# DNA Base Modifications Induced in Isolated Human Chromatin by NADH Dehydrogenase-Catalyzed Reduction of Doxorubicin†

Steven A. Akman,*,‡ James H. Doroshow,‡ Thomas G. Burke,‡,∥ and Miral Dizdaroglu§

*Department of Medical Oncology and Therapeutics Research, City of Hope National Medical Center, Duarte, California 91010, and Chemical Science and Technology Laboratory, National Institute of Standards and Technology, Gaithersburg, Maryland 20899*

*Received November 7, 1991; Revised Manuscript Received January 24, 1992*

ABSTRACT: The antineoplastic benzanthroquinone drug doxorubicin can undergo flavoenzyme-catalyzed one-electron reduction which, in an aerobic environment, leads to the generation of oxygen-derived species. We therefore sought to determine whether doxorubicin in the presence of NADH dehydrogenase and the transition metal ions Fe(III) or Cu(II) induces DNA base modifications in isolated human chromatin. NADH dehydrogenase-catalyzed reduction of doxorubicin (25–100 μM) caused hydroxyl radical production detected as methane generated from dimethyl sulfoxide; addition of isolated human chromatin to the system produced a concentration-dependent quenching of detectable hydroxyl radical formation. Doxorubicin (5–50 μM)-stimulated enzyme-catalyzed oxidation of NADH was also diminished, but still detectable, in the presence of chromatin. Doxorubicin-induced DNA base modifications in chromatin were measured by gas chromatography/mass spectrometry with selected-ion monitoring. Production of modified bases required the addition of transition metal ion and was enhanced by the addition of active flavoenzyme. The non-redox cycling analogue 5-iminodaunorubicin induced significantly less base modification than did doxorubicin. In the presence of Fe(III), NADH dehydrogenase-catalyzed reduction of doxorubicin caused enhancement in the content of all modified bases over control levels. Substitution of Cu(II) for Fe(III) altered both the degree and the pattern of doxorubicin/NADH dehydrogenase-induced base modifications. The scavengers of hydroxyl radical mannitol and dimethyl sulfoxide or catalase did not significantly affect doxorubicin/NADH/NADH dehydrogenase/transition metal ion-induced base modifications. Superoxide dismutase further enhanced production of all base modifications. The data demonstrate that flavoenzyme-catalyzed redox cycling of doxorubicin generates typical hydroxyl radical-induced base modifications in the DNA of isolated human chromatin, suggesting a possible mechanism for the mutagenicity of doxorubicin in vivo.

Doxorubicin[1] (Figure 1) is a benzanthroquinone drug which is useful in the treatment of several types of human malignancies (Young et al., 1981). It is cytotoxic and mutagenic (Marquardt et al., 1976; Au et al., 1981), both in bacterial and in mammalian test systems.

The mutagenicity of doxorubicin has been attributed to interaction(s) of the drug with DNA. One type of interaction is associated with the production of reactive free radicals (Lown et al., 1978). The quinone moiety of doxorubicin may undergo flavoenzyme-catalyzed one-electron reduction to a semiquinone (Handa & Sato, 1975; Bachur et al., 1978; Pan et al., 1981). Under aerobic conditions, the semiquinone rapidly reduces molecular oxygen, causing production of superoxide anion radical ($O_2^-$)[2] while regenerating the quinone (Goodman & Hochstein, 1977; Bachur et al., 1978, 1979; Doroshow, 1983). Thus, doxorubicin may serve as an electron shuttle between NAD(P)H and oxygen. It has been established that this type of enzyme-catalyzed cyclic reduction–oxidation of doxorubicin occurs in mammalian nuclei (Bachur et al., 1982; Mimnaugh et al., 1985).

Dismutation of $O_2^-$ yields hydrogen peroxide [for a review see Halliwell and Gutteridge (1989)]. The latter compound can cause formation of modified bases from pyrimidines and purines in the presence of metal ions in isolated DNA (Aruoma et al., 1989; Blakely et al., 1990; Aruoma et al., 1991), in isolated mammalian chromatin (Dizdaroglu et al., 1991b; Nackerdien et al., 1991), and in intact mammalian cells (Dizdaroglu et al., 1991a). These base modifications have been attributed to attack by highly reactive species with the characteristics of the hydroxyl radical (•OH). Some of these base modifications are promutagenic when studied in site-specific mutagenicity assays (Basu et al., 1989; Wood et al., 1990; Moriya et al., 1991). Therefore, it is possible that the mutagenicity of doxorubicin might be due, in part, to enzyme-catalyzed one-electron reduction of doxorubicin in close proximity to DNA, leading to metal ion-dependent DNA base modifications.

The production of modified bases by doxorubicin semiquinone might involve •OH as an intermediate. However, it

† This work was supported by the City of Hope Cancer Center Support Grant CA33572-09. M.D. acknowledges partial support by the Office of Health and Environmental Research, Office of Energy Research, U.S. Department of Energy, Washington, DC.

* Address correspondence to this author at the Department of Medical Oncology and Therapeutics Research, City of Hope National Medical Center, 1500 E. Duarte Rd., Duarte, CA 91010.

‡ City of Hope National Medical Center.

∥ Present address: Division of Pharmaceutics, College of Pharmacy, The Ohio State University, Columbus, OH 43210.

§ National Institute of Standards and Technology.

[1] Doxorubicin and daunorubicin are also commonly referred to as adriamycin and daunomycin, respectively.

