Iron(III)–Adriamycin and Iron(III)–Daunorubicin Complexes: Physicochemical Characteristics, Interaction with DNA, and Antitumor Activity†

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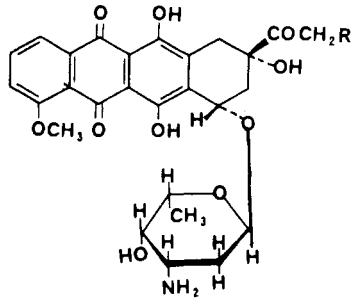
ABSTRACT: Fe(III) complexes of two anthracyclines, adriamycin and daunorubicin, have been studied. Using potentiometric and spectroscopic measurements, we have shown that adriamycin and daunorubicin form two well-defined species with Fe(III), which can be formulated as respectively $Fe(HAd)_3$ and $Fe(HDr)_3$. In these formulas, HAd and HDr stand for adriamycin and daunorubicin in which the 1,4-dihydroxyanthraquinone moiety is half-deprotonated. Both complexes are six-membered chelates. The stability constant is $\beta = (2.5 \pm 0.5) \times 10^{28}$ for both complexes. Interaction with DNA has been studied showing that, despite strong coordination to Fe(III), anthracyclines are able to intercalate between DNA bases pairs, releasing the metal. These complexes display antitumor activity against P 388 leukemia that compares with that of the free drug. $Fe(HAd)_3$, unlike adriamycin, does not catalyze the flow of electrons from NADH to molecular oxygen through NADH dehydrogenase. Moreover, it is shown that the triferric adriamycin compound so called "quelamycin" is in fact a mixture of $Fe(HAd)_3$ and polymeric ferric hydroxide.

The anthracycline antibiotics adriamycin (Adr) and daunorubicin (Dr) are highly efficient antineoplastic agents; their



R = H Daunorubicin Dr

R = OH Adriamycin Adr

clinical use is limited due to clinical and histopathologic evidence of cardiotoxicity (Ferrans, 1978). Although the mechanism of anthracycline cardiac toxicity remains incompletely understood, recent studies have suggested that the cytotoxic effects of these agents may be related to the formation of semiquinone free-radical intermediates in vivo (Sato et al., 1977). Both cardiac sarcosomes (Doroshow, 1981) and mitochondria (Doroshow, 1981; Thayer, 1977) can reduce Adr and Dr to their respective semiquinones, initiating a free-radical cascade. Thayer (1977) suggested initially and Davies et al. (1983) demonstrated latter that a component of mitochondrial NADH dehydrogenase actively reduces Adr.

The hope of finding a noncardiotoxic yet active anthracycline antibiotic has spurred the search for new naturally occurring anthracyclines (Bhuyan & Smith, 1975; Hori et al., 1977; Nettleton et al., 1977) and the development of a large number of semisynthetic analogues (Arcamone, 1977; Israël et al., 1975; Maral et al., 1979); one of these semisynthetic compounds has been named quelamycin by Gosalvez et al. (1978); quelamycin is reported to consist of a single adriamycin molecule chelated to three iron atoms. Gosalvez et al. (1978) reported that the adriamycin iron compound lacks cardiotoxicity while other workers have found that it yields increased cardiac toxicity as compared to adriamycin alone (Young et al., 1978; Bono, 1980; Egorin et al., 1983).

Other observations have focused attention on the interaction of these anthracyclines with iron: May et al. (1980) and Kiraly & Martin (1982) have determined the stability constants; Sugioka et al. (1981) have studied the decomposition of unsaturated phospholipid by iron–ADP–adriamycin complex as well as the mechanism of phospholipid peroxidation induced by this complex (Sugioka & Nakano, 1982); Samuni et al. (1984) studied the interaction of the adriamycin–iron complex with model membrane systems, and Myers et al. (1982) examined the oxidative destruction of erythrocyte ghost membranes catalyzed by the adriamycin–iron complex. This last team reported a 1:3 Fe(III)–adriamycin complex. On the other hand, Bachur et al. studied the tissue distribution and metabolism of a wide variety of freshly prepared and lyophilized iron–adriamycin perparations in mice (Egorin et al., 1983). They also investigated the physicochemical characteristics of ferric adriamycin complexes (Bachur et al., 1984).

