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Thiol-Dependent DNA Damage Produced by Anthracycline-Iron Complexes

The Structure-Activity Relationships and Molecular Mechanisms

JOSEPHIA MUINDI, BIRANDRA K. SINHA, LUCA GIANNI, AND CHARLES MYERS

Biochemical Pharmacology Section, Clinical Pharmacology Branch, Clinical Oncology Program, Division of Cancer Treatment, National Cancer Institute, Bethesda, Maryland 20205

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SUMMARY

Doxorubicin (Adriamycin) and daunomycin analogs have been examined for their ability to chelate iron and catalyze the oxidative cleavage of DNA. The results show that the C-11-hydroxyl group is essential for iron binding and DNA damage. Thus, the iron complexes of doxorubicin, daunomycin, carminomycin, and 4-demethoxydaunomycin are potent redox catalysts capable of reducing molecular oxygen in the presence of physiologic concentrations of glutathione. They are also effective catalysts of hydroxyl radical formation from hydrogen peroxide. With the exception of daunomycin, generation of hydroxyl radical from hydrogen peroxide is stimulated by greater than 200% by DNA addition. Analogs that lack the C-11-hydroxyl group are relatively inefficient at oxygen reduction, hydroxyl radical formation, and DNA cleavage. The potencies of the anthracycline analogs tested in the H_2O_2-dependent DNA cleavage reaction correlated well with their relative cardiac toxicities.

INTRODUCTION

A variety of flavin-dependent reductases such as cytochrome P-450 reductase and xanthine oxidase have been shown to catalyze the reduction of the anthraquinone anticancer drug doxorubicin (Adriamycin) to a semiquinone free radical (1). This drug radical, in turn, rapidly reacts with molecular oxygen to form superoxide, hydrogen peroxide and hydroxyl radicals (2). Doxorubicin-mediated production of these reactive oxygen species has been shown to initiate lipid peroxidation in a variety of experimental systems (3, 4). Recently, it has been shown that doxorubicin-mediated oxygen radical production only results in lipid peroxidation in the presence of iron (5–8). Thus, the specific ferric ion chelator desferrioxamine will block lipid peroxidation without altering the rate of oxygen consumption (6, 7). The role of iron in such systems is not clear, but has been attributed to the role of iron in catalyzing the Haber-Weiss reaction.

However, doxorubicin itself chelates Fe(III) with an overall association constant of $10^{33.4}$ (9). Because of this affinity, doxorubicin can abstract iron from ferritin, the major intracellular form of iron (10). In addition, ICRF-159 and 187, which can be hydrolyzed to yield iron-chelating EDTA derivatives, have been shown to block effectively the cardiac toxicity of doxorubicin and to increase dramatically urinary iron excretion (11–13). In the presence of thiols, the doxorubicin-iron complex has been shown to catalyze oxygen consumption and oxidative destruction of erythrocyte ghost membranes (14). Proof of catalysis in this case included evidence that 1) the reaction required the presence of both iron and drug for maximal rates, 2) the drug was not irreversibly altered in the process, and 3) the reaction gave linear Lineweaver-Burk plots for oxygen and glutathione as substrates from which K_m and V_{max} values were estimated. This complex also binds to DNA to form a stable ternary complex which cleaves DNA in the presence of thiols or hydrogen peroxide (15). Hydroxyl radicals recently have been shown to be the reactive oxygen species involved in the oxidative destruction of DNA by the doxorubicin-iron complex (16). Thus, it is not clear at present whether the influence of iron on doxorubicin-mediated oxygen radical reactions involves a drug-metal chelate or adventitial iron (17).

In order to help clarify the role of iron in anthracycline radical chemistry, we have investigated the structural requirements for iron to bind to the chromophore of these drugs and the ability of the resulting drug-metal chelates to catalyze hydroxyl radical-mediated DNA cleavage. The results show that the presence of the C-11-hydroxyl group is required for spectral evidence of iron binding, thiol-dependent oxygen consumption, hydroxyl radical formation, and DNA destruction. These findings are consistent with predictions made based upon the X-ray crystal structure of daunomycin and carminomycin (18–20) (see Fig. 1). In addition, changes at C-

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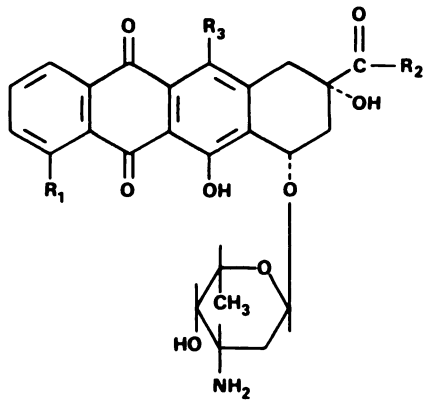

	R_1	R_2	R_3
Adriamycin	OCH_3	CH_2OH	OH
Daunomycin	OCH_3	CH_3	OH
Carminomycin	OH	CH_3	OH
4-demethoxydaunomycin	H	CH_3	OH
4-demethyl-11-deoxyadriamycin	OH	CH_2OH	H
11-deoxyadriamycin	OCH_3	CH_2OH	H
11-deoxydaunomycin	OCH_3	CH_3	H
11-o-methydaunomycin	OCH_3	CH_3	OCH_3

FIG. 1. *Structures of the anthracyclines studied*

4 on the drug chromophore have a major impact on potency. Furthermore, the structure-activity relationships observed in this study correlate well with the reported biologic activity of these analogs.

MATERIALS AND METHODS

Doxorubicin HCl, daunomycin HCl, and carminomycin HCl were supplied by the Drug Development Branch, Division of Cancer Treatment, National Cancer Institute, Bethesda, MD. 4-Demethoxydaunomycin, 11-deoxydaunomycin, 11-*O*-methyldaunomycin, 11-deoxydoxorubicin, and 4-demethyl-11-deoxydoxorubicin were a gift from Dr. F. Arcamone, Farmitalia C. Erba, Milano, Italy. $FeCl \cdot 6H_2O$, 99.5% pure (Allied Chemical Co.) and $Fe(NH_4)_2(SO_4)_2 \cdot 6H_2O$, 99.999% pure (Aldrich Chemical Co.) were used as sources of Fe^{3+}. GSH[1] was obtained from Sigma Chemical Co. Glutathione solutions were prepared at pH 6 using 1 M NaOH, passed over Chelex-100 resin (Bio-Rad Co.) to remove any contaminating transition metal ions prior to estimating the concentration of reduced thiols by the 5,5-dithiobis-(2-nitrobenzoic acid) reaction (21). H_2O_2 (30%) was obtained from Fisher Chemical Co. Diethylenetriaminepentaacetic acid and the spin trapping agent DMPO were from Aldrich Chemical Co. DMPO was purified by two vacuum distillations before use. Desferrioxamine mesylate (Desferal) was purchased from Ciba Pharmaceutical Co. SDS was bought from BDH Chemicals Ltd. Poole, U. K. SV-40 [^{3}H]DNA (>95% form 1) was supplied by Bethesda Research Laboratories and used without further purification. Highly polymerized calf thymus DNA (Sigma Chemical Co.) for ESR studies was freed of metal ions by dialysis for 24 hr against 0.1% diethylenetriaminepentaacetic acid followed by a 48-hr dialysis against metal ion-free buffer at 4° (16). All other reaction solutions and buffers were freed of metal ions by passage over Chelex-100 resin. Reagent-grade chemicals were used whenever available.