[2] Abbreviations: $O_2^-$, superoxide anion radical; •OH, hydroxyl radical; GC/MS-SIM, gas chromatography/mass spectrometry with selected-ion monitoring; EDTA, ethylenediaminetetraacetic acid; 5-OH-5-MeHyd, 5-hydroxy-5-methylhydantoin; 5-OH-Hyd, 5-hydroxyhydantoin; 5-OHMe-Ura, 5-(hydroxymethyl)uracil; 5,6-diOH-Cyt, 5,6-dihydroxycytosine; FapyAde, 4,6-diamino-5-formamidopyrimidine; FapyGua, 2,6-diamino-4-hydroxy-5-formamidopyrimidine; 8-OH-Ade, 8-hydroxyadenine; 8-OH-Gua, 8-hydroxyguanine.

0006-2960/92/0431-3500$03.00/0 

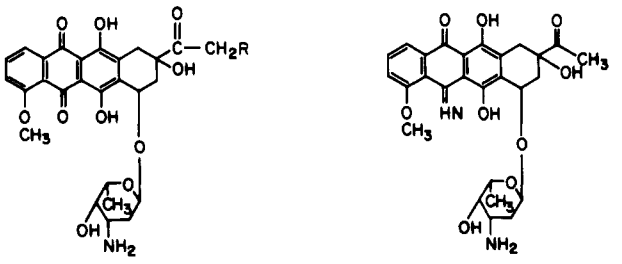


FIGURE 1: Structural formulas of the anthracycline antitumor antibiotics used in these studies.

has been argued that doxorubicin bound to DNA is not available to serve as a substrate for enzyme-catalyzed one-electron reduction (Rowley & Halliwell, 1983). DNA-bound doxorubicin may not necessarily need to undergo enzyme-catalyzed reduction in order to cause metal ion-dependent base modifications. Under appropriate conditions, DNA-bound doxorubicin can form a complex with Fe(III) (Eliot et al., 1984) which stimulates oxygen-dependent free radical production (Zweier, 1983; Gianni et al., 1985).

In the present study, in order to determine under what conditions doxorubicin might cause the occurrence of promutagenic base modifications in chromatin DNA, we have measured modified DNA bases in isolated human chromatin which was exposed to mixtures of doxorubicin, NADH, and the flavoenzyme NADH dehydrogenase in the presence of Cu(II) or Fe(III). The technique of GC/MS-SIM was used for measurement of modified bases in chromatin DNA.

## Materials and Methods

*Materials.*[3] Doxorubicin, NADH, EDTA, mannitol, dimethyl sulfoxide, copper–zinc superoxide dismutase, and NADH dehydrogenase (EC 1.6.99.3) were purchased from Sigma Chemical Co., St. Louis, MO. NADH dehydrogenase was Chelex-treated prior to use; activity was determined at 25 °C by modification of the method of Mahler (1955) using nonacetylated cytochrome *c* as the electron acceptor. Enzymatic activity was assayed in a reaction mixture containing 50 mM Tris-HCl, pH 8.5, 100 μM cytochrome *c*, and 0.2 mM NADH; reduction of cytochrome *c* was measured spectrophotometrically at 550 nm. One unit of NADH dehydrogenase activity was defined as that amount of enzyme capable of reducing 1 μmol of cytochrome *c*/min under the reaction conditions outlined above. Catalase (EC 1.11.1.6) was obtained from Boehringer Mannheim Biochemicals, Indianapolis, IN. Reagents for electrophoresis and Chelex 100 resin (100–200 mesh, sodium salt) were purchased from Bio-Rad, Richmond, CA. Dialysis membranes with a molecular weight cutoff of 6000–8000 were purchased from Fisher Scientific, Fairlawn, NJ. Acetonitrile and bis(trimethylsilyl)trifluoroacetamide containing 1% trimethylchlorosilane were obtained from Pierce Chemical Co., Rockford, IL.

*Measurement of Hydroxyl Radical Production.* The production of hydroxyl radical by NADH dehydrogenase in the presence of doxorubicin with or without chromatin was measured by the formation of methane from dimethyl sulfoxide using a modification of the method of Repine et al. (1979) as previously described (Doroshow, 1983).

Methane concentrations were determined using a Varian Model 3700 gas chromatograph (Varian Associates, Palo Alto, CA) equipped with a flame ionization detector and a $^1/_8$-in. by 6-ft stainless steel column packed with 80/100 mesh Carbosieve II (Supelco, Inc., Bellefonte, PA). The methane content of the samples was determined by comparison with a standard curve that was linear over the range of 0.05–10 nmol of methane. Results were expressed as nmol of methane/mL of headspace gas.

*Measurement of NADH Oxidation.* NADH oxidation was measured at room temperature spectrophotometrically as a decrease in $A_{340}$. Reaction cuvettes of 1 mL contained 0–50 μM doxorubicin with or without 50–100 μg/mL chromatin and 120 milliunits/mL NADH dehydrogenase, in 1 mM phosphate buffer, pH 7.6. Reactions were initiated by the addition of 0.2 mM NADH. An absorption coefficient of $6.18 \times 10^3$ $mol^{-1}$ $cm^{-1}$ (Ziegenhorn et al., 1976) was used to calculate nmol of NADH oxidized/s.