These reports led us to examine the iron–anthracylcine systems in more detail. In this paper, we report the following experiments. (i) The complexes that are formed between Fe(III) and adriamycin and Fe(III) and daunorubicin, respectively, have been accurately identified: we have shown that Fe(III) binds three molecules of drug with the release of one proton per molecule; the stability constants of these complexes as well as their spectral pattern have been determined, and two conformers named A and D have been identified. (ii) The interaction of these complexes with deoxyribonucleic acid

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(DNA) has been studied: in the presence of DNA, Fe(III) is slowly released from the complex, and the drug is intercalated between the base pairs. (iii) The ferric complexes of these two anthracyclines inhibit P 388 leukemia cell growth in vitro and display antitumor activity against P 388 leukemia. (iv) The ferric adriamycin complex, unlike free adriamycin, does not catalyze the flow of electrons from nicotinamide adenine dinucleotide (NADH) to molecular oxygen through NADH dehydrogenase.

Materials and Methods

Purified adriamycin and daunorubicin were kindly provided by Laboratories Roger Bellon and Rhône Poulenc, respectively. Concentrations were determined by diluting stock solutions to approximately 10 μM and by using ϵ_{480} = 11 500 M^{-1} (Chaires et al., 1982a). As anthracycline solutions are sensitive to light and oxygen, stock solutions were prepared just before use. Standard Fe(II) and Fe(III) solutions were prepared from reagent-grade $FeSO_4(NH_4)_2SO_4 \cdot 6H_2O$ and $Fe(SO_4)_2NH_4 \cdot 12H_2O$, respectively. Calf thymus DNA, cytochrome *c* (type VI from horse heart), NADH (grade III), and cardiac NADH dehydrogenase were purchased from Sigma Chemical Co., superoxide dismutase (SOD) was from Miles, and apotransferrin was from Behring. All other reagents were of the highest quality available, and deionized bidistilled water was used throughout the experiments. Unless otherwise stated, buffer solutions were 0.05 M Hepes [*N*-(2-hydroxyethyl)-piperazine-*N'*-2-ethanesulfonic acid].

Absorption spectra were recorded on a Cary 219 spectrophotometer; circular dichroism (CD) spectra were recorded on a Jobin Yvon dichrograph 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] and [Dr], molar concentrations of adriamycin and daunorubicin, respectively. Raman spectra were measured on a Coderg D 800 spectrometer with the exciting lines of Ar^+ and Kr^+ Spectra Physics lasers; 90° scattering was used throughout. Frequencies are reported with ± 2-cm^{-1} accuracy. Potentiometric measurements were obtained with a Methrom pH meter, Model E 603, at 25 °C with a Methrom EA 147 combined glass electrode.

Tumor Systems. P 388 leukemia provided by the Mason Research Institute (Worcester, MA) is maintained and used for in vivo antitumor testing in accordance with the protocols described by the National Cancer Institute (Geran et al., 1972). Male B6D2FI (19–21 g) are used in experiments and are implanted ip with 10^6 P 388 cells on day 0 (five mice per group). Compounds are prepared in physiological saline and are injected ip on days 1–4 (20 mL/kg). The response is measured in median survival time (in days). Results are expressed by $T/C \times 100$ (T = median survival time of treated animals; C = median survival time of control animals). The criteria for significant activity is $T/C \times 100 > 120$.

In Vitro Inhibition of P 388 Leukemia Cell Growth. P 388 cells can be grown in vitro in RPMI 1640 medium supplemented with fetal calf serum (10%) and 10 μM 2-mercaptoethanol. For the growth studies, tubes are seeded with 4.5 mL of cells (approximately 5×10^4 cells/mL); compounds prepared in whole medium are added under a final volume of 0.5 mL (three tubes per concentration). Tubes are incubated at 37 °C for 4 days, and cell numbers are then determined with a Coulter counter. Drug effect is expressed by inhibitory dose (ID_{50}), which is obtained by plotting the logarithms of drug concentration against percent inhibition of cell growth and extrapolating the concentration required to inhibit 50% of cell growth.

NADH Dehydrogenase Assay. NADH dehydrogenase activity was determined at 25 °C by modification of a method described previously (Malher, 1955) with cytochrome *c* as the electron acceptor. Adr, Dr, $Fe(HDr)_3$, and $Fe(HAd)_3$ were assayed for their NADH–cytochrome *c* oxidoreductase activity by following cytochrome *c* reduction at 550 nm with the extinction coefficient for cytochrome *c* (reduced minus oxidized being 19 600. The reaction mixture contained Hepes buffer (0.05 M), pH 7.2, cytochrome *c* (80 μM), NADH (160 μM), NADH dehydrogenase (3.5 units/L), and either 0 or C μM free or complexed anthracycline (C was varied from 0 to 250). The reaction was initiated by the addition of the enzyme. Enzymatic activity has been expressed in units, where 1 unit of activity was that amount of enzyme capable of reducing 1 μM cytochrome *c*/min at pH 7.2 and 25 °C under the reaction conditions outlined above. The production of superoxide anion in the experimental samples was calculated from the rate of cytochrome *c* reduction inhibited by SOD (20 μg/mL).