Optical studies. Absorption spectra were obtained by a Hewlett-Packard 8450A diode array spectrophotometer equipped with a Hewlett-Packard 7470A plotter and 89100A temperature controller. All spectrophotometric titrations were performed at 24°. Aliquots of 2.5 mM freshly prepared aqueous solution of $Fe(NH_4)_2(SO_4)_2$ were progressively added to 1 ml of an aerated solution containing 30 μM of an anthracycline antibiotic in 20 mM NaCl, 10 mM Tris HCl buffer, pH 7.5, and spectrum recorded 3 min later in 1-cm path length cells. In aerated solution, Fe^{3+} is rapidly oxidized to Fe^{3+}. The addition of microliter quantities of unbuffered iron solution did not change the pH of the antibiotic solution.

Oxygen consumption studies. Oxygen consumption studies were performed using a Clark-type electrode (Yellow Springs Instruments; No. 5331 on a Gilson model 5/6 oxygraph as previously described) (14). The complexes were prepared by mixing equal volumes of 5 mM aqueous solutions of anthracycline antibiotic and $FeCl_3$ at pH 2. The reaction mixture consisted of 0–20 mM GSH and 30 μM of 1:1 anthracycline-iron complex in 20 mM NaCl, 10 mM Tris HCl buffer, pH 7.5. The reaction was initiated by the addition of the complex and the rate of oxygen consumption was followed at room temperature in a final volume of 2 ml. For the purpose of calculations, the reaction mixture was assumed to contain the same amount of dissolved oxygen as plain water (251 μM at 25°).

DNA cleavage studies. The DNA cleavage reaction consisted of either 2.5 mM GSH or 0–1 mM H_2O_2, 0.25 μg (5000 cpm) of SV-40 [^{3}H]DNA and 0–1 mM of 1:1 anthracycline-Fe^{3+} complex in a final volume of 100 μl of 20 mM NaCl, 10 mM Tris HCl buffer, pH 7.5. The reaction was initiated by adding the complex and following a 10-min incubation at room temperature, it was terminated by adding excess Desferal, 1% SDS, and adjusting the pH to 5 with HCl. Desferal and SDS stop the reaction by dissociating the DNA-drug-iron ternary complex. At pH 5.0, the dissociation of the ternary complex is completed in less than 1 min.

Gel filtration with prepacked Sephadex G-25 columns (PD-10 columns from Pharmacia Fine Chemicals) was used to separate DNA from the rest of the assay reactants. The DNA recovery from these columns, using SSC buffer, pH 7.1, was routinely greater than 95%. This step is essential because the presence of anthracyclines interferes with the estimation of nicked DNA by nitrocellulose filters.

The percentage of nicked DNA was quantitated after a denaturation-renaturation cycle using Schleicher and Schuell BA 85 nitrocellulose filters (17). These filters allow unnicked double-stranded DNA (form 1) to pass through while retaining nicked (linear and circular) single-stranded DNA. Alkaline denaturation was achieved with 0.9 M NaCl, 0.1 M K_2HPO_4, and 0.025 M EDTA buffer adjusted to pH 12.15 with NaOH while HCl (2 M) was used for renaturation. Filters were dried at 60° and then allowed to dissolve in Hydrofluor (National Diagnostics) scintillation liquid prior to counting. Filter counts (nicked DNA) were expressed as a percentage of total counts in the assay after subtracting the background counts.

Hydroxyl radical formation studies. Hydroxyl radical formation was assayed by ESR spectrometry using DMPO as a spin-trapping agent (23). A Varian E-104 spectrometer operating at X-band (9.5 GHz) and employing 100-kHz modulation was used for ESR measurements. The assay mixture in 20 mM NaCl, 10 mM Tris HCl buffer, pH 7.5, contained 10 μM 1:1 anthracycline-Fe^{3+} complex, 100 μg/ml calf thymus DNA, and 100 mM DMPO. The reaction was started by adding 90 μM H_2O_2 and followed for 15 min at room temperature (24°).

RESULTS

Spectral evidence for iron binding. The spectral evidence for doxorubicin-Fe^{3+} complex formation is shown in Fig. 2 and has previously been reported (14). The characteristic changes include a hypochromic shift of the peak at 480 nm and the appearance of a new band at

[1] The abbreviations used are: GSH, reduced glutathione; SSC, standard saline citrate; DMPO, 5,5-dimethyl-1-pyroline oxide; SDS, sodium dodecyl sulfate.

