the deprotonation scheme proposed by these authors, which is different from ours. We think, however, that our spectrophotometric and potentiometric data unambiguously show that the first proton is fully released below pH 8 and that only above this pH the 1,4-dihydroxyanthraquinone moiety begins to deprotonate (see Figures 2 and 4).

CD Spectra of H_2Ad and Ad. As has already been reported, the CD spectrum of adriamycin is largely dependent on the association state of the drug.[11,12] At pH 7.4 and 10 μM concentration, adriamycin is fully in the monomeric form and the visible CD spectrum exhibits one positive band at 465 nm. When the concentration is increased to 1 mM, this band splits into a doublet characteristic of the associated form (see Table I).[11] As is shown in Figure 5, this pattern is not observed at basic pH (since the spectra evolved rapidly at high pH, they were recorded about 2 min after raising the pH). The CD visible spectrum at pH 11.2 is characterized by positive bands around 550 nm, and this occurs at concentrations ranging from 50 μM to 1 mM, although Δε at 550 nm increases with increasing adriamycin concentration. This spectrum, in fact, evolves very rapidly, and after 1 h, the visible bands change sign without any further evolution. This inversion in the visible CD spectrum strongly suggests that mutarotation of the amino sugar moiety takes place as pH increases.

Potentiometric and Spectrophotometric Titrations of the Cu(II)–Adriamycin System at 1:2 and 1:1 [Cu]:[Adr] Molar

(12) Barthelemy-Clavey, V.; Maurizot, J. C.; Dimicoli, J. L.; Sicard, P. *FEBS Lett.* **1974**, *46*, 5.

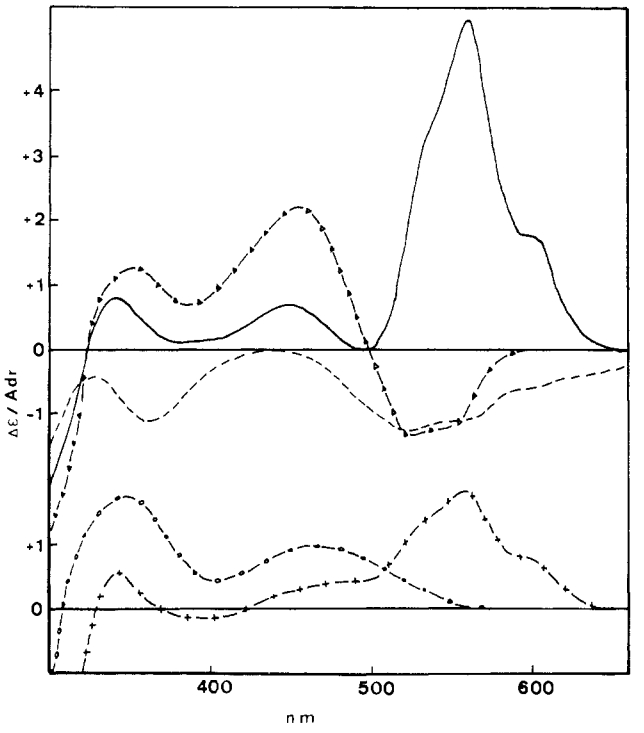

Figure 5. CD spectra of adriamycin: [Adr] = 10 μM, pH 7.4 (-○-); [Adr] = 50 μM, pH 11.2, Δt = 2 min (-+-); [Adr] = 10^{-3} M, pH 7.4 (-△-); [Adr] = 10^{-3} M, pH 11.2, Δt = 2 min (—) and 60 min (---).

Ratios. The formation of Cu(II)–adriamycin complexes as a function of [Cu]:[Adr] molar ratio has been followed by visible absorption and CD spectroscopy. The addition of Cu(II) to adriamycin at pH 7.4 (HEPES buffer 0.05 M) yields a CD spectrum exhibiting two characteristic bands: a negative one at 490 nm and a positive one at 550 nm. The value $\Delta\epsilon$/[Adr] of both bands increases as the molar ratio [Cu]:[Adr] increases reaching a maximum of respectively −6 and +8.5 at [Cu]:[Adr] = 1:2. At [Cu]:[Adr] = 1:1 the CD spectrum is remarkably different exhibiting, in the visible region, two positive bands at 570 nm ($\Delta\epsilon$/[Adr] = +4.5) and 605 nm ($\Delta\epsilon$/[Adr] = +6.2). Addition of 1 or 2 mol more of copper/mol of adriamycin does not greatly modify this spectrum. The conclusion therefore follows that two different copper complexes are formed depending on molar ratio: one at [Cu]:[Adr] = 1:2 with the metal bound to two adriamycins and the other at [Cu]:[Adr] = 1:1 with the metal bound to only one. The absorption spectra of both species are also shown in Figure 6. It must be pointed out, however, that formation of these complexes is rather slow: about 30 min is necessary to reach the maximal amplitude of the CD bands. Moreover, the same spectra are obtained whatever the concentration of adriamycin used, if ranging from 50 μM to 1 mM, indicating that the formation of both complexes is independent of the initial state of aggregation of the drug.