*Analysis of Drug Binding by Fluorescence Titration.* Drug binding was studied using the doxorubicin analogue daunorubicin, which has similar DNA binding properties (Chaires, 1990), and plasmid pCMVβgal (MacGregor & Caskey, 1989) DNA. pCMVβgal, received as a gift from Dr. Grant MacGregor, was amplified in *Escherichia coli* strain DH5 and purified by the alkaline lysis technique. Plasmid DNA was either supercoiled or relaxed by digestion with *Eco*RI. Equilibrium binding studies concerning the interaction of daunorubicin with DNA were performed on an SLM Model 4800S spectrofluorometer at ambient temperature. An excitation wavelength of 470 nm was used, and the emission from intrinsically fluorescent daunorubicin was observed through 550-nm long-pass filters (Oriel Corp., Stratford, CT). Sample volumes of 3 mL were used, each sample containing 4 μM daunorubicin in 1 mM phosphate buffer, pH 7.6, and the specified DNA concentrations.

*Cell Culture and Isolation of Chromatin.* The cells used for isolation of human chromatin were human K562 leukemia cells. These cells were grown in liquid suspension culture in RPMI 1640 medium (Gibco, Grand Island, NY), pH 7.4, supplemented with 10% heat-inactivated fetal bovine serum in a humidified 5% $CO_2$ atmosphere at 37 °C. Late-log phase cells [$(0.5–1.0) \times 10^9$] were harvested by centrifugation at 280*g*, 4 °C; chromatin was isolated according to Gajewski et al. (1990). Chromatin, which was obtained in 1 mM Tris-HCl, pH 7.4, was dialyzed extensively against 1 mM phosphate buffer, pH 7.4, which had been treated with Chelex resin. The entire isolation procedure was carried out at 4 °C. After dialysis, chromatin was homogenized briefly with 4–5 strokes in a glass homogenizer.

The absorption spectrum of chromatin purified from K562 cells resembled absorption spectra characteristic of mammalian chromatin (Bonner et al., 1968; Gajewski et al., 1990) with the following characteristics: $A_{258}/A_{280}$ ranged from 1.50 to 1.67, $A_{258}/A_{230}$ from 0.80 to 1.17, and $A_{258}/A_{320}$ from 8.6 to 12.6.

The protein components of chromatin were analyzed by sodium dodecyl sulfate–acrylamide gel electrophoresis by the method of Laemmli (1970). Stacking gels (4%) and resolving gels (18%) were prepared from a stock solution of 29.8% acrylamide and 0.2% bis(acrylamide). The banding pattern of chromatin, which was compared to that of a commercial histone standard (Sigma Chemical Co., St. Louis, MO) by

[3] Certain commercial equipment or materials are identified in this paper in order to specify adequately the experimental procedure. Such identification does not imply recommendation or endorsement by the National Institute of Standards and Technology, nor does it imply that the materials or equipment identified are necessarily the best available for the purpose.

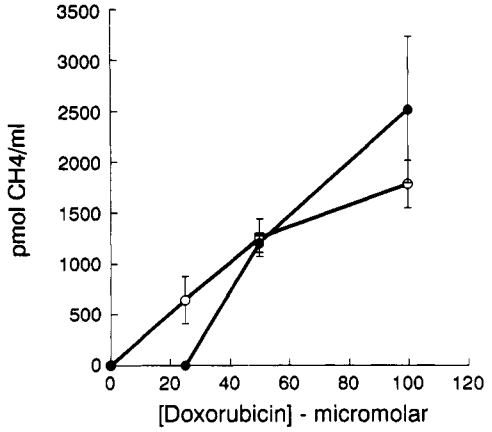


FIGURE 2: Hydroxyl radical production as measured by methane production from dimethyl sulfoxide caused by aerobic NADH dehydrogenase-catalyzed reduction of doxorubicin in the absence (○) or presence (●) of 50 μg/mL isolated human chromatin. Each data point represents the mean ● standard deviation of triplicate determinations. No hydroxyl radical production was detected if NADH dehydrogenase was not present in the reaction mixture (data not shown).

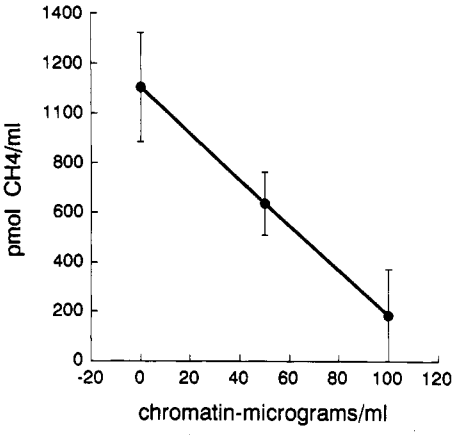


FIGURE 3: Hydroxyl radical production as measured by methane production from dimethyl sulfoxide caused by aerobic NADH dehydrogenase-catalyzed reduction of 50 μM doxorubicin in the presence of various amounts of isolated human chromatin.

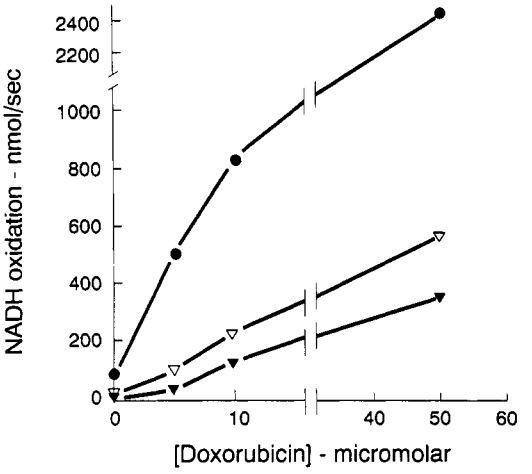


FIGURE 4: NADH oxidation as measured by decrease in absorbance at 340 nm. Reactions were carried out at room temperature in cuvettes containing the indicated concentrations of doxorubicin, 120 milliunits/mL NADH dehydrogenase, 0.2 mM NADH, and either 0 (●), 50 (▽), or 100 (▼) μg/mL chromatin. The scales of both the *x* and *y* axes have been altered for better graphic visualization of the rate of NADH oxidation at low doxorubicin.

silver staining of the gel, confirmed the presence of histones H1, H2A, H2B, H3, and H4 (data not shown).