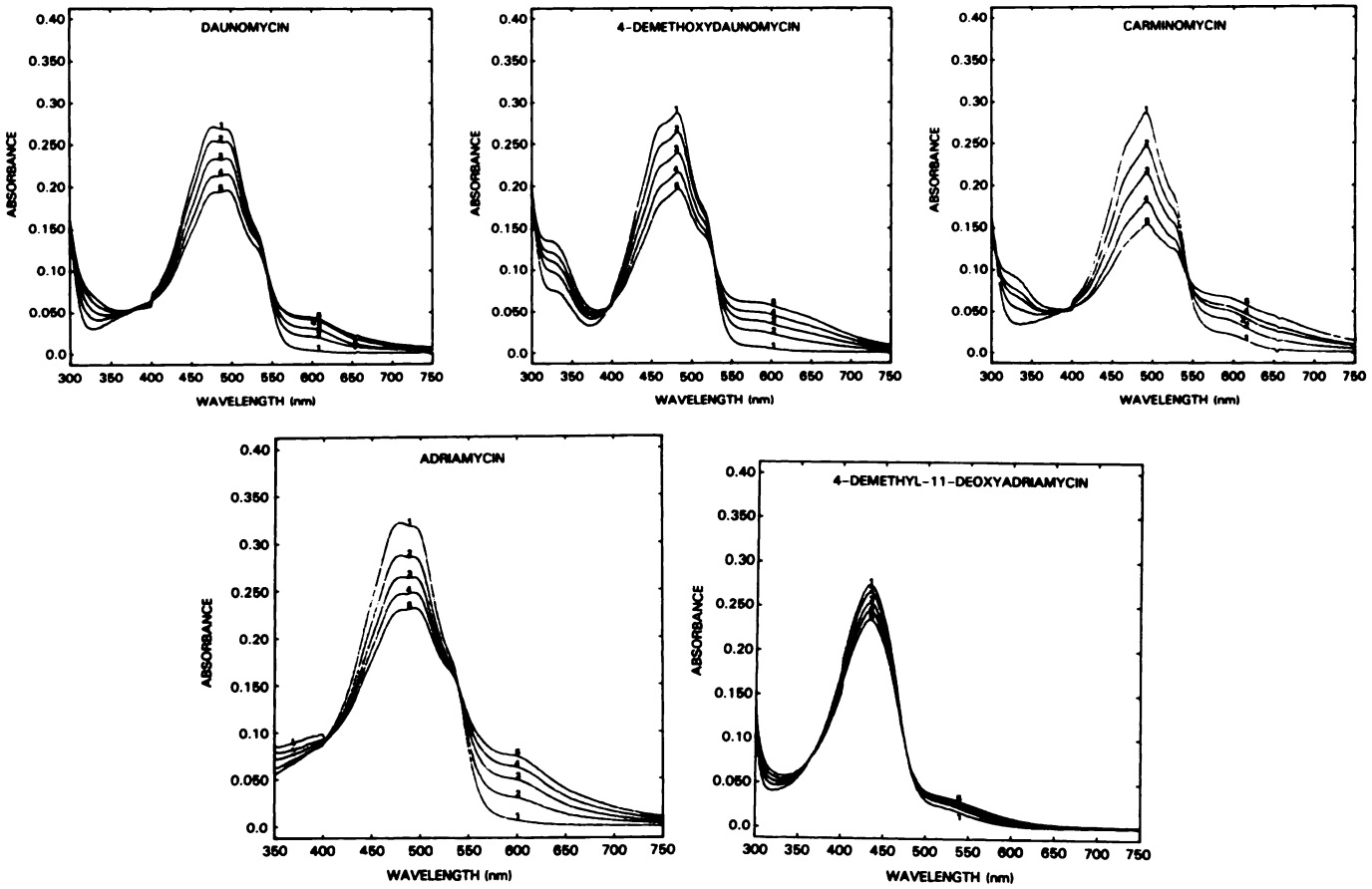

FIG. 2. *Titration of anthracycline with* $Fe(NH_4)_2(SO_4)_2$

Final concentration of the anthracycline antibiotic in the assay was 30 μM. Curve *1* was anthracycline alone. Curves *2–5* represent 12:1, 6:1, 4:1, and 3:1 anthracycline/metal ion ratios in that order.

lower energy. The iron-binding site, however, remains uncertain. In order to clarify this issue, we have examined the ability of various anthracyclines to chelate iron. The structural modifications on the anthracycline chromophore investigated are shown in Fig. 1. The addition of small aliquots of Fe^{2+} to 30 μM aerated daunomycin solution in 20 mM NaCl, 10 mM Tris HCl buffer, pH 7.5 produced spectral changes parallel to those seen with doxorubicin (Fig. 2). Similarly, the substitution of the C-4-methoxy group of daunomycin with either a hydroxyl group (carminomycin) or hydrogen (4-demethoxydaunomycin) did not impair iron chelation (Fig. 2). With all of these anthracycline antibiotics, the new absorption band is located between 550 and 620 nm. The intensity of this absorption band has allowed us to determine the stoichiometry of iron binding as we had done earlier for doxorubicin (14). We were able to reconfirm the drug:iron ratio of 3:1 for doxorubicin. In the case of daunomycin, carminomycin, and 4-demethoxydaunomycin, a ratio of 2:1 was obtained, suggesting that the differences in the side chain between doxorubicin and these daunomycin derivatives may cause steric problems with the binding of the third drug molecule.

The titration of solutions of 11-deoxydaunomycin, 11-*O*-methyldaunomycin, and 11-deoxydoxorubicin with iron did not produce a significant change in their absorption spectra. Thus, methylation or removal of the C-11-hydroxyl group of daunomycin and doxorubicin resulted in complete loss of iron binding to the drug chromophore. However, combined removal of the C-11-hydroxyl group in conjunction with demethylation of the C-4-methoxy group resulted in an antibiotic (4-demethyl-11-deoxydoxorubicin) which did exhibit a minor spectral shift upon iron addition.

Catalysis of thiol-dependent oxygen consumption. We have previously reported that the doxorubicin-Fe^{3+} complex catalyzes thiol-dependent oxygen consumption and that reactive oxygen species generated are responsible for the oxidative destruction of both cell membranes and DNA (14). For this reason, we have examined the ability of the various anthracycline-Fe^{3+} complexes to catalyze this thiol-dependent oxygen consumption. This activity was observed with Fe^{3+} complexes of doxorubicin, daunomycin, carminomycin, and 4-demethoxydaunomycin. Stimulation of oxygen consumption by daunomycin and carminomycin-Fe^{3+} complexes using physiological GSH concentrations (0–20 mM) is shown in Fig. 3. Using 10 mM GSH, the complexes of daunomycin and doxorubicin were four times more potent than Fe^{3+} alone (Table 1). Thus, hydroxylation of the methyl side chain had no influence on oxygen consumption in the presence of GSH. The presence of the C-4-hydroxyl group instead of