In order to accurately characterize both species, potentiometric and spectrophotometric titrations of the Cu(II)–adriamycin system at molar ratios equal to 1:1 and 1:2 were performed with 50 μM adriamycin and KCl (0.05 M). In Figure 7 are illustrated the visible CD spectra of one Cu-

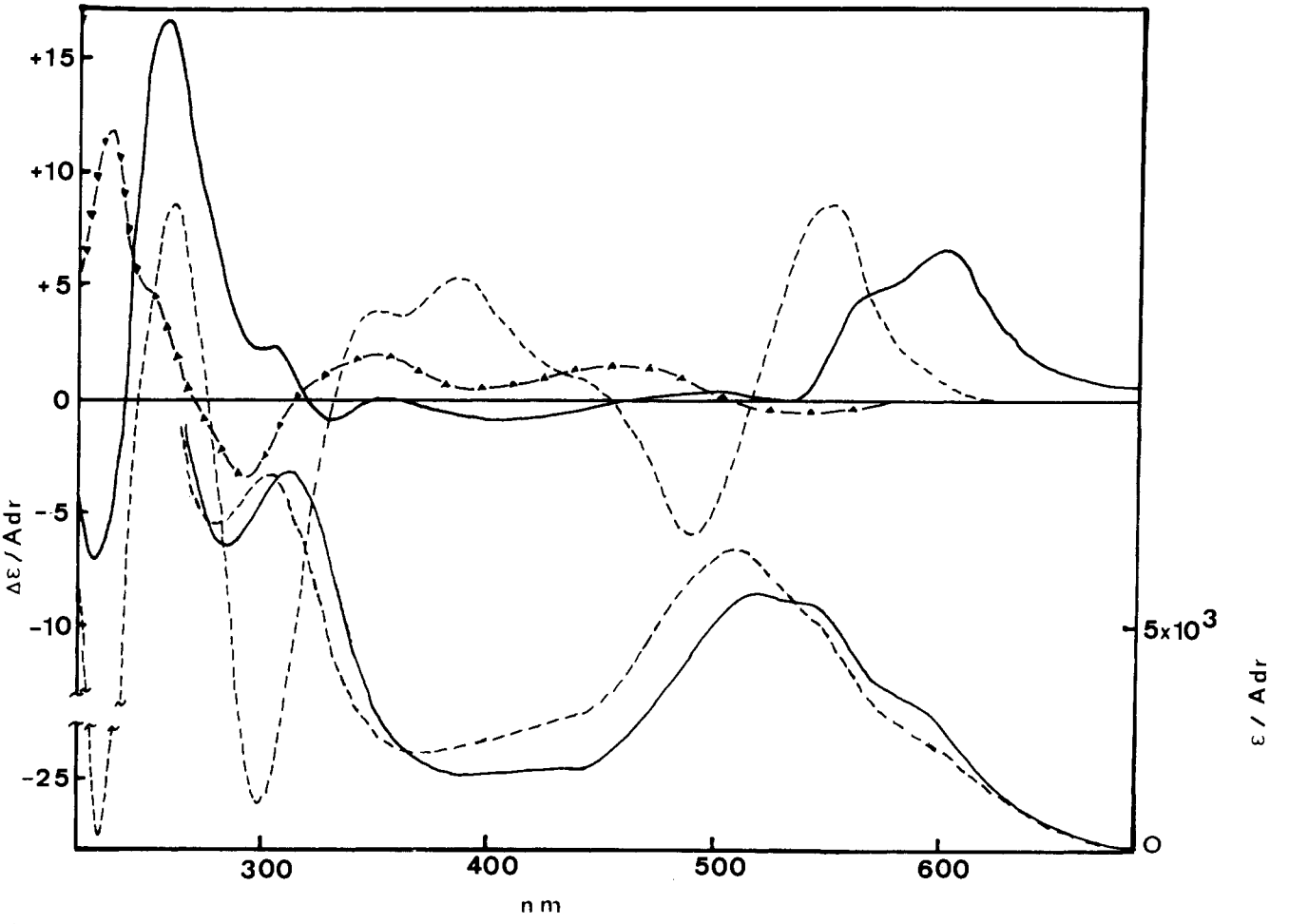

Figure 6. Absorption and CD spectra of the Cu(II)–adriamycin system (HEPES buffer 0.05 M, pH 7.4, [Adr] = 50 μM): [Cu]:[Adr] = 1:2, $Cu(HAd)_2$ complex (---); [Cu]:[Adr] = 1:1, Cu(Ad) complex (—). For comparison, the CD spectrum of adriamycin has been plotted (-▲-).