*Doxorubicin Treatment of Chromatin.* Reaction mixtures contained the following compounds, where appropriate, in a final volume of 1–1.5 mL of Chelex-treated 1 mM phosphate buffer, pH 7.5: 100 μg of chromatin dialyzed against phosphate buffer, 25 μM $FeCl_3$, 25 μM $CuSO_4$, 100 μM EDTA, 0.5 mM NADH, 15 milliunits/mL NADH dehydrogenase, 5–500 μM doxorubicin, 4500 units/mL catalase, copper–zinc superoxide dismutase (EC 1.15.1.1, 20 μg/mL), 50 mM mannitol, 50 mM dimethyl sulfoxide, and 1 mM glutathione. Where indicated, $FeCl_3$ and $CuSO_4$ were mixed with EDTA prior to addition to the reaction mixture. Upon addition of >100 μM doxorubicin, chromatin took on an intense red color and visibly condensed. Mixtures were incubated, protected from light, at 37 °C for 1 h. After incubation, reactions were quenched by addition of 200 μM desferrioximine, followed by exhaustive dialysis against 100 volumes of distilled water at 4 °C. After dialysis, reaction mixtures were held at 37 °C overnight after addition of 1% (w/v) sodium dodecyl sulfate to dissolve any chromatin precipitated by interaction with doxorubicin. Aliquots (50 μL) were removed and extracted twice with chloroform/methanol (2:1 v/v) to remove chromatin-bound doxorubicin, and then DNA contents were quantified by $A_{258}$. 6-AzaThy (2.5 nmol) and 8-azaAde (5.0 nmol) were added as internal standards to each chromatin sample containing 100 μg of DNA, after which samples were dried under vacuum.

*Hydrolysis, Derivatization, and Gas Chromatography/Mass Spectrometry.* Chromatin samples were hydrolyzed with 60% formic acid in evacuated and sealed tubes at 140 °C for 30 min. Samples were lyophilized and trimethylsilylated with 0.12 mL of a mixture of *N,O*-bis(trimethylsilyl)trifluoroacetamide and acetonitrile (2:1 v/v) at 130 °C for 30 min. GC/MS-SIM analysis of derivatized samples and quantification of modified bases were performed as described previously (Gajewski et al., 1990).

## RESULTS

*Hydroxyl Radical Production.* Hydroxyl radical production due to reduction of doxorubicin (25–100 μM) by NADH dehydrogenase was detectable as methane generated from dimethyl sulfoxide (Figure 2). Addition of chromatin containing 50 μg of DNA/mL completely quenched detectable •OH production induced by 25 μM doxorubicin. However, a doxorubicin dose-dependent increase in •OH formation occurred at higher doxorubicin concentrations when the amount of chromatin was held constant. Detectable •OH production induced by 50 μM doxorubicin was quenched by chromatin in a concentration-dependent fashion (Figure 3). Under the experimental conditions of these studies, no doxorubicin-mediated •OH production was detected in the absence of active enzyme.

*NADH Oxidation.* NADH dehydrogenase-catalyzed oxidation of NADH stimulated by doxorubicin (≥5 μM) was detectable as a decrease in $A_{340}$ (Figure 4). Chromatin partially quenched NADH oxidation; however, even at the lowest concentration of doxorubicin tested, 5 μM, doxorubicin-stimulated NADH oxidation was still detectable. NADH was oxidized at a rate <16 nmol/min in reactions containing all of the reaction components (including 100 μg/mL chromatin) except for doxorubicin. It should be noted that, while the concentration of enzyme used in this set of experiments was 8-fold higher than that used in the base modification experiments, these results suggest that some doxorubicin was available as a substrate for NADH dehydrogenase in the presence of chromatin.

Table I: Yields (nmol/mg of DNA) of DNA Base Products Produced by the Doxorubicin/Fe(III) ± EDTA System in Isolated Human Chromatin[a]