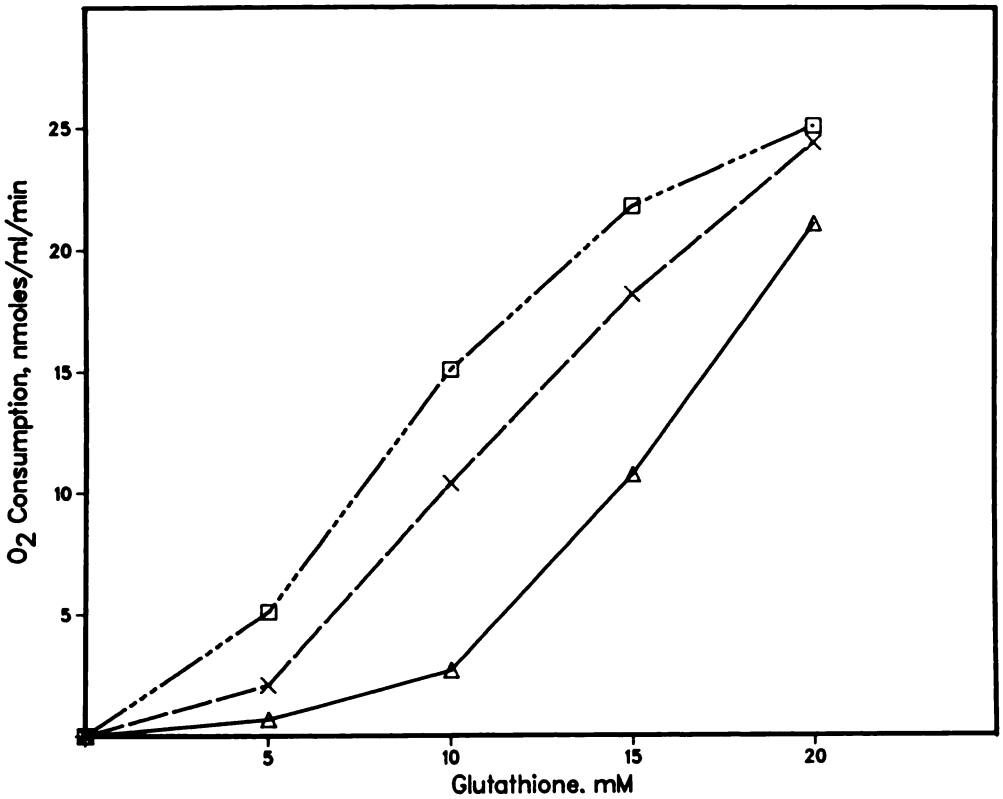

FIG. 3. *Catalysis of thiol-dependent oxygen consumption by 30 μM carminomycin-Fe^{3+} complex (□-···-□) daunomycin-Fe^{3+} complex (×---×), and Fe^{3+} (△——△)*

The anthracyclines in the absence of iron did not catalyze oxygen consumption.

TABLE 1

Catalysis of oxygen consumption by the anthracycline-iron complexes

Oxygen consumption was assayed at 4° using 30 μM 1:1 anthracycline-ferric ion complex and 10 mM GSH in 20 mM NaCl, 10 mM Tris HCl buffer, pH 7.5. The complex was prepared from $FeCl_3$ at Ph 2. Oxygen consumption is expressed as a mean (n = 3) ± the standard deviation. All of the anthracyclines tested exhibited no activity in the absence of iron.

Drug-Fe^{3+} complexes	Oxygen consumption
	nmol/ml/min
Iron only	2.3 ± 1.3
11-*O*-Methyldaunomycin	2.4 ± 1.5
11-Deoxydaunomycin	2.3 ± 1.9
11-Deoxydoxorubicin	3.1 ± 1.9
4-Demethyl-11-deoxydoxorubicin	4.0 ± 2.0
Daunomycin	9.4 ± 2.7
Doxorubicin	9.6 ± 2.9
4-Demethoxydaunomycin	12.8 ± 1.2
Carminomycin	14.8 ± 3.0

the methoxy group (carminomycin) or the removal of C-4-methoxy group (4-demethoxydaunomycin), however, resulted in the formation of Fe^{3+} complexes that were superior in this catalysis (Table 1).

The removal of the hydroxyl group at C-11 or its methylation produced antibiotics (11-deoxydoxorubicin, 11-deoxydaunomycin, and 11-*O*-methyldaunomycin) which were no better than iron alone in oxygen consumption (Table 1). These three antibiotics also gave no spectral evidence of iron binding. Although 4-demethyl-11-deoxydoxorubicin exhibited a minor spectral shift upon iron addition, the resulting mixture had marginal activity as a catalyst of the thiol-dependent oxygen consumption. These results suggest that the presence of C-11-hydroxyl is a prerequisite for activity. Additional changes at C-4 will significantly increase activity only if the C-11-hydroxyl is already present. Fe^{3+} is unstable in aqueous solution at physiologic pH and polymerizes to form insoluble aggregates. The activity reported here for Fe^{3+} is for freshly prepared Fe^{3+}. Over the course of 2–3 hr, Fe^{3+} loses most of its catalytic activity. The iron complexes of the active anthracyclines are much more stable, and so with time the differences between Fe^{3+} and the drug-metal chelates widen beyond that shown here.

Fig. 4 shows the relationship between the drug to iron ratio and the rate of oxygen consumption for those anthracyclines active in catalyzing this reaction. In each case, the maximal rate of oxygen consumption is seen at drug:iron ratios of 2–3:1. These results are consistent with the spectrally determined stoichiometry of iron binding presented earlier in this paper.

Thiol-dependent DNA cleavage. We have previously shown that the doxorubicin-Fe^{3+} chelate forms a ternary complex with DNA which can react with thiols to cleave DNA (15). In the present study, we have correlated the concentration of 1:1 anthracycline-iron complexes with the percentage of viral DNA nicked in the presence of 2.5 mM GSH. The results in general followed the pattern seen above for the thiol-dependent oxygen consumption