Results

Iron–Adriamycin in Hepes Buffer: Spectroscopic Studies. The addition of Fe(II) to an aqueous adriamycin solution at pH 7.2 (Hepes buffer, 0.05 M) in the presence of oxygen yields a brown-violet complex. In order to determine the stoichiometry of the complex, increasing quantities of Fe(II) were added to an adriamycin solution, at molar ratios of Fe to Adr varying from 0:1 to 3:1. The experiments were monitored by absorption and CD spectroscopy. The addition of Fe(II) gives rise to a characteristics CD spectrum exhibiting ellipticities both positive at 510, 575, and 620 nm and negative at 400 and 460 nm with an isodichroic point at 480 nm. The visible absorption spectrum of this complex is characterized by a new absorption band at 600 nm and the decrease in the absorbance at 480 nm; an isosbestic point is at 535 nm. The values of $\Delta\epsilon$/[Adr] of the bands at 620 and 575 nm and ϵ/[Adr] at 600 nm increase as the molar ratio of Fe to Adr increases, reaching maximum values of 3.7, 3.2, and 3000, respectively, at [Fe]:[Adr] = 1.3.

The conclusion therefore follows that an iron complex is formed in which one metal atom is bound to three adriamycin molecules. It must be pointed out that formation of this complex is rather slow and that several hours are necessary for equilibration. The absorption and CD spectra of this species are shown in Figure 1. As can be noticed, this absorption spectrum is similar to that reported by Myers et al. (1982). The typical CD spectrum thus obtained will thereafter be labeled A.

The same experiments have been performed with Fe(III), and analogous results have been obtained although, due the rapid formation of ferric hydroxide at this pH value, the rate formation of the complex in this case is considerably slower (about 1 day).

Iron–Daunorubicin in Hepes Buffer: Spectroscopic Studies. The same kind of experiment was performed with daunorubicin, and similar results are obtained. As for the Fe–Adr system, one may conclude that an Fe(III) complex is formed in which one metal atom is bound to three daunorubicin molecules. The CD spectrum of the Fe–Dr (1:3) complex thus obtained is different from that of Fe–Adr (1:3) with a characteristic intense band at 660 nm ($\Delta\epsilon$ = 14). This typical CD spectrum will thereafter be labeled D. It is shown in Figure 1, as well as the corresponding absorption spectrum.

Fe(III)–Dr (1:3) and Fe(III)–Adr (1:3) Complexes: Spectroscopic and Potentiometric Titrations. In order to accurately characterize the above species, potentiometric and

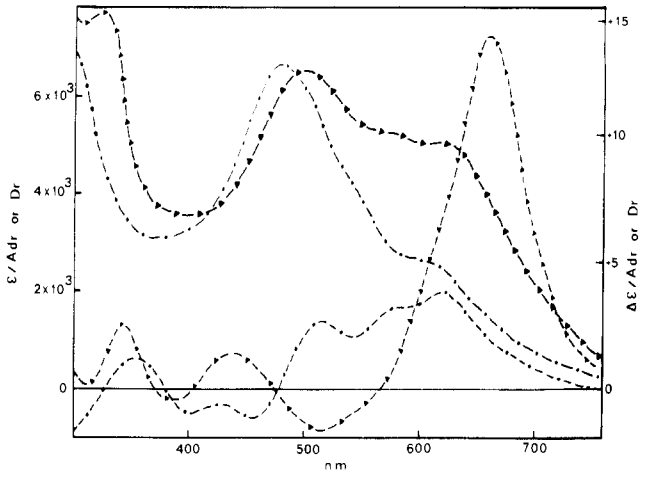

FIGURE 1: Absorption and CD spectra of $Fe(HDr)_3$ and $Fe(HAd)_3$ complexes in the D and A conformations respectively: for $Fe(HDr)_3$, absorption (▲—▲) and CD (▲--▲); for $Fe(HAd)_3$, absorption (●—●) and CD (●--●).