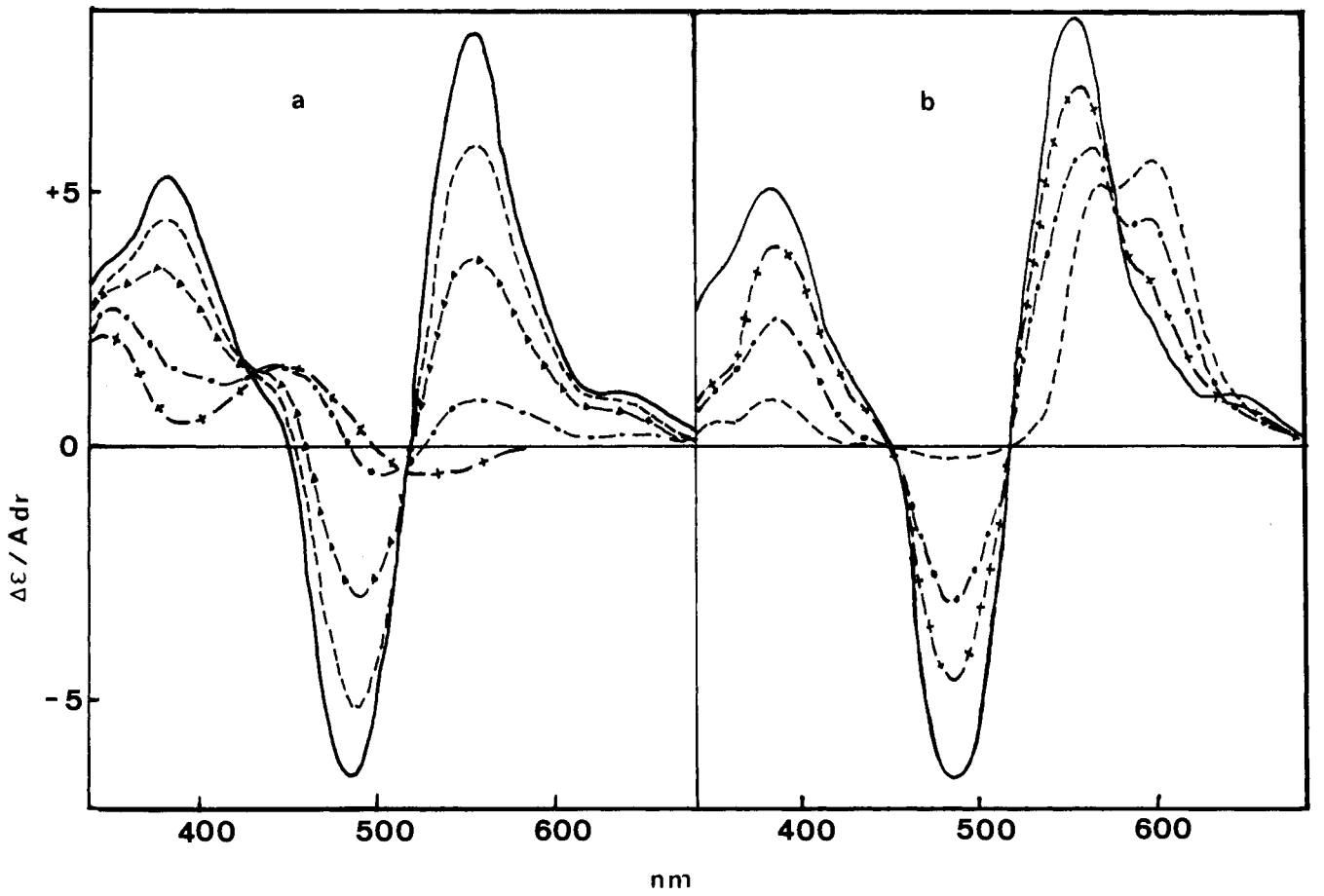

Figure 7. CD spectrum of the 1:1 Cu(II)–adriamycin system at different pH values ([Adr] = 50 μM, [KCl] = 0.05 M). Curves a: pH 4.2 (–+–); pH 4.5 (–•–); pH 4.9 (–▲–); pH 5.2 (---); pH 5.6 (—). Curves b: pH 5.6 (—); pH 6.4 (–+–); pH 6.6 (–•–); pH 7.0 (---).

(II)–adriamycin 1:1 solution at various pH. As pH increases from 4 to 5.8 (Figure 7a), the spectral pattern characteristic of the Cu(II)–adriamycin 1:2 complex becomes clearly apparent. When the pH is further increased from 5.8 to 7.2, striking changes are noticeable, and at pH 7.3, the spectrum of the Cu(II)–adriamycin 1:1 complex is finally obtained (Figure 7b). An isodichroic point is observed at 570 nm.

Figure 8 shows the potentiometric titration of the same Cu(II)–adriamycin 1:1 solution (curve a), the plot of $\Delta\epsilon$ at 490 nm (curve b), and that of $\Delta\epsilon$ at 605 nm (curve c) against pH. The first $\Delta\epsilon$ value has been taken as a measure of the Cu–adriamycin 1:2 complex concentration and the second as that of the Cu–adriamycin 1:1 complex. As can be seen, in the pH range 4.5–5.8 one proton per adriamycin is released; $\Delta\epsilon$ at 490 nm increases and reaches a maximum (–6) at pH 5.8. Then, from pH 5.8 to 7.3, two additional protons per adriamycin are released with the concomitant decrease of $\Delta\epsilon$ at 490 nm and the increase of $\Delta\epsilon$ at 605 nm, a pattern characteristic of the Cu–adriamycin 1:1 complex.