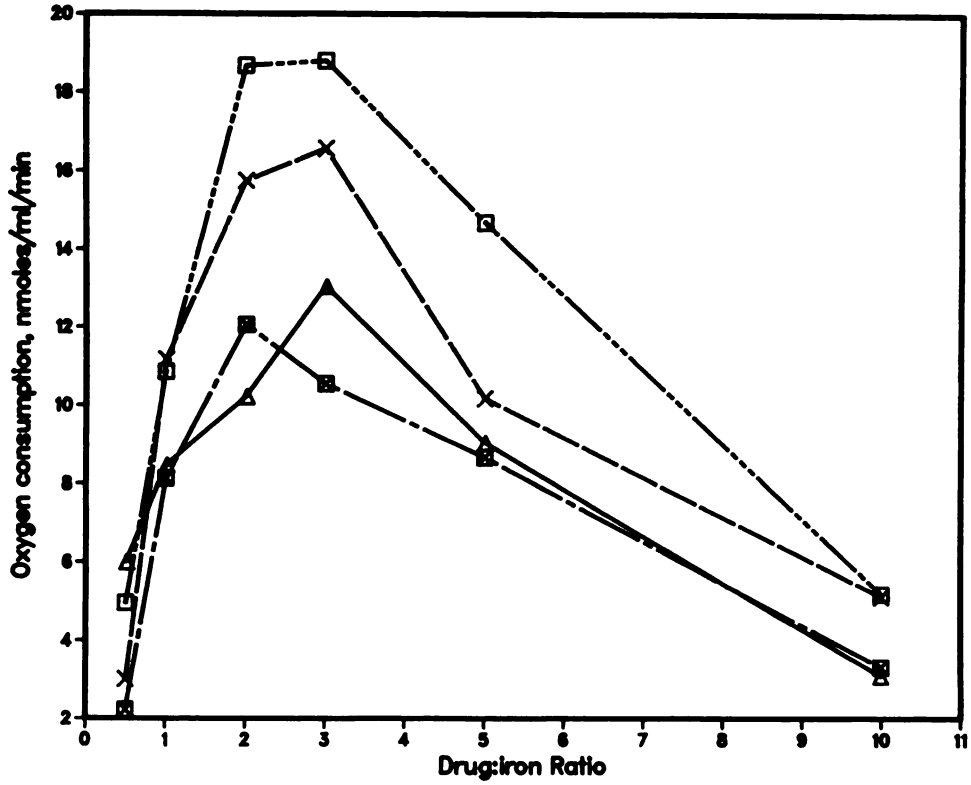

FIG. 4. *Thiol-dependent oxygen consumption as a function of anthracycline:iron ratio*

The concentrations of Fe^{3+} and reduced glutathione were kept constant at 30 μM and 10 mM, respectively. Experiments were performed at 24° as described in Materials and Methods. Carminomycin (□–·–□), doxorubicin (⊠–···–⊠), daunomycin (△——△), and 4-demethoxydaunomycin (×– – –×).

reaction but the difference between the analogs is of much greater magnitude. The concentration-response curves obtained in these assays with iron complexes of carminomycin, daunomycin, and 11-*O*-methyldaunomycin and iron alone are shown in Fig. 5, and the IC_{50} values for the rest of the analogs are given in Table 2. Doxorubicin and daunomycin gave identical values, indicating that hydroxylation of the daunomycin methyl side chain has no effect on this reaction. Removal of the C-11-hydroxyl group from doxorubicin (11-deoxydoxorubicin) and daunomycin (11-deoxydaunomycin) resulted in a 20-fold decrease in DNA cleavage potency (Table 2). Methylation of the C-11-hydroxyl group of daunomycin (11-*O*-methyldaunomycin) also caused a 20-fold drop in activity. The lack of potency for the 11-deoxy and 11-*O*-methyl analogs is consistent with their inability to chelate iron and the inactivity of these compounds as catalysts of thiol-dependent oxygen consumption.

Here again, the C-4-position was of interest. While the 4-demethyl-11-deoxydoxorubicin-Fe^{3+} complex was no more active than iron alone in the thiol catalysis of oxygen consumption, it was significantly more active than iron alone in the thiol-dependent DNA cleavage assay. Similarly, carminomycin-Fe^{3+} complex was superior to the daunomycin-Fe^{3+} chelate at cleaving DNA (Table 2). These results point to the importance of the C-4 position in the thiol-dependent DNA cleavage reaction.

Hydrogen peroxide-dependent DNA cleavage. We have previously demonstrated that the thiol-dependent DNA cleavage reaction is a complex process involving first the reduction of oxygen to hydrogen peroxide. The second step was a reaction between the DNA-bound drug-metal ternary complex and hydrogen peroxide which led to DNA cleavage (15). Because of this, it is possible that an anthracycline-iron complex might possess the capacity to catalyze the thiol-dependent reduction of oxygen but not be able to form a ternary complex with DNA or utilize hydrogen peroxide to cleave DNA. With that in mind, we next examined the ability of the various anthracycline-Fe^{3+} complexes to catalyze the hydrogen peroxide-dependent DNA cleavage reaction. The titration curves for a series of representative anthracyclines covering the spectrum of activities observed are shown in Fig. 6. At the highest hydrogen peroxide concentration used (1 mM), the 4-demethyl-11-deoxydoxorubicin-Fe^{3+} complex cleaved only 30% of the DNA while the analogs which did not bind iron (the other 11-deoxy and 11-*O*-methyl analogs) were even less active, cleaving less than 10%. Table 3 summarizes the results for those analogs that exhibited significant activity in this reaction. Hydroxylation of the daunomycin methyl side chain (doxorubicin) resulted in a 20-fold decrease in the hydrogen peroxide requirement, while replacement of the C-4-methoxy with the hydroxyl group reduced it by two-thirds. Doxorubicin-Fe^{3+} complex without an exogenous source of hydrogen peroxide cleaved 30% of the DNA.

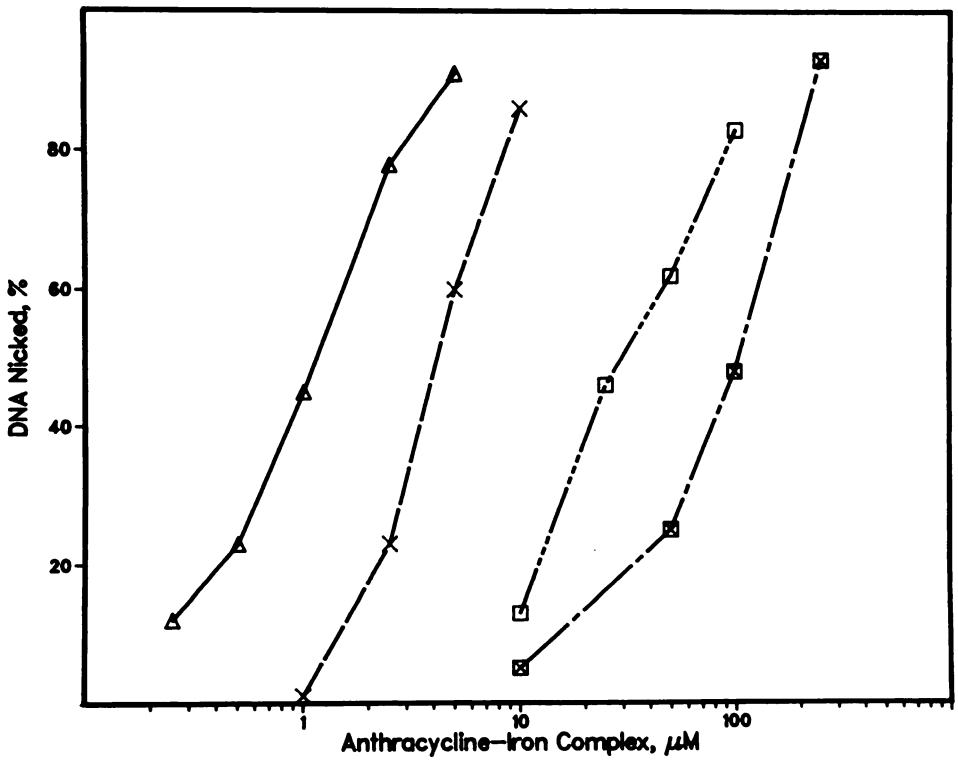

FIG. 5. *Dose-response curves for thiol-dependent DNA cleavage catalyzed by anthracycline-Fe^{3+} complexes*
Carminomycin-Fe^{3+} (△——△), daunomycin-Fe^{3+} (×– – –×), Fe^{3+} (□-·-·-□), 11-*O*-methyldaunomycin-Fe^{3+} (⊠-·-⊠). In the above reactions, the reduced glutathione concentration was 2.5 mM. The individual anthracyclines did not catalyze thiol-dependent DNA cleavage in the absence of iron.