| product | chromatin (chr) | chr/dox[b]/Fe | chr/dox/ Fe-EDTA | chr/dox/ Fe/NADH | chr/dox/Fe/ NADH/ enzyme | chr/dox/ Fe-EDTA/ NADH/ enzyme | chr/dox/ Fe-EDTA/ NADH/heat-inactivated enzyme | chr/5-imino-daunorubicin/ Fe-EDTA/ NADH/ enzyme |
|---|---|---|---|---|---|---|---|---|
| column no. | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 |
| 5-OH-5-MeHyd | 0.269 ± 0.058 | 0.227 ± 0.011 | 0.342 ± 0.104 | 0.295 ± 0.074 | 0.577 ± 0.084 | 0.562 ± 0.017 | 0.357 ± 0.129 | 0.941 ± 0.320 |
| 5-OH-Hyd | 0.310 ± 0.158 | 0.432 ± 0.116 | 0.368 ± 0.034 | 0.378 ± 0.004 | 1.23 ± 0.175 | 0.960 ± 0.321 | 0.866 ± 0.071 | 1.300 ± 0.127 |
| 5-OHMe-Ura | nd[c] | nd | nd | 0.185 ± 0.015 | 0.89 ± 0.112 | 0.933 ± 0.312 | 0.402 ± 0.035 | 0.395 ± 0.139 |
| Cyt glycol | 0.323 ± 0.187 | 0.520 ± 0.231 | 0.548 ± 0.150 | 0.549 ± 0.119 | 0.611 ± 0.102 | 0.777 ± 0.033 | 0.232 ± 0.053 | 0.546 ± 0.178 |
| Thy glycol | 0.158 ± 0.016 | 0.312 ± 0.04 | 0.324 ± 0.095 | 0.292 ± 0.008 | 0.836 ± 0.113 | 0.720 ± 0.144 | 0.492 ± 0.047 | 0.127 ± 0.016 |
| 5,6-diOH-Cyt | 0.129 ± 0.055 | 0.116 ± 0.019 | 0.137 ± 0.020 | 0.130 ± 0.020 | 0.275 ± 0.091 | 0.526 ± 0.096 | 0.228 ± 0.037 | 0.252 ± 0.066 |
| FapyAde[d] | 0.238 ± 0.003 | 0.205 ± 0.056 | 0.201 ± 0.037 | | | | | |
| 8-OH-Ade[d] | 0.272 ± 0.036 | 0.443 ± 0.085 | 0.523 ± 0.065 | | | | | |
| FapyGua | 0.179 ± 0.027 | 0.364 ± 0.101 | 0.266 ± 0.098 | 0.353 ± 0.015 | 1.11 ± 0.462 | 0.915 ± 0.177 | 0.316 ± 0.073 | 0.629 ± 0.059 |
| 8-OH-Gua | 0.593 ± 0.257 | 1.28 ± 0.097 | 1.31 ± 0.206 | 1.32 ± 0.206 | 1.95 ± 0.269 | 1.62 ± 0.338 | 1.10 ± 0.12 | 0.610 ± 0.075 |

[a] All values represent the mean ± standard deviation from triplicate measurements. [b] 50 μM doxorubicin was used in all experiments listed in this table. [c] nd = below detection limit. [d] These bases are not reported in samples containing NADH.

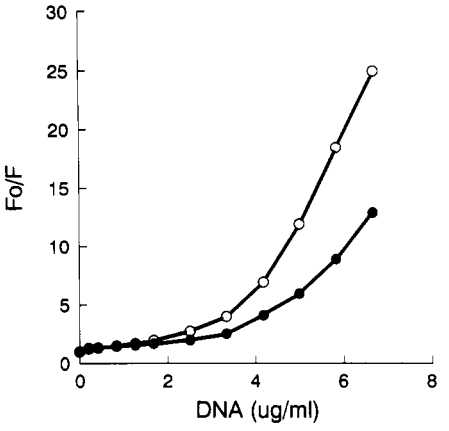


FIGURE 5: The ratio of the fluorescence intensity of the drug in the absence of DNA ($F_0$) and in the presence of DNA ($F$) is shown. Data are shown for both the supercoiled (●) as well as the relaxed (○) forms. In the presence of large excesses of DNA, the $F_0/F$ values for the relaxed and supercoiled forms of DNA were 50 and 43, respectively.

*Drug Binding.* The DNA binding characteristics of the closely related analogue daunorubicin to plasmid DNA were analyzed by the quenching of intrinsic drug fluorescence upon binding. Quenching of intrinsic drug fluorescence was observed in the presence of as little as 2 μg/mL DNA (Figure 5). Relaxed DNA bound drug with greater affinity than did supercoiled DNA. In the presence of large excesses of DNA (≥50 μg/mL), intrinsic drug fluorescence was quenched by 50- and 43-fold by relaxed and supercoiled DNA, respectively.

*Base Modification by Doxorubicin in the Presence of Fe(III).* Chromatin samples were analyzed by GC/MS-SIM after hydrolysis and derivatization. The yields of modified bases under various conditions of exposure of chromatin to doxorubicin ± Fe(III) ± NADH ± NADH dehydrogenase are given in Table I. The yields of Ade modifications are not reported in samples containing NADH because, despite extensive dialysis of the reaction mixtures prior to hydrolysis, Ade modifications were detected in control samples not containing chromatin, presumably due to residual modified NADH. All of the modified bases assayed were detected in control chromatin samples. Consistent with a previous report (Dizdaroglu et al., 1991b), Fe(III) alone did not increase the content of base modifications in purified chromatin (data not shown). Doxorubicin + Fe(III) with or without EDTA or NADH caused minor (≤2-fold) increases in Thy glycol, 8-OH-Ade, and 8-OH-Gua in the absence of active NADH dehydrogenase (columns labeled 1–4, Table I). Addition of active enzyme to doxorubicin/Fe(III)/NADH caused increases in the contents of all modified bases (column labeled 5). Doxorubicin/Fe(III)/NADH/NADH dehydrogenase-mediated increases over control levels ranged from 2-fold for the Cyt-derived products, 4–5-fold for the Thy-derived products, to 3–6-fold for the Gua-derived products (compare columns 1 and 5). The chelator EDTA did not significantly affect modified base production by doxorubicin/Fe(III)/NADH/NADH dehydrogenase (compare columns 5 and 6).

The non-redox cycling analogue 5-iminodaunorubicin was markedly less effective than doxorubicin as an inducer of most of the modified DNA bases in reaction mixtures containing Fe(III)-EDTA/NADH/NADH dehydrogenase (compare columns 6 and 8, Table I). Only the hydantoins 5-OH-5-MeHyd and 5-OH-Hyd were induced comparably by 5-iminodaunorubicin and doxorubicin.