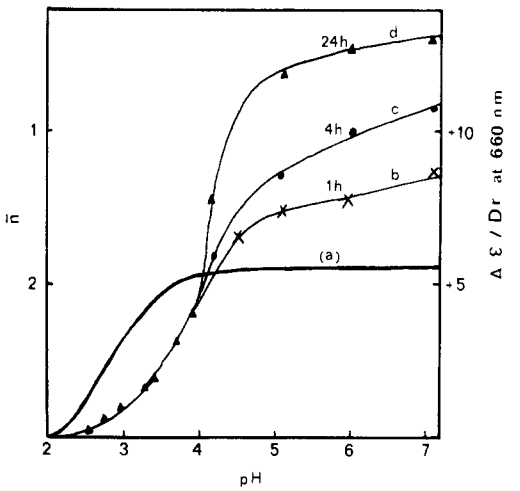

FIGURE 2: Potentiometric and spectroscopic titrations of the 1:3 Fe(III)–daunorubicin system ([Dr] = 10^{-3} M; [KCl] = 0.05 M). (Curve a) $\bar{n}$, the number of protons released per daunorubicin, as a function of pH. Three independent measurements have been performed. (Curves b–d) $\Delta\epsilon$ at 660 nm as a function of pH at Δt equal respectively to 1, 4, and 48 h.

spectroscopic titrations of the Fe(III)–Dr and Fe(III)–Adr complexes at molar ratios equal to 1:3 were performed in 10^{-3} M antibiotic solution in the presence of KCl, 0.05 M. The pH of the antibiotic solution was first adjusted to 2 by the addition of HCl, Fe(III) was subsequently added, and the solution was very slowly titrated with NaOH, 0.1 M. Titration from pH 2 to pH 8 took about 3 h.

Figure 2 shows the number $\bar{n}$ of protons released per daunorubicin. As can be seen, in the pH range 2–4, one proton per daunorubicin is released with a pK of 2.7. On the same figure, $\Delta\epsilon$ at 660 nm has been plotted as a function of pH. In fact, as has been previously mentioned, the spectra evolve as time elapses; they have thus been recorded at various periods of time ranging from 1 h to 4 days. The important point that emerges from these experiments is the following: at pH higher than 4.5, after 1 h the spectra can be accounted for a mixture of A and D types, and after 1 day the spectra ultimately reach the D type shown in Figure 1.

The studies of Fe(III)–Adr (1:3) complex have been performed in exactly the same conditions. The potentiometric titration indicates that in the pH range 2–4 one proton per adriamycin is released with a pK of 2.7 (Figure 3). The spectra have also been recorded as a function of pH at various

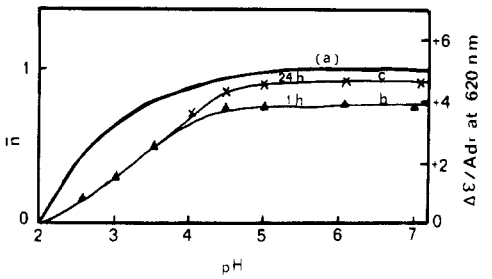

FIGURE 3: Potentiometric and spectroscopic titrations of the 1:3 Fe(III)–adriamycin system ([Adr] = 10^{-3} M; [KCl] = 0.05 M). (Curve a) $\bar{n}$, the number of proton released per adriamycin, as a function of pH. Three independent measurements have been performed. (Curves b and c) $\Delta\epsilon$ at 620 nm as a function of pH at Δt equal respectively to 1 and 24 h.

periods of time, and Figure 3 shows the plot of $\Delta\epsilon$ at 620 nm as a function of pH. At pH higher than 4.5, after 1 h the spectra can be accounted for by a mixture of A and D types, and after 1 day the spectra ultimately reach the A type shown in Figure 1.

From the foregoing results we can state that Fe(III)–Adr (1:3) and Fe(III)–Dr (1:3) complexes are formed at pH 4 with the release of one proton per anthracycline due to phenolate binding to iron (this is confirmed by resonance Raman study), and this is true also in the presence of an excess of iron. These complexes may then be formulated as $[Fe^{3+}(Ad\text{-}NH_3^+,\text{-}OH,O^-)_3]^{3+}$ and $[Fe^{3+}(Dr\text{-}NH_3^+,OH,O^-)_3]^{3+}$. For the sake of simplicity, we will refer to them as $Fe(HAd)_3$ and $Fe(HDr)_3$, where HAd and HDr stand for adriamycin and daunorubicin with the 1,4-dihydroxyanthraquinone moiety half-protonated, respectively.