TABLE 2

Catalysis of thiol-dependent DNA cleavage by anthracycline-iron complexes

The DNA cleavage reaction mixture consisted of 1:1 drug-iron complex, 2.5 mM GSH, and 0.25 μg of SV-40 [^{3}H]DNA in a final volume of 100 μl. Experiments were performed at room temperature in 20 mM NaCl, 10 mM Tris HCl buffer, pH 7.5. The concentration at 50% DNA cleavage is expressed as a mean (n = 3) ± standard deviation. The anthracyclines did not exhibit activity in the absence of iron.

Drug-Fe^{3+} complex	Concentration of complex cleaving 50% of DNA
	μM
Iron	35.0 ± 3.0
Carminomycin	2.0 ± 0.6
Daunomycin	4.5 ± 0.6
Doxorubicin	4.7 ± 0.5
4-Demethoxydaunomycin	6.5 ± 0.4
4-Demethyl-11-deoxydoxorubicin	13.0 ± 1.0
11-Deoxydoxorubicin	120 ± 20
11-Deoxydaunomycin	105 ± 20
11-*O*-Methyldaunomycin	100 ± 10

This background cleavage was blocked by catalase (400 units/ml) and probably represents H_2O_2 generated by the direct reduction of iron by doxorubicin (5, 24, 25).

For the sake of comparison, we also determined the efficiency of iron-EDTA complex in this assay. Fe^{3+}-EDTA does not react with hydrogen peroxide and so was not tried (26). In contrast, 120 μM Fe^{3+}-EDTA complex and 100 μM hydrogen peroxide were required to cause 50% DNA cleavage. This is 12-fold more complex and over 100 times the peroxide concentration required of the doxorubicin-iron complex. These observations are of particular interest, in that iron-EDTA was used to prove that iron was required for the formation of hydroxyl radical from superoxide and hydrogen peroxide (27). These results suggest that more is involved here than simply the chemistry of chelated iron, but rather the reactivity of the iron complexes is also determined by the nature of the ligand.

Hydroxyl radical formation. In our earlier report, we had shown that the hydrogen peroxide-dependent DNA cleavage reaction of the doxorubicin-iron complex was partially inhibited by very high concentrations of the hydroxyl radical scavengers dimethyl sulfoxide and mannitol (15). These results led us to suggest a role for the hydroxyl radical in the DNA damage which resulted. We have subsequently demonstrated that DNA stimulates hydroxyl radical formation by doxorubicin-Fe^{3+} complex from hydrogen peroxide using ESR spin-trapping techniques (16). With these results in mind, we have examined the relationship between hydroxyl radical production by the various anthracycline-Fe^{3+} complexes and their ability to catalyze the hydrogen peroxide-dependent DNA cleavage reaction. In these studies, hydroxyl radical formation was quantitated via the formation of the DMPO-OH spin adduct by ESR spectrometry. As reported previously (16), when hydrogen peroxide was added to the doxorubicin-Fe^{3+} complex, a small but detectable ESR spectrum with the characteristics of the DMPO-OH adduct, namely a 1:2:2:1 quartet, hyperfine

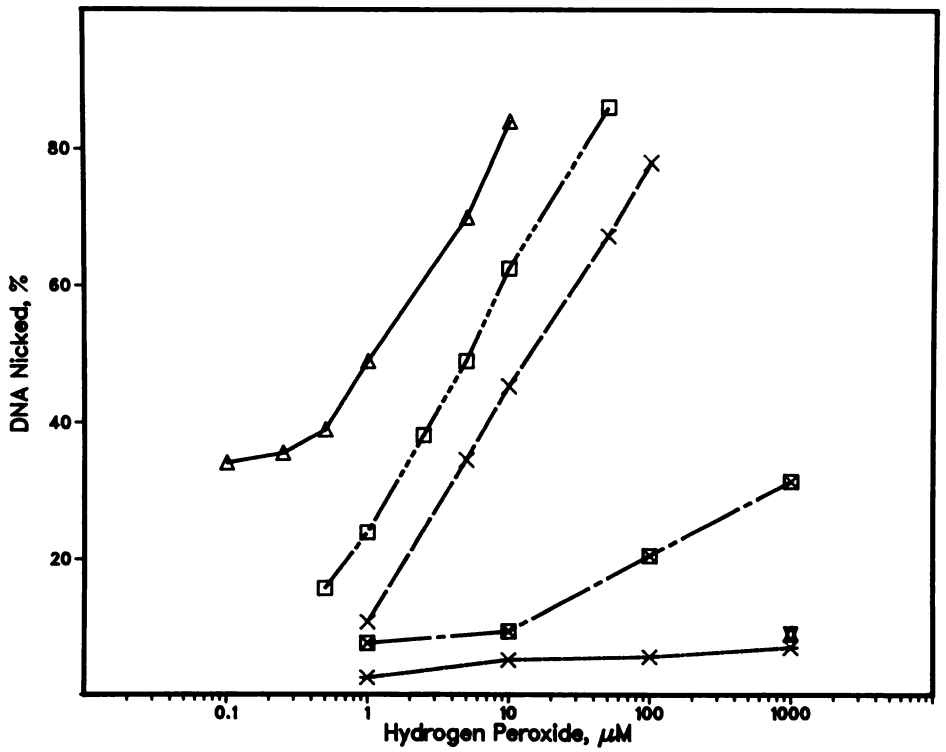

FIG. 6. *Hydrogen peroxide dose-response curves for DNA cleavage catalyzed by anthracycline-Fe^{3+} complexes*
Doxorubicin-Fe^{3+} (△——△), carminomycin-Fe^{3+} (□-·-·-□), daunomycin-Fe^{3+} (×——×), 4-demethyl-11-deoxydoxorubicin (⊠-·-⊠), 11-*O*-methyldaunomycin (×---×), and 11-deoxydaunomycin (XX). The concentration of the anthracycline 1:1 complexes was 10 μM. In the absence of iron, all of the above anthracyclines exhibited no hydrogen peroxide-dependent DNA cleavage.