From the foregoing results we can infer that the Cu(II)–adriamycin 1:2 complex is formed at pH 5.8 with the release of one proton per adriamycin due to phenolate binding to copper, and this even in the presence of an excess of copper. At pH 7.3 the Cu–adriamycin 1:1 complex is formed by the removal of three protons per adriamycin suggesting that two of them arise from phenolate binding to copper and that the third is released by the ammonium group. As we have shown above, the ammonium group deprotonates in this pH range independently of the presence of copper and most probably does not bind to the metal. These complexes may then be formulated as $[Cu^{2+}(AdNH_3^+,OH,O^-)]_n^{2+}$ and $[Cu^{2+}(AdNH_2,O^-,O^-)]_p$. The values of n and p will be discussed below. For the sake of simplicity, using the above-mentioned convention, we will refer to them as $Cu(HAd)_2$ and Cu(Ad). Their spectroscopic data are collected in Table I.

Similar experiments performed with a Cu(II)–adriamycin 1:2 solution at pH 5.8 yield the spectral pattern characteristic of the $Cu(HAd)_2$ complex. No further modification of these spectra are observed by increasing the pH up to 7.4.

Determination of $Cu(HAd)_2$ and CuAd Stability Constants. Stability constants were obtained by two different methods: (i) spectrometric titrations and (ii) the method of competing equilibria.[13]

The formation constants β_I and β_{II} are defined by the equilibria

$$Cu^{2+} + 2HAd \rightleftharpoons Cu(HAd)_2$$

$$\beta_I = \frac{[Cu(HAd)_2]}{[Cu^{2+}][HAd]^2} \quad (1)$$

and

$$Cu^{2+} + Ad \rightleftharpoons Cu(Ad)$$

$$\beta_{II} = \frac{[Cu(Ad)]}{[Cu^{2+}][Ad]} \quad (2)$$

In addition, one can state

$$Cu^{2+} + 2H_2Ad \rightleftharpoons Cu(HAd)_2 + 2H^+$$

$$^*\beta_I = \frac{[Cu(HAd)_2][H^+]^2}{[Cu^{2+}][H_2Ad]^2} \quad (3)$$

(13) Rossotti, F. J. C.; Rossotti, H. "The Determination of Stability Constants"; McGraw-Hill: New York, 1961; p 78.

and

$$Cu^{2+} + Cu(HAd)_2 \rightleftharpoons 2Cu(Ad) + 2H^+$$

$$^*\beta_{II} = \frac{[Cu(Ad)]^2[H^+]^2}{[Cu^{2+}][Cu(HAd)_2]} \quad (4)$$

Taking into account the two-step phenolic deprotonation k_1 and k_2, it follows that

$$\beta_I = \frac{^*\beta_I}{k_1^2} \qquad \beta_{II} = \frac{(^*\beta_I{}^*\beta_{II})^{1/2}}{k_1k_2}$$

With use of the first method, $^*\beta_I$ and $^*\beta_{II}$ have been determined for various pH values by means of the spectrophotometric and potentiometric titration data of Figure 8. As we have indicated previously, $[Cu(HAd)_2]$ and $[Cu(Ad)]$ have been measured by $\Delta\epsilon$ at 490 and 605 nm, respectively. On the other hand, one can state

$$[Adr]_T = [H_2Ad] + [HAd] + [Ad] + 2[Cu(HAd)_2] + [Cu(Ad)] \quad (5)$$

Owing to the low value of the deprotonation constants k_1 and k_2, [HAd] and [Ad] are negligible in comparison with $[Cu(HAd)_2]$ and [Cu(Ad)] below pH 7.4. Hence, $[H_2Ad]$ can be calculated from eq 5. The stability constants thus obtained are $\beta_I = 3.6 \times 10^{16}$ and $\beta_{II} = 1.2 \times 10^{12}$.

For the second method, diethyldithiocarbamate (DTC) and arginine were chosen as competing ligands and added to a solution containing Cu(Ad) in HEPES buffer (0.05 M, pH 7.4). The addition of 1 mol of DTC/mol of Cu(Ad) yields the $Cu(HAd)_2$ species according to

$$Cu(Ad) + DTC = {}^1/_2Cu(DTC)_2 + {}^1/_2Cu(HAd)_2$$

When the second mole is added, free adriamycin is obtained:

$${}^1/_2Cu(HAd)_2 + DTC = {}^1/_2Cu(DTC)_2 + {}^1/_2H_2Ad$$

A similar step reaction is obtained by addition of increasing concentrations of arginine although in this case a large excess of competing ligand is necessary. The $Cu(HAd)_2$ complex is fully formed with [Adr] = 100 μM and [Arg]/[Adr] = 10. An isodichroic point is noticeable in the CD spectra of this system where, one must recall, the CD signal of $Cu(Arg)_2$ is negligible in comparison with those of CuAd and $Cu(HAd)_2$. With further addition of arginine the signal of free adriamycin is recovered.