Determination of $Fe(Adr)_3$ and $Fe(HDr)_3$ Stability Constants. Stability constants were obtained from the potentiometric titration results. The formation constants β_1 and β_1^* are defined by the following equilibria:

$$Fe^{3+} + 3HAd \rightleftarrows Fe(HAd)_3$$

with

$$\beta_1 = \frac{[Fe(HAd)_3]}{[Fe^{3+}][HAd]^3}$$

and

$$Fe^{3+} + 3H_2Ad \rightleftarrows Fe(HAd)_3 + 3H^+$$

with

$$\beta_1^* = \frac{[Fe(HAd)_3][H^+]^3}{[Fe^{3+}][H_2Ad]^3}$$

where H_2Ad stands for adriamycin with the 1,4-dihydroxyanthraquinone moiety fully protonated. Taking into account the first phenolic deprotonation $k_1 = [HAd][H^+]/[H_2Ad]$ with $k_1 = 1.1 \times 10^{-9}$ (Beraldo et al., 1983; Kiraly & Martin, 1982), it follows that $\beta_1 = \beta_1^*/k_1^3$. On the other hand, one can state

$$[Adr]_t = [H_2Ad] + [HAd] + [Ad] + 3[Fe(HAd)_3]$$

Owing to the low value of the deprotonation constants k_1 and k_2 of the two phenolic deprotonations, [HAd] and [Ad] are negligible in comparison with $[Fe(HAd)_3]$ and $[H_2Ad]$ below pH 7.4. Hence, $[H_2Ad]$ can be calculated from the above equation. The stability constant thus obtained is $\beta_1 = (2.5 \pm 0.5) \times 10^{28}$. The same scheme applies for the Fe^{3+}–Dr system, and we obtained the same value for the stability constant.

It must be pointed out that these compounds once lyophilized are very easily redissolved in water or buffer solution.

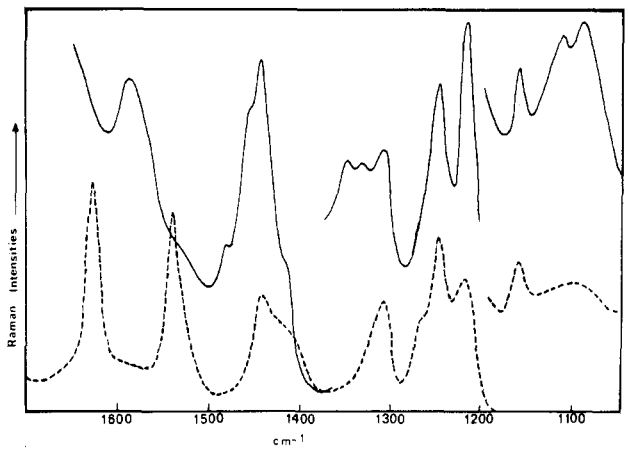

FIGURE 4: Resonance Raman spectra of $Fe(HAd)_3$ (--) and Adr (—) with the 647.1- and 457.9-nm laser line, respectively ([Adr] = 200 μM; $[Fe(HAd)_3]$ = 100 μM; Hepes buffer, pH 7.2). Experimental conditions were as follows: laser power 60 mW; slit width 6 cm^{-1}; scan rate 50 cm^{-1}/min.

The solutions thus obtained are very stable and can be kept several weeks without the occurrence of any precipitate.

Fe(III)–Adr (3:1) System. This system has also been studied in order to identify accurately the species present in the quelamycin derivative prepared by Gosalvez et al. (1978). The compound was prepared under exactly the same conditions indicated by these authors; i.e., three molecular equiv of ferric chloride was added to a pH 3 adriamycin solution, and the mixture was subsequently neutralized slowly to pH 7 by means of NaOH. The CD spectrum of the resultant solution thus obtained is strictly analogous to the A type obtained by us at pH higher than 4.0 (see above, Figure 1). As has been reported by Gosalvez et al. (1978), quelamycin solution is only stable for a few hours because it tends to form polymers. We think that this polymer formation may be accounted for by polymerization of ferric hydroxide, which is formed at this pH by the excess of Fe(III) present in the solution. Our results thus provide suggestive evidence concerning the species present in quelamycin that, in fact, appears to be a mixture of the $Fe(HAd)_3$ complex and ferric hydroxide.