TABLE 3

Catalysis of hydrogen peroxide-dependent DNA damage by drug-iron complexes

The assays were performed with 10 μM 1:1 drug-iron complex at room temperature. The results are the mean of two experiments. The anthracyclines in the absence of iron did not exhibit activity.

Drug-iron complex	[H_2O_2] required to cleave 50% of DNA
	μM
Doxorubicin	0.7
Carminomycin	5.0
Daunomycin	14.0
4-Demethoxydaunomycin	18.0
EDTA (120 μM)	100.0

TABLE 4

Stimulation of hydroxyl radical production by anthracycline-iron complexes in the presence of DNA

The details of the reaction conditions are in Materials and Methods. The anthracyclines in the absence of either iron or hydrogen peroxide did not produce a detectable spin adduct spectrum.

Drug-iron complexes	·OH production[a]	
	Complex + H_2O_2 −DNA	Complex + H_2O_2 +DNA
Iron		100
Doxorubicin	193	533
Carminomycin	110	256
4-Demethoxydaunomycin	133	256
Daunomycin	184	184

[a] Hydroxyl radical production is expressed as the relative signal intensity of the low-field line of the DMPO-OH adduct spectrum with iron + DNA + H_2O_2 set to a value of 100.

splitting constants $a^N = a^H = 14.9$ G, and significant quenching of the signal by ethyl alcohol and dimethyl sulfoxide was observed. The addition of DNA significantly stimulated (>250%) hydroxyl radical formation by the doxorubicin-Fe^{3+} complex, thus confirming our previous observations (16).

In the absence of DNA, the relative hydroxyl radical production was similar for the Fe^{3+} complexes of doxorubicin, daunomycin, carminomycin, and 4-demethoxydaunomycin (Table 4) and greater than iron alone. The addition of DNA caused marked stimulation of radical production from the iron complexes of carminomycin and 4-demethoxydaunomycin similar to that observed earlier for the doxorubicin-Fe^{3+} complex. On the other hand, hydroxyl radical production by the daunomycin-Fe^{3+} complex was not stimulated by DNA addition, although this complex did generate more ·OH than did Fe^{3+}-DNA alone (Table 4). The anthracycline antibiotics which did not give a spectral shift with iron addition exhibited hydroxyl radical production similar to that of Fe^{3+} alone. Thus, the analogs active in the hydrogen peroxide-dependent DNA damage reaction was characterized by spectral evidence of iron binding, enhanced hydroxyl radical production when compared to iron alone, and, with the exception of daunomycin, hydroxyl radical production which was stimulated by DNA addition.

As mentioned earlier, doxorubicin-Fe^{3+} forms a stable ternary complex with DNA which can be isolated from free doxorubicin-Fe^{3+} by chromatography on Sephadex G-25 (11). This drug-iron-DNA complex was an active redox catalyst (15) and formation of this ternary complex may result in the enhanced hydroxyl radical formation observed following DNA addition. For this reason, we have examined the ability of the active analogs listed in Table 3 to form ternary complexes stable enough to be detected by gel permeation chromatography using our previously published techniques (15). Doxorubicin, carminomycin, 4-demethoxydaunomycin, and daunomycin all produced a ternary complex by this criterion with equivalent yield. Therefore, each of the analogs active in the H_2O_2-dependent DNA cleavage reaction was characterized by the ability both to form a ternary complex and to catalyze hydroxyl radical formation from hydrogen peroxide. Thus, the stimulation of hydroxyl radical formation seen upon the addition of DNA to the iron complexes of carminomycin, doxorubicin, and 4-demethoxydaunomycin might well be due to ternary complex formation. On the other hand, daunomycin clearly forms a ternary complex, yet DNA addition had no significant effect on hydroxyl radical formation, so that it appears that the redox activity of a given drug-iron-DNA complex was influenced by the nature of the drug ligand.

DISCUSSION

In this paper, we have initially examined the structural requirements for iron binding to the anthracycline chromophore, using a spectrophotometric technique and a number of anthracycline analogs with specific chromophore structural modifications. The spectral criteria for iron binding to the drug chromophore have been previously described (14). Whereas all anthracyclines having a C-11-hydroxyl group showed spectral evidence of complex formation upon iron addition, those lacking this group did not. This observation suggests that a C-11-hydroxyl is involved in complex formation and that the most likely iron-binding site is between C-12-carbonyl and C-11-hydroxyl. X-ray crystal structure studies of daunomycin have found the distance between C-12-carbonyl oxygen and C-11-hydroxyl oxygen to be 2.53 and 2.59 Å(18–20). Since strong bidentate ligands of Fe^{3+} in which the binding occurs between two oxygens, such as acetohydroxamic acid, have been reported to have 2.53 to 2.56 Å spacing, the C-12-carbonyl and C-11-hydroxyl oxygens of anthracycline antibiotics are well suited for iron binding. The space between C-5-carbonyl and C-6-hydroxyl oxygens in daunomycin measured 2.46 to 2.47 Å and may thus not be ideal for iron chelation. This would provide a rationale for the lack of iron chelation noted with most of the 11-deoxy or 11-*O*-methyl analogs. With 4-demethyl-11-deoxydoxorubicin, iron can potentially bind via the C-4-hydroxyl and C-5-carbonyl oxygens but the removal of C-4-methoxy group may also alter the C-5-carbonyl to C-6-hydroxyl oxygen distance making it more suitable for iron binding. X-ray crystal structure studies would be required to confirm this conjecture. As a test of this hypothesis, it would have been valuable to examine the behavior of the C-6-deoxy analogs. Unfortunately, these were not available to us. Other potential iron-binding sites on these antibiotics, besides the quinone-hydroquinone functionalities, include the sugar amino group and the side chain carbonyl group of doxorubicin and its derivatives. Binding to these sites may not be detectable by the spectrophotometric techniques used here.