In Figure 9, the percentages of Cu(Ad) and $Cu(HAd)_2$ present in the solution, as determined by $\Delta\epsilon$ at 605 and 490 nm, respectively, have been plotted against [Arg]/[Cu]. In this case the following equilibria can be taken into consideration:

$$2Cu(Ad) + 2HArg \overset{K_1}{\rightleftharpoons} Cu(HAd)_2 + Cu(Arg)_2$$

$$K_1 = \frac{[Cu(Arg)_2][Cu(HAd)_2]}{[Cu(Ad)]^2[HArg]^2} \quad (6)$$

$$Cu(HAd)_2 + 2HArg \overset{K_2}{\rightleftharpoons} Cu(Arg)_2 + 2H_2Ad$$

$$K_2 = \frac{[H_2Ad]^2[Cu(Arg)_2]}{[Cu(HAd)_2][HArg]^2} \quad (7)$$

$$Cu^{2+} + 2HArg \overset{^*\beta_A}{\rightleftharpoons} Cu(Arg)_2 + 2H^+$$

$$^*\beta_A = \frac{[Cu(Arg)_2][H^+]^2}{[Cu^{2+}][HArg]^2} \quad (8)$$

In these equations HArg stands for arginine with the α-amino group protonated. The guanidine moiety is protonated in the pH range under study and is not taken into account since it does not intervene in complexation.

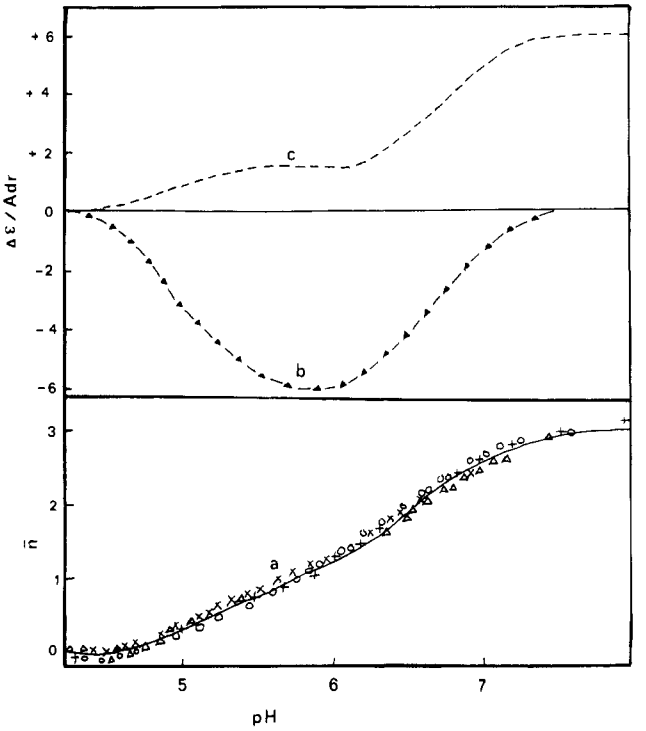

Figure 8. Potentiometric and spectrophotometric titrations of the 1:1 Cu(II)–adriamycin system ([KCl] = 0.05 M, [Adr] = 50 μM). Curve a: $\bar{n}$, the number of protons released per adriamycin, as a function of pH. Ten independent measurements have been performed. Curve b: $\Delta\epsilon$ at 490 nm as a function of pH. Curve c: $\Delta\epsilon$ at 605 nm as a function of pH.

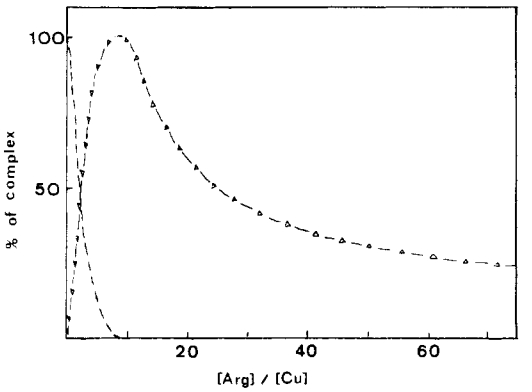

Figure 9. Percentage of complex present as a function of [Arg]:[Cu] molar ratio for the Cu(II)–adriamycin–arginine system ([Adr] = 100 μM, HEPES buffer 0.05 M, pH 7.4): percentage of Cu(Ad) (---), percentage of $Cu(HAd)_2$ (-Δ-).

From (6)–(8) it follows $^*\beta_I = {}^*\beta_A/K_2$ and $^*\beta_{II} = {}^*\beta_A/K_1$. Here again owing to the small deprotonation constant of arginine (14), [Arg] is negligible as compared to [HArg] and $[Cu(Arg)_2]$ at pH 7.4 in the equation

$$[Arg]_T = [HArg] + [Arg] + 2[Cu(Arg)_2]$$

β_I and β_{II} are thus calculated from the values of k_1 and k_2 previously determined and that of $^*\beta_A$ from ref 14, giving $\beta_I = 5.7 \times 10^{16}$ and $\beta_{II} = 2.5 \times 10^{12}$. The stability constants obtained by these two independent methods are in quite good agreement. Thus, taking the mean of them, we finally obtain $\beta_I = (4.6 \pm 1.1) \times 10^{16}$ and $\beta_{II} = (1.8 \pm 0.7) \times 10^{12}$.

(14) Phan, C. V.; Tosi, L.; Garnier, A. *J. Inorg. Nucl. Chem.* **1975**, *37*, 2385.

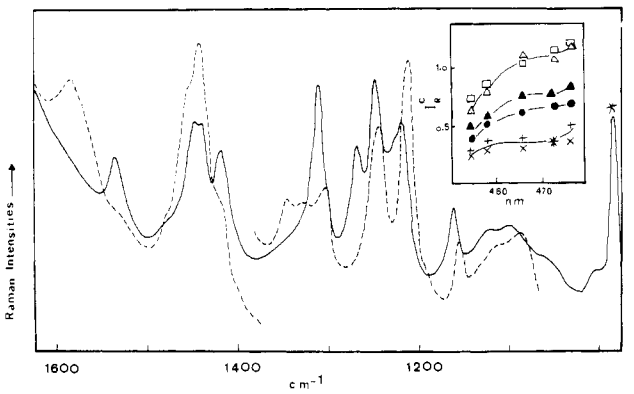

Figure 10. Resonance Raman spectra of $Cu(HAd)_2$ (—) and adriamycin (---) using the 457.9-nm laser line ([Adr] = 200 μM, $[Cu(HAd)_2]$ = 100 μM, HEPES buffer, pH 7.4, $[SO_4^{2-}]$ = 0.05 M). Inset: excitation profiles of the bands at 1535 cm^{-1} (×), 1418 cm^{-1} (●), 1310 cm^{-1} (△), 1268 cm^{-1} (▲), 1248 cm^{-1} (□), 1162 cm^{-1} (+). Experimental conditions: laser power 60 mW, slit width 6 cm^{-1}, scan rate 50 cm^{-1}/min.

Resonance Raman (RR) Spectra. The RR spectrum of the $Cu(HAd)_2$ complex in the 950–1600-cm^{-1} region, upon excitation with 457.9-nm laser radiation, is illustrated in Figure 10. For comparison, the RR spectrum of adriamycin is also represented. The region below 1000 cm^{-1} has been omitted since the spectrum of the complex exhibits only weak bands that are not enhanced in the excitation range under study. As is known, adriamycin is strongly fluorescent in aqueous solution so that a good RR spectrum can only be obtained by exciting with the 454.5- and 457.9-nm laser lines.[15,16] Coordination to copper quenches partially this fluorescence, and four more exciting lines can be explored at 465.8, 472.7, 476.5, and 488.0 nm. It is impossible to go beyond that limit, and with use of the last line, intensity measurements are very inaccurate.

In addition, adriamycin is very sensitive to laser radiation at 457.9 nm when in the monomeric form but becomes more stable as more associated form is present in the solution. A concentration of 200 μM is enough to record a quite good spectrum in an interval of 15–20 min. Coordination to copper to form the $Cu(HAd)_2$ complex stabilizes the drug, and several scans can be performed with the same solution without appreciable alteration. This is not true for the Cu(Ad) complex, which under the effect of laser radiation rapidly transforms in a mixture of both species. Hence, intensity measurements were performed with the $Cu(HAd)_2$ complex. In fact, the RR patterns of both systems are analogous (see below).

As can be noticed by inspection of Figure 10, striking changes take place in the RR spectrum of the drug upon complexation. Three regions are particularly affected: 1500–1590, 1300–1450, and 1200–1270 cm^{-1}. The band at 1587 cm^{-1} shifts to 1532 cm^{-1}, the strong and broad band at 1445 cm^{-1} splits in two overlapping peaks at 1448–1438 cm^{-1}, and the weak shoulder at 1420 cm^{-1} as well as the band at 1310 cm^{-1} are considerably enhanced. The band at 1215 cm^{-1} shifts to 1220 cm^{-1} and decreases in intensity whereas a new band appears at 1268 cm^{-1}. The same spectral pattern is obtained with the $Cu(HAd)_2$–Cu(Ad) mixture, although the bands at 1220, 1268, and 1310 cm^{-1} are somewhat less intense.

Discussion

From the foregoing results it appears now clearly established that, depending on pH, adriamycin forms two well-defined complexes with Cu(II): $Cu(HAd)_2$ and Cu(Ad).

The visible CD spectrum of adriamycin gives additional information on the stacking of the monomers. In aqueous solution, at 1 mM concentration and at pH lower than 8 so that adriamycin is in the H_2Ad form, the self-association of Adr gives rise to a visible CD spectrum that is conservative (couplet type).[17]

The amplitude of the CD signal contains information on the stacking geometry and can be interpreted in terms of the angle ω between the transition dipole moments μ of the 480-nm transition (the long axis $^1L_b \rightarrow {}^1A$ transition[18–20]). We have adopted the model of a vertical stack with R_{12} perpendicular to the rings and equal to the van der Waals distance of closest approach (3.4 Å); ω has been calculated by using the expression[17,21]

$$\sin \omega = 0.88 \frac{\Delta_0}{\pi} \frac{1}{R_{12}} \frac{1}{|\mu|^2} |\Delta\epsilon|_0$$

where Δ_0 is the half-bandwidth at $\Delta\epsilon = 0.368\Delta\epsilon_M$ of the monomeric absorption spectrum. With use of the values determined from the monomer absorption spectrum, Δ_0 = 60 nm and μ = 4.9 D, a value of approximately 24° is obtained (or 180° + 24°). We cannot arrive at a unique geometry on the basis of the CD data alone. Of the four possible geometries (head-to-head and head-to-tail each with ω = 24 or 204°), it appears that the head-to-tail with ω = 204° is the most likely. In this geometry, the repulsion between the permanent dipole moments as well as the steric hindrance is minimized.