Heat inactivation of NADH dehydrogenase by autoclaving (121 °C for 15 min) markedly reduced base modification, as compared to active enzyme, in reaction mixtures containing doxorubicin/Fe(III)-EDTA/NADH/enzyme (compare columns 6 and 7, Table I). The amount of base modification caused by doxorubicin/Fe(III)-EDTA/NADH in the presence of heat-inactivated enzyme was comparable to that caused by doxorubicin/Fe(III)-EDTA without additional enzyme (compare columns 3 and 7, Table I), the lone exception being 5-OH-Hyd.

The hydroxyl radical scavengers mannitol and dimethyl sulfoxide had only minor and variable effects on modified base production by doxorubicin/Fe(III)/NADH/NADH dehydrogenase with or without EDTA (compare columns 5 and 6, Table I, with columns 1–4, Table II). Catalase was also ineffective as an inhibitor of doxorubicin/Fe(III)-EDTA/NADH/NADH dehydrogenase-mediated base modification (compare column 6, Table I, with column 5, Table II). Inclusion of copper–zinc superoxide dismutase enhanced the production of all of the base modifications (compare column 6, Table I, with column 6, Table II). The thiol glutathione inhibited the production of Cyt glycol and partially inhibited production of the Thy-derived products Thy glycol and 5-OHMe-Ura, but did not affect production of Gua-derived products (compare column 6, Table I, with column 7, Table II).

*Base Modification by Doxorubicin in the Presence of Cu(II).* As has been reported previously (Dizdaroglu et al., 1991b), Cu(II) alone caused some base modification (column

Table II: Yields (nmol/mg of DNA) of DNA Base Products Produced by the Doxorubicin/Fe(III) ± EDTA System in Isolated Human Chromatin in the Presence of •OH Scavengers/Antioxidants[a]

| product | chr/dox[b]/Fe/ NADH/ enzyme/ mannitol | chr/dox/Fe/ NADH/enzyme/ dimethyl sulfoxide | chr/dox/ Fe-EDTA/ NADH/ enzyme/ mannitol | chr/dox/ Fe-EDTA/ NADH/enzyme/ dimethyl sulfoxide | chr/dox/ Fe-EDTA/ NADH/ enzyme/ catalase | chr/dox/ Fe-EDTA/ NADH/enzyme/ superoxide dismutase | chr/dox/ Fe-EDTA/ EDTA/NADH/ glutathione |
|---|---|---|---|---|---|---|---|
| column no. | 1 | 2 | 3 | 4 | 5 | 6 | 7 |
| 5-OH-5-MeHyd | 0.783 ± 0.174 | 0.687 ± 0.232 | 0.386 ± 0.173 | 0.466 ± 0.162 | 0.717 ± 0.052 | 0.956 ± 0.217 | 0.534 ± 0.027 |
| 5-OH-Hyd | 1.520 ± 0.215 | 1.130 ± 0.405 | 0.967 ± 0.287 | 0.712 ± 0.201 | 1.05 ± 0.134 | 2.22 ± 0.330 | 0.953 ± 0.172 |
| 5-OHMe-Ura | 0.976 ± 0.147 | 0.933 ± 0.312 | 1.098 ± 0.092 | 0.808 ± 0.329 | 0.787 ± 0.010 | 1.744 ± 0.335 | 0.474 ± 0.079 |
| Cyt glycol | 0.887 ± 0.090 | 0.777 ± 0.033 | 0.739 ± 0.117 | 0.431 ± 0.128 | 0.599 ± 0.033 | 1.42 ± 0.193 | 0.363 ± 0.072 |
| Thy glycol | 0.130 ± 0.464 | 0.720 ± 0.144 | 0.801 ± 0.064 | 0.362 ± 0.016 | 0.708 ± 0.052 | 1.03 ± 0.188 | 0.420 ± 0.124 |
| 5,6-diOH-Cyt | 0.235 ± 0.044 | 0.526 ● 0.096 | 0.197 ± 0.010 | 0.609 ± 0.259 | 0.291 ± 0.064 | 0.537 ± 0.163 | 0.155 ± 0.040 |
| FapyAde[c] | | | | | | | |
| 8-OH-Ade[c] | | | | | | | |
| FapyGua | 1.200 ± 0.250 | 0.915 ± 0.177 | 1.100 ± 0.020 | 0.689 ± 0.179 | 0.550 ± 0.060 | 1.20 ± 0.165 | 0.990 ± 0.150 |
| 8-OH-Gua | 1.060 ± 0.150 | 1.62 ± 0.338 | 1.20 ± 0.250 | 1.44 ± 0.276 | 1.40 ± 0.160 | 1.70 ± 0.20 | 1.50 ± 0.20 |

[a] All values represent the mean ± standard deviation from triplicate determinations. [b] 50 μM doxorubicin was used in all experiments listed in this table. [c] These bases are not reported in samples containing NADH.