We have next investigated the chemical reactivity of these complexes with oxygen and DNA in the presence of GSH. Only anthracycline analogs which gave spectral evidence of iron chelation catalyzed the thiol-dependent oxygen consumption reaction. These results also suggest that Fe^{3+} chelated to anthracyclines is easily reduced to Fe^{2+} by glutathione and is subsequently involved in oxygen reduction. The poor catalysis by free Fe^{3+} of the thiol-dependent oxygen consumption reaction may occur because Fe^{3+} ions are unstable in aqueous solutions at physiological pH and rapidly form insoluble ferric hydroxide polymers (28). One important role of anthracyclines in the oxygen consumption reaction is therefore probably to chelate iron so as to prevent the formation of insoluble and therefore less reactive ferric hydroxide polymers. From the oxygen consumption studies, we conclude that only the Fe^{3+} chelated between C-11-hydroxyl and C-12-carbonyl oxygens appears to be important in the thiol-dependent oxygen consumption catalysis and that these studies did not reveal evidence of any additional catalytically important iron-binding sites. It is also possible that the redox activity of the drug-iron complexes might alter the course of the reaction or its rate.

H_2O_2-dependent hydroxyl radical production by the anthracycline-iron complexes exhibits several unusual properties. Iron catalysis of hydroxyl radical production from hydrogen peroxide is usually envisioned as occurring via the Fenton reaction (26): $Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + {}^{\cdot}OH + OH^-$. Where Fe^{3+} rather than Fe^{2+} has been added to the reaction mixture, some means has usually been found to reduce Fe^{3+} to Fe^{2+}. In the Haber-Weiss reaction, this reduction is accomplished by superoxide (26): $O_2^- + Fe^{3+} \rightarrow Fe^{2+} + O_2$.

In the case of doxorubicin, several mechanisms have been proposed to accomplish production of hydroxyl radical from H_2O_2. The reduced semiquinone formed via the action of flavin-dependent enzymes such as xanthine oxidase can react with oxygen to yield the superoxide anion (2, 29). In the presence of iron, this can trigger the Haber-Weiss reaction. Alternatively, the drug semiquinone could react with Fe^{3+} as follows: $DOX^{\cdot -} + Fe^{3+} \rightarrow DOX + Fe^{2+}$, where DOX is doxorubicin. The Fe^{2+} thus formed can initiate the Fenton reaction. In each of the above systems, hydroxyl radical formation is iron dependent. Winterbourn and others (30–34) have proposed yet a third, mechanism which is iron independent: $DOX^{\cdot -} + H_2O_2 \rightarrow DOX + {}^{\cdot}OH + OH^-$.

None of the above mechanisms fit the results of the present study. All that is required for hydroxyl radical production is Fe^{3+}, H_2O_2, and the appropriate anthracycline. Each of these components appears to be required. First, neither iron salts nor iron chelated to EDTA exhibits significant activity. Second, the anthracyclines

in the absence of iron exhibit no activity. Third, hydrogen peroxide at these concentrations does not damage DNA. The Haber-Weiss reaction is ruled out in that there are no means in this system for the generation of superoxide. Neither thiols nor Fe^{2+} reduces doxorubicin to its semiquinone (14). In addition, a reaction between Fe^{3+} and hydrogen peroxide under the conditions used seems unlikely (26, 35). The only possibility which remains is the reduction of Fe^{3+} to Fe^{2+} by the anthracycline. Preliminary evidence for Fe^{3+} reduction by doxorubicin has already been published and this may be a general property of the anthracyclines (24, 25).

The H_2O_2-dependent DNA cleavage studies were undertaken not only to determine the relative efficiency of the various anthracycline-Fe^{3+} complexes to cleave DNA, but also to assess their effectiveness at catalyzing hydroxyl radical formation from H_2O_2 in the presence of DNA. All of the anthracycline analogs active in the H_2O_2-dependent DNA cleavage reaction exhibited enhanced hydroxyl radical production when compared to iron alone, both in the presence and absence of DNA. In an earlier report, we showed that the doxorubicin-iron complex bound to DNA to form a ternary complex capable of catalyzing the reduction of O_2 by thiols (14). In that paper, we proved catalysis by showing 1) the drug is not irreversibly altered in the reaction, 2) the reaction gave linear Lineweaver-Burk plots for oxygen and glutathione from which K_m and V_{max} values were estimated, and 3) the reaction required both the presence of iron and drug for maximal reaction rates. In the present study, we have found that DNA addition markedly increased hydroxyl radical production from the doxorubicin, carminomycin, and 4-demethoxydaunomycin iron complexes, but not from the daunomycin-iron complex. In contrast to the redox activity of the anthracycline-iron-DNA ternary complex, DNA-intercalated doxorubicin is devoid of redox activity (36). This effect of DNA on iron chemistry is not without precedence in that DNA addition has also been observed to enhance the generation of hydroxyl radicals from hydrogen peroxide in the presence of ferrous ion. In this latter case, DNA apparently bound Fe^{2+} in a state which protected the ferrous ion from reaction with oxygen but not hydrogen peroxide (37). One possible explanation that may be offered is that these drugs may reduce Fe^{3+} to Fe^{2+} which is then stabilized by DNA. The ability of DNA addition to increase the yield of hydroxyl radicals in the presence of anthracycline-iron complexes might then be due to the same stabilization of Fe^{2+} observed earlier by Floyd and Lewis (37). Studies to confirm this sequence of events are currently in progress in this laboratory.

Finally, it is interesting to note that animal and clinical studies have reported that doxorubicin is more cardiotoxic than carminomycin, which is in turn more cardiotoxic than daunomycin and 4-demethoxydaunomycin (38–40). Ths ranking of cardiotoxic potential parallels the results obtained with their complexes in the hydrogen peroxide-dependent DNA cleavage reaction reported in this paper. In this regard, it should be noted that substantial amounts of hydrogen peroxide are produced as a normal product of myocardial electron transport. In addition, the anthracyclines can be expected to markedly stimulate H_2O_2 production because the enzymatic machinery for doxorubicin reduction is abundantly present in cardiac tissue (7). The latter observation has led us to propose that metabolic reduction of the drug to a semiquinone anion and the chemistry of the drug-metal complex could potentiate one another in that the former may act as a source of hydrogen peroxide and the latter as an effective means of utilizing the hydrogen peroxide to cause tissue injury (15). The major limitation to the above hypothesis is likely to be the limited availability of iron *in vivo* (9). In this regard, we note that Demant (10) has recently shown that doxorubicin is able to abstract iron from ferritin, the major intracellular storage form of iron. This result would seem to ensure that doxorubicin would have access to iron *in vivo*. In support of this concept, the EDTA derivatives ICRF-159 and 187 have been shown to both markedly increase renal iron clearance and lessen the cardiac toxicity of doxorubicin (11–13). These results suggest that the role of anthracycline-iron complexes in the cardiac toxicity of the anthracyclines may be worthy of investigation.

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Send reprint requests to: Josephia Muindi, Building 10, Room 6N119, National Institutes of Health, Bethesda, MD 20205.