Resonance Raman Spectra (RR). The RR spectrum of the $Fe(HAd)_3$ complex in the 950–1600-cm^{-1} region upon excitation with the 647.1-nm laser radiation is illustrated in Figure 4. The RR spectrum of adriamycin with the 457.9-nm radiation is also illustrated for the sake of comparison. This difference in the choice of the exciting radiation is due to fluorescence, which is strong in the 640–490-nm region in the case of adriamycin. The spectrum of the $Fe(HDr)_3$ complex being essentially analogous to that of the adriamycin complex is not represented. The spectra of two vibrational regions, i.e., 1500–1600- and 1200–1300-cm^{-1} region, show appreciable changes upon complexation. They correspond to the stretching vibrations $\nu_{C=O}$ of the quinone carbonyl and ν_{C-O} of the phenol, respectively. As can be noticed, the band at 1587 cm^{-1} in the spectrum of adriamycin, assigned mostly to the hydrogen-bonded $\nu_{C=O}$ mode of anthraquinone (Beraldo et al., 1983), shifts to 1542 cm^{-1}, confirming binding of the carbonyl oxygen to the metal. A shift of 45 cm^{-1} seems quite reasonable. The band at 1625 cm^{-1} not observed in the spectrum of adriamycin owing to strong fluorescence in this region is due mainly to a $\nu_{C=C}$ stretching vibration of anthraquinone (Smulevich et al., 1982).

The decrease in intensity of the peak at 1218 cm^{-1} and the concomitant appearance of the shoulder at 1263 cm^{-1} is also indicative of complexation through phenolate oxygen. Phenol deprotonation and coordination to metals is characterized by the shift of ν_{C-O} to higher frequencies. The phenolate CO stretch has been found around 1260–1288 cm^{-1} in metal transferrins (Tomimatsu et al., 1976) and at 1272 cm^{-1} in Cu(II)–tyrosinato complexes (Garnier-Suillerot et al., 1981). Similar spectral patterns have been observed by us in the case of Cu(II)–adriamycin complex (Beraldo et al., 1983).

$Fe(HDr)_3$ and $Fe(HAd)_3$. (A) Transferrin and Plasma Interaction. The first barrier encountered by the drug being plasma, it is an important point to determine the behavior of the complexes in face of the various components of plasma. As far as Fe(III) complexes are concerned, it is obvious that the plasma compound that is the most able to perturb the complexes is transferrin, which binds Fe(III) in two different sites; at pH 7.4 and atmospheric P_{CO_2}, the apparent stability constants are 4.7×10^{20} and 2.4×10^{19}, respectively (Aisen et al., 1978). In order to determine what will be the devenir of these complexes in the presence of transferrin, the following experiments were performed: $Fe(HDr)_3$ or $Fe(HAd)_3$ (10^{-4} M) was added to apotransferrin (3×10^{-4} M) in Hepes in the presence of carbonate as sinergistic anion at a concentration of an air-equilibrated solution, 3×10^{-4} M (Aisen et al., 1978). Despite the facts that in our experiments the transferrin concentration was much higher than in plasma (3.3×10^{-5} M), we did not observe any modification of the CD spectra of the complexes nor did we see any appearance of the charge-transfer band at 460 nm characteristic of the complexation of Fe(III) to the protein, over a period of several days. Moreover, to check whether any other components of plasma could interact with these complexes, they were directly added to plasma, and their CD spectra were recorded after several hours of incubation; no modification has thus been detected. The interesting point that emerges from this study is that, since Fe(III) is not removed from the complexes by plasma components, the possibility of this being transported in undissociated form in plasma must be taken into consideration.

(B) DNA Interactions. From the studies on the mechanism of action of anthracyclines in vivo, it appears reasonable to assume that the site of action of these complexes is probably nuclear DNA; therefore, an examination of the interaction of these complexes with DNA was undertaken. The following experiments were carried out under conditions for total binding of the free drug to DNA, i.e., at a nucleotide to drug ratio higher than 7 (Chaires et al., 1982b). The results obtained with both anthracycline complexes are identical. Only the experiment for the $Fe(HDr)_3$–DNA system is reported here. in a typical experiment, DNA and $Fe(HDr)_3$ concentrations were respectively 8×10^{-4} and 2.2×10^{-5} M. The interaction between the complex and DNA is time dependent and is characterized by the decrease of the CD band at 660 nm. The reaction versus time can be followed conveniently by monitoring this band. Figure 5 shows the evolution of the CD spectra, and in the insert the $\Delta\epsilon_{660nm}$ has been plotted as a function oif time. The CD spectrum obtained after several days exhibits two bands in the visible region at 490 and 380 nm, characteristic of daunorubicin intercalated between DNA base pairs (Gabbay et al., 1976). From these results we may state that the free drug is intercalated between the base pairs after the release of Fe(III) from $Fe(HDr)_3$. This process is most probably facilitated by the presence of DNA phosphate groups, which will bind to ferric ion.