It is interesting to compare these data with those obtained for the $Cu(HAd)_2$ complex. As can be seen in Figure 6, the visible CD spectrum of this complex is also of the couplet type. In this case, however, it is more difficult to determine the angle between the transition dipole moments since we ignore which values to adopt for the monomer. In fact, as we have calculated that the transition moment for the species Ad (at pH 11.2) is not far from that of H_2Ad (~4.9 D), we can assume a similar value for the species HAd. In that case using the above expression, it turns out that $\sin \omega > 1$. This result means that, in the case of $Cu(HAd)_2$, R_{12} cannot be taken as the van der Waals distance of closest approach. If, as this result suggests, the presence of the metal separates the two molecules of adriamycin, one might infer that it is located between the two molecular planes.

As can be seen in Figure 6, the CD spectrum of the Cu(Ad) complex differs considerably from that of $Cu(HAd)_2$. From the absence of splitting of the visible CD band we can immediately infer that in this complex there is no stacking of molecules. The same observation holds for the CD spectrum of adriamycin at 1 mM concentration at pH 11.2, which exhibits in the visible only positive bands and no couplet. This fact strongly suggests that the presence of phenolic groups or, alternatively, coordination to a copper ion is necessary in order to ensure self-association. In fact, the CD spectrum of the drug at pH 11.2 compares with that of the complex Cu(Ad) exhibiting positive bands in the 550–600-nm region.

One may postulate that in the $Cu(HAd)_2$ and Cu(Ad) complexes the ligand coordinates to the metal through a carbonyl and a phenolate oxygen forming a six-membered chelate. The strong shift observed at 1587 cm^{-1} upon coordination as well as the appearance of the bands at 1420 and 1268 cm^{-1} corroborates this assumption. The band at 1587 cm^{-1} is primarily assigned either to a pure ring CC stretch[22]

(15) Manfait, M.; Bernard, L.; Theophanides, T. *J. Raman Spectrosc.* **1981**, *11*, 68.
(16) Angeloni, L.; Smulevich, G.; Marzocchi, M. P. *Spectrochim. Acta, Part A* **1982**, *38A*, 213.
(17) Bayley, P. M. *Prog. Biophys. Mol. Biol.* **1973**, *27*, 3.
(18) Sidman, J. W. *J. Am. Chem. Soc.* **1956**, *78*, 4567.
(19) Capps, R. N.; Vala, M. *Photochem. Photobiol.* **1981**, *33*, 673.
(20) Bell, M. J.; Buchanan, G. W.; Hollebone, B. R.; Jones, D. *Can. J. Chem.* **1982**, *60*, 291.
(21) Heyn, M. P.; Bretz, R. *Biophys. Chem.* **1975**, *3*, 35.
(22) Smulevich, G.; Angeloni, S.; Giovannardi, S.; Marzocchi, M. P. *Chem. Phys.* **1982**, *65*, 313.

or to a ring CC stretch coupled with the hydrogen-bonded carbonyl CO mode.[15] It must contain, however, a strong contribution of the CO vibration; otherwise, a smaller shift should be expected. A very strong shift has been observed in the infrared band assigned to the stretching mode of the hydrogen-bonded carbonyl in the case of several hydroxy-*p*-quinones upon chelation to metal ions to form five- and six-membered rings via the carbonyl and phenolate oxygens.[23] This shift ranges from 52 to 146 cm^{-1} in the case of Cu(II) complexes. Similar results were obtained with the insoluble polymeric Cu(II) complex of adriamycin.[8]

In the $Cu(HAd)_2$ complex there must be two carbonyls not coordinated to the metal. Accordingly one might expect to find the peak at 1587 cm^{-1} still present in the spectrum although somewhat less intense. As a matter of fact, we were unable to detect this peak in our spectra. The only explanation to this fact is that the nonbonded carbonyl groups are not hydrogen bonded in the complex. The free carbonyl stretch is very weak in the Raman spectrum and lies at much higher frequencies where it is very difficult to detect owing to the overlapping of the HOH bending of water. In D_2O solution this band appears to be strongly coupled with the C=O stretching vibration.[15]

As we have shown above, the presence of phenolic groups are necessary for self-association and this suggests that intermolecular hydrogen bonding takes place at higher concentrations of the drug. In addition, as our calculations suggest, if the metal intercalates between the molecular planes or, alternatively, if complexation separates them, intermolecular hydrogen bonding, if any, should be weakened. This fact could explain why the carbonyl not coordinated to the metal is free.