Table III: Yields (nmol/mg of DNA) of DNA Base Products Produced by the Doxorubicin/Cu(II) System in Isolated Human Chromatin[a]

| product | chromatin (chr) | chr/Cu | chr/Cu/ NADH | chr/Cu/dox[b] | chr/Cu/ NADH/dox | chr/NADH/ enzyme/dox | chr/Cu/ NADH/ enzyme/dox | chr/dox/ Cu-EDTA/ NADH/ enzyme |
|---|---|---|---|---|---|---|---|---|
| column no. | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 |
| 5-OH-5-MeHyd | 0.168 ± 0.042 | 0.219 ± 0.052 | 0.040 ± 0.005 | 0.074 ± 0.024 | 0.084 ± 0.009 | 0.168 ± 0.046 | 0.184 ± 0.020 | 0.115 ± 0.014 |
| 5-OH-Hyd | 0.457 ± 0.109 | 0.498 ± 0.082 | 0.216 ± 0.018 | 0.505 ± 0.009 | 0.186 ± 0.046 | 0.217 ± 0.019 | 0.611 ± 0.070 | 0.393 ± 0.022 |
| 5-OHMe-Ura | 0.092 ± 0.029 | 0.216 ± 0.051 | 0.260 ± 0.075 | 0.292 ± 0.035 | 0.173 ± 0.037 | 0.259 ± 0.040 | 0.432 ± 0.009 | 0.375 ± 0.117 |
| Cyt glycol | 0.149 ± 0.055 | 0.540 ± 0.139 | 0.683 ± 0.014 | 0.659 ± 0.036 | 0.252 ± 0.045 | 0.156 ± 0.030 | 0.810 ± 0.066 | 0.458 ± 0.058 |
| Thy glycol | 0.132 ± 0.004 | 0.206 ± 0.083 | 0.110 ± 0.036 | 0.200 ± 0.057 | 0.136 ± 0.183 | 0.237 ± 0.085 | 0.165 ± 0.020 | 0.255 ± 0.102 |
| 5,6-diOH-Cyt | 0.121 ± 0.026 | 0.093 ± 0.014 | 0.083 ± 0.021 | 0.109 ± 0.015 | 0.076 ± 0.028 | 0.101 ± 0.015 | 0.192 ± 0.053 | 0.160 ± 0.062 |
| FapyAde[c] | 0.125 ± 0.027 | 0.178 ± 0.023 | | 0.087 ± 0.010 | | | | |
| 8-OH-Ade[c] | 0.242 ± 0.050 | 0.359 ± 0.073 | | 0.188 ± 0.016 | | | | |
| FapyGua | 0.156 ± 0.011 | 0.105 ± 0.034 | 0.100 ± 0.015 | 0.180 ± 0.003 | 0.227 ± 0.030 | 0.269 ± 0.038 | 2.46 ± 0.640 | 0.159 ± 0.038 |
| 8-OH-Gua | 0.550 ± 0.145 | 0.735 ± 0.083 | 0.740 ± 0.047 | 0.592 ± 0.128 | 0.418 ± 0.088 | 0.529 ± 0.062 | 7.56 ± 0.178 | 0.419 ± 0.046 |

[a] All values represent the mean ± standard deviation from triplicate measurements. [b] 50 μM doxorubicin was used in all experiments listed in this table. [c] These bases are not reported in samples containing NADH.

Table IV: Yields (nmol/mg of DNA) of DNA Base Products Produced by the Doxorubicin/Cu(II) System in Isolated Human Chromatin[a]

| product | chr/Cu/NADH/ enzyme/5 μM dox | chr/Cu/NADH/ enzyme/10 μM dox | chr/Cu/NADH/ enzyme/50 μM dox | chr/Cu/NADH/ enzyme/50 μM dox/mannitol |
|---|---|---|---|---|
| column no. | 1 | 2 | 3 | 4 |
| 5-OH-5-MeHyd | 0.118 ± 0.017 | 0.178 ± 0.023 | 0.184 ± 0.020 | 0.115 ± 0.027 |
| 5-OH-Hyd | 0.290 ± 0.025 | 0.344 ± 0.054 | 0.611 ± 0.070 | 0.384 ± 0.042 |
| 5-OHMe-Ura | 0.440 ± 0.030 | 0.384 ± 0.077 | 0.432 ± 0.009 | 0.452 ± 0.085 |
| Cyt glycol | 1.29 ± 0.175 | 1.37 ± 0.448 | 0.810 ± 0.066 | 0.918 ± 0.190 |
| Thy glycol | 0.200 ± 0.020 | 0.220 ± 0.027 | 0.165 ± 0.020 | 0.186 ± 0.021 |
| 5,6-diOH-Cyt | 0.111 ± 0.017 | 0.156 ± 0.025 | 0.192 ± 0.053 | 0.137 ± 0.041 |
| FapyAde[b] | | | | |
| 8-OH-Ade[b] | | | | |
| FapyGua | 0.444 ± 0.020 | 1.67 ± 0.180 | 2.46 ± 0.640 | 1.29 ± 0.301 |
| 8-OH-Gua | 3.54 ± 1.69 | 6.79 ± 1.09 | 7.56 ± 0.178 | 7.39 ± 0.792 |

[a] All values represent the mean ± standard deviation from triplicate measurements. [b] These bases are not reported in samples containing NADH.

2, Table III). Addition of NADH and/or doxorubicin (50 μM) did not significantly enhance the yield of base modifications further (compare columns 2, 3, 4, and 5, Table III). When both Cu(II) and NADH/NADH dehydrogenase were present in the reaction mixture, marked enhancement of the amounts of modified bases was observed (compare columns 5 and 7, Table III). The contents of 8-OH-Gua and FapyGua were enhanced by up to 15-fold, and the contents of Cyt-derived products Cyt glycol, 5,6-diOH-Cyt, and 5-OH-Hyd, by approximately 3-fold. The amounts of Thy-derived products 5-OH-5-MeHyd and 5-OHMe-Ura were enhanced by approximately 2-fold; Thy glycol was not affected.

Chelation of Cu(II) with EDTA diminished the production of all of the doxorubicin/NADH dehydrogenase-mediated base modifications, back to control levels in most instances (compare columns 7 and 8, Table III).