At $Fe(HDr)_3$ of 2.2×10^{-5} M and nucleotide of 8×10^{-4} M, the half-lifetime of the reaction is 18 h. The same experiment performed with a larger excess of DNA, i.e., nu-

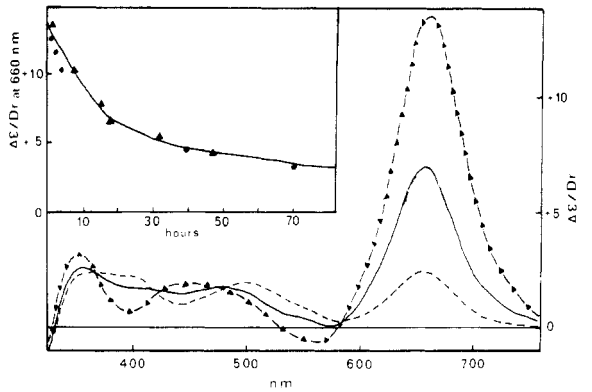

FIGURE 5: CD spectra of the $Fe(HDr)_3$–DNA system as a function of time. Experimental conditions were as follows: $[Fe(HDr)_3] = 22$ μM; [nucleotide] = 0.8×10^{-3} M; 0.05 M Hepes buffer, pH 7.2. Time elapsed after addition of the complex to DNA was 5 min (–▲–), 2 h (—), and 4 days (– –). (Insert) $\Delta\epsilon/[Dr]$ at 660 nm has been plotted as a function of time. $[Fe(HDr)_3] = 22$ μM; [nucleotide] = 0.8×10^{-3} M (●) and 3.6×10^{-3} M (▲).

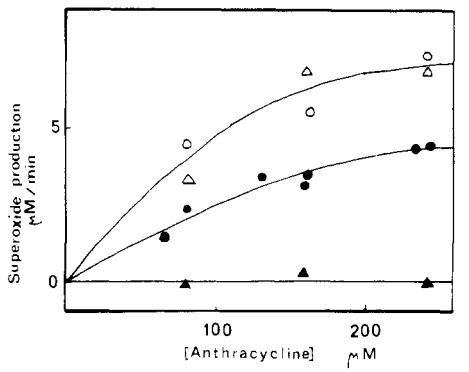

FIGURE 6: Effect of drug concentration on superoxide formation by NADH dehydrogenase. Superoxide formation was determined spectrophotometrically by the rate of SOD-inhibitable cytochrome *c* reduction as described under Materials and Methods. The reaction mixture contained Hepes buffer (0.05 M), pH 7.2, cytochrome *c* (80 μM), NADH dehydrogenase (3.5 units/L), 0 or 20 μg/mL SOD, and the indicated amount of (△) adriamycin, (○) daunorubicin, (▲) $Fe(HAd)_3$, on (●) $Fe(HDr)_3$.

Table I: Antitumor Activity of $Fe(HDr)_3$ and $Fe(HAd)_3$ Complexes[a]

compd	ID_{50} (pM)[b]	dose per injection (mg/kg)[c]	body wt change from day 1 to day 7 (g)[c]	$T/C \times 100$[c]
daunorubicin	6.2	5	−0.4	143
		2.5	0.9	140
$Fe(HDr)_3$	18	7.5	−0.4	144
		3.75	0.7	141
adriamycin	23	5	−0.1	161
		2.5	1.3	145
$Fe(HAd)_3$	54	7.5	0.8	150
		3.75	0.2	143

[a] The experimental conditions are described under Materials and Methods. [b] In vitro inhibition of P 388 leukemia cell growth by daunorubicin, adriamycin, $Fe(HDr)_3$, and $Fe(HAd)_3$. [c] Antitumor activity against P 388 leukemia of daunorubicin, adriamycin, $Fe(HDr)_3$, and $Fe(HAd)_3$.

cleotide of 3.6×10^{-3} M, gives similar results.