The phenolate CO stretching mode has been found around 1260–1288 cm^{-1} in metal transferrins[24,25] and at 1272 cm^{-1} in Cu(II)–tyrosinato complexes.[26] Accordingly, we propose to assign the new band at 1268 cm^{-1} primarily to this mode. As can be noticed, the strong peak at 1215 cm^{-1} in the spectrum of adriamycin decreases appreciably in intensity in that of the complex (see Figure 10). Since in the $Cu(HAd)_2$ complex one phenolic group per adriamycin remains, we propose to assign this peak mainly to the CO stretch of the phenolic group that shifts to higher frequencies when free. In the infrared spectrum of phenol, this vibration lies at 1249 cm^{-1}.[27] A shift of 53 cm^{-1} upon complexation appears quite strong indeed but comparable to that of the carbonyl stretch.

The strong and broad band at 1445 cm^{-1} with two weak shoulders at 1457 and 1420 cm^{-1} has been attributed either to a CC skeletal mode[22] or to a coupling of CC and carbonyl stretches.[15] On the other hand, the band at 1310 cm^{-1} has been assigned either to the phenolic CO stretch[22] or to the CC plus CO stretches.[15] Since neither of them is shifted upon complexation, a pure CC stretch mode seems more plausible for both bands. As far as they are strongly enhanced upon complexation, a major contribution of the CC stretches of the chelate ring in both vibrations may be postulated. In fact, a neat increase in intensity at 1420 cm^{-1} has also been observed in the DNA–adriamycin complex without significant change at 1310 cm^{-1}. Since in that case the drug is protonated, an involvement of the stretch corresponding to the C–C bond that contains the phenolic carbon is assignable to the band at 1310 cm^{-1}. Further investigations are under way in order to study the validity of these assumptions.

As we have stated above, in the spectra of the $Cu(HAd)_2$–Cu(Ad) mixture the bands at 1220, 1268, and 1310 cm^{-1} are less intense. We expect, in fact, to find in this region the only difference between the RR spectra of $Cu(HAd)_2$ and Cu(Ad). However, as it is difficult to ascertain the percentage of $Cu(HAd)_2$ in the mixture, it is impossible to draw conclusions. Anyhow, taking into account that in Cu(Ad) adriamycin is fully deprotonated, the complex must be of the long-chain polymeric type. Bottei and McEachern[23] have shown that dihydroxy-*p*-quinones able to form five- or six-membered chelates in opposite sides of benzene or naphthalene rings tend always to form long-chain polymers.

The excitation profiles of the resonance-enhanced bands are presented in the Figure 10 inset. For the sake of clarity, those of the bands at 1448–1438 and 1220 cm^{-1} are not represented since they follow the same trend of the others. The intensity values in arbitrary units are the mean of three measurements. All the bands show a similar enhancement pattern, reaching a plateau at approximately 460 nm. There is about a twofold increase in intensity (except at 1535 and 1162 cm^{-1}) and a subsequent enhancement seems to take place beyond 475 nm. In the RR spectra of adriamycin in the solid state, the Raman bands occurring in the 1550–1200-cm^{-1} range are in resonance with the absorption at 485 nm. This peak corresponds to a Franck–Condon progression roughly involving an overlapping contribution of several skeletal modes with ν_m = 1530 cm^{-1}.[16] The enhancement pattern of the Figure 10 inset could also be interpreted as arising from one maximum of a vibronic progression.

Alternatively, the RR pattern most probably arises from two different electronic transitions: the π–π^* one due to the quinoid structure and a second transition due to the ligand-to-metal charge transfer (LMCT). Unfortunately, the strong fluorescence of the complex precludes the unambiguous assignment of the latter. The phenolate-to-copper(II) LMCT transition lies around 390–440 nm.[24,26] In the six-membered chelates $Cu(HAd)_2$ and Cu(Ad), strong delocalization is not expected since, as we have shown, the carbonyl and phenolate stretching vibrations lie in a well-defined spectral region characteristic of these modes. Hence, a moderate shift to longer wavelengths should result; 20–30 nm does not seem unreasonable.

Three conclusions may be drawn from these data: (i) participation of the amino sugar moiety in bonding via the NH_2 group, if any, is negligible even at pH 7; (ii) at pH around 7 and low [Cu(II)]:[Adr] molar ratio, which are probably physiological conditions, the species preferentially formed in $Cu(HAd)_2$; (iii) in the presence of competing ligands, even at higher [Cu(II)]:[Adr] molar ratios (1:1), the complex that may be actually formed is again $Cu(HAd)_2$.

Acknowledgment. We thank the Laboratoire Roger Bellon for providing the adriamycin used in this work. H.B. is grateful to CAPES (Brazil) for a fellowship.

Registry No. Cu(Ad), 87861-42-5; $Cu(HAd)_2$, 87861-43-6.

(23) Bottei, R. S.; McEachern, C. P. *J. Inorg. Nucl. Chem.* **1970**, *32*, 2653.
(24) Gaber, B. P.; Miskowski, V.; Spiro, T. G. *J. Am. Chem. Soc.* **1974**, *96*, 6868.
(25) Tomimatsu, Y.; Kint, S.; Scherer, J. M. *Biochemistry* **1976**, *15*, 4918.
(26) Garnier-Suillerot, A.; Albertini, J. P.; Collet, A.; Faury, L.; Pastor, J. M.; Tosi, L. *J. Chem. Soc., Dalton Trans.* **1981**, 2544.
(27) Pinchas, S. *Spectrochim. Acta, Part A* **1972**, *28*, 801.