Enhancement of the content of these base modifications was observed after reaction with as low as 5 μM doxorubicin (the lowest concentration tested) (compare column 1, Table III, with columns 1, 2, and 3, Table IV). A concentration dependence was observed with 8-OH-Gua and FapyGua; however, this was not the case with the pyrimidine-derived products. The •OH scavenger mannitol did not inhibit doxorubicin/Cu(II)/NADH/NADH dehydrogenase-induced base

modifications (column 4, Table IV). Dimethyl sulfoxide was similarly ineffective (data not shown).

## Discussion

Our data demonstrate that biologically-relevant concentrations of doxorubicin can cause DNA base modifications in chromatin similar to those caused by •OH-generating systems [for reviews see Dizdaroglu (1991) and Halliwell and Aruoma (1991)]. The mechanism(s) of doxorubicin-mediated DNA base modification is (are) dependent upon the presence of flavoenzyme and transition metal ion and appears (appear) to involve a doxorubicin semiquinone intermediate. 5-Iminodaunorubicin, which is not reduced to a semiquinone (Lown et al., 1979; Pietronigro et al., 1979), does not cause nearly the amount of DNA base modification as does doxorubicin. Under aerobic conditions, doxorubicin semiquinone generates $O_2^{\cdot-}$, which can lead to •OH formation via the metal ion-catalyzed Haber–Weiss reaction [for a review see Halliwell and Gutteridge (1989)]. We observed significant enzyme-dependent production of •OH caused by doxorubicin in the absence of chromatin; however, detectable production of •OH was quenched by chromatin. This observation, plus the incomplete inhibition of doxorubicin-mediated base modification by catalase or scavengers of •OH, strongly suggests that •OH generated "free" in solution by reduction of doxorubicin is not the major oxidation pathway responsible for the observed base modifications. Our data suggest that doxorubicin-mediated base modifications occur, in large part, via site-specific generation of either •OH (Samuni et al., 1983; Goldstein & Czapski, 1986) or a similarly-reactive species. In support of this hypothesis are the following results: (i) fluorescence titration experiments suggested that >97% of 5 μM doxorubicin is bound by 50 μg/mL DNA, yet this concentration of doxorubicin caused significant base modification; (ii) complete quenching of detectable •OH generation due to doxorubicin (≤25 μM) by 50 μg/mL chromatin also suggests that most of the doxorubicin molecules were bound under these conditions; and (iii) there is a marked preference for Cyt and Gua modification by doxorubicin/Cu(II)/NADH/NADH dehydrogenase. Cu(II) is well-known to preferentially form coordination complexes with C·G base pairs in DNA (Pezzano & Podo, 1980).

It has been argued that doxorubicin, when intercalated in DNA, can not undergo flavoenzyme-catalyzed reduction (Rowley & Halliwell, 1983). The site-specific nature of the flavoenzyme-dependent base modifications caused by doxorubicin would seem to suggest otherwise, especially in light of the fact that doxorubicin-induced, enzyme-dependent NADH oxidation was detectable in the presence of excess chromatin, albeit at a 10-fold reduced rate.

However, we cannot rule out an alternative hypothesis that flavoenzyme-catalyzed reduction of nonintercalated doxorubicin (bound electrostatically) takes place exterior to chromatin-associated DNA and that an electron is transferred from the semiquinone by an undetermined mechanism to a DNA-associated molecule capable of generating •OH. Recent pulse radiolysis studies have demonstrated that doxorubicin semiquinone, when bound to DNA, can disproportionate along an intramolecular path across DNA over a distance of approximately 100 base pairs (Houee-Levin et al., 1991); the mechanism of electron transfer is similar to that which occurs in doped organic polymers. Therefore, it is not inconceivable that long-range electron transfer from doxorubicin semiquinone is part of the mechanism of base modification.

There were marked differences in the pattern of Cu(II)- and Fe(III)-dependent DNA base modifications. Cu(II) was associated with a significantly greater amount of base modification than was Fe(III). Cu(II) was also associated with a marked preference for Cyt and Gua modification, whereas Fe(III) was not. It is in accord with previous observations that Cu(II)–hydrogen peroxide-induced DNA damage exhibits a strong preference for C·G (Sagripanti & Kraemer, 1989; Yamamoto & Kawanishi, 1989; Dizdaroglu et al., 1991b). Doxorubicin/Cu(II) was not able to stimulate base damage over the level caused by Cu(II) alone in the absence of active NADH dehydrogenase, whereas doxorubicin/Fe(III) caused marked increases in the amount of some modified bases over control levels. This may reflect the ability of doxorubicin to form complexes with Fe(III) (Sugioka & Nakano, 1982; Zweier, 1983; Eliot et al., 1984) from which •OH can be generated by nonenzymatic electron transfer from doxorubicin to Fe(III) (Gianni et al., 1985).

Another marked difference between the effects of Cu(II) and Fe(III) was that EDTA inhibited Cu(II)-mediated formation of modified bases, whereas the effect of Fe(III) was unchanged by EDTA. Among the amounts of modified bases measured, the highest ratio of increase over the background level was observed in the amount of FapyGua in samples treated by the doxorubicin/Fe(III)/NADH/NADH dehydrogenase and doxorubicin/Cu(II)/NADH/NADH dehydrogenase systems. This high ratio of increase and the high yield of FapyGua, which was comparable to the yield of 8-OH-Gua in the case of the latter system, is remarkable because the formation of 8-OH-Gua is almost exclusively preferred over that of FapyGua when