Antitumor Activity. The in vitro inhibition of P 388 leukemia cell growth by $Fe(HAd)_3$ and $Fe(HDr)_3$ was compared with that induced by the free drugs. The data summarized in Table I show that both complexes inhibit cell growth at a concentration slightly higher than that of the parent compounds and display antitumor activity against P 388 leukemia. No significant differences from the free drugs in terms of therapeutic efficacy and of general toxicity were observed.

Effect of $Fe(HAd)_3$ and $Fe(HDr)_3$ Complexes on Superoxide Production by NADH Dehydrogenase. To examine the role of oxygen radical metabolism in anthracycline-induced mitochondrial injury, we investigated the effect of the anthracycline iron complexes on superoxide anion formation by mitochondrial NADH dehydrogenase. This effect was compared with that of the free drugs. As it can be seen in Figure 6, Adr and Dr increased superoxide formation by NADH dehydrogenase in a dose-dependent fashion that appeared to follow saturation kinetics. These results compared with those of Doroshow (1983). $Fe(HDr)_3$ complex also increased the superoxide formation but to a lesser extent than the free drug (about 65%). On the contrary, $Fe(HAd)_3$ does not increase superoxide formation over control levels. These experiments strongly suggest that, although the flow of electrons from NADH to molecular oxygen through NADH dehydrogenase is enhanced significantly by the free drugs, it occurs to a much lesser extent by $Fe(HDr)_3$ and not at all by $Fe(HAd)_3$.

Discussion

There has been a continuing interest for the improvement of the chemotherapeutic index of adriamycin and daunorubicin via analogue synthesis. Our approach for this goal has been mainly to form complexes and is based on the finding of Gosalvez et al. (1978), which suggests that the triferric adriamycin derivative quelamycin might reduce cardiotoxicity of adriamycin in patients.

Our data appear to establish that adriamycin and daunorubicin form two well-defined complexes, namely, $Fe(HAd)_3$ and $Fe(HDr)_3$; this stoichiometry is in good agreement with that reported by Myers et al. (1982) for the Fe(III)–Adr complex. Potentiometric titrations indicate that these complexes are formed by the release of one proton per molecule of anthracycline; this together with the resonance Raman measurements suggests that one carbonyl and one phenolate oxygen are bound to one Fe(III), possibly forming a six-membered chelate. If the assumptions made on the molecular formulas of the complexes are correct, then it is highly likely that the structures of both complexes are octahedral. At this moment, this assignment is only tentative.

On the other hand, our CD data substantiate the occurrence of two different conformations, which can be adopted by each one of these complexes. These conformations labeled A and D are respectively characterized by the A and D CD spectra. The preferred conformation adopted by the $Fe(HAd)_3$ complex at pH 7 is A. However, this same complex is able to adopt the conformation D in special conditions. Thus, if Fe(II) is rapidly added in excess to a concentrated (higher than 10^{-3} M) solution of adriamycin in Hepes buffer, the D conformation can be obtained. This form is very stable and can be kept unchanged for a long period of time. Accordingly, we may state that, depending on experimental conditions, the $Fe(HAd)_3$ complex is able to adopt both conformations. Similar conclusions cannot be drawn for the $Fe(HDr)_3$ complex; i.e., so far we have not been able to find the right experimental condition in which the $Fe(HDr)_3$ species of the A form can be formed and stabilized at pH 7.

Another important result to be stressed concerns the stability constant of these compounds, i.e., 2.5×10^{28}, one of the highest shown by Fe(III) complexes. It compares with the value for the overall formation constant of the tris complex of Fe(III) with acetohydroxamic acid, 2×10^{28} (Raymond, 1978). Due to this high stability constant, Fe(III) is not dissociated from these complexes by plasma components, e.g., transferrin,

suggesting that they are able to reach their target unmodified. Notwithstanding this high stability constant, anthracyclines are displaced from these complexes by DNA. This is quite an interesting result as far as DNA is assumed to be one of the targets of these drugs.

As we have demonstrated, the compound "quelamycin" is a mixture of the $Fe(HAd)_3$ complex and of polymeric ferric hydroxide, which obviously represents the major problem in its chemical use. Since our antitumor activity data indicate that the active species is the complex, one might expect that treatment with the hydroxide-free complex will improve its efficiency. Moreover, the observations that (i) $Fe(HAd)_3$, unlike Adr, does not participate to any significant extent in NADH dehydrogenase mediated free-radical redox cycles and (ii) $Fe(HAd)_3$ retains antitumor activity against P 388 leukemia suggest that $Fe(HAd)_3$ could be an effective noncardiotoxic alternative to other currently available anthracycline anticancer agents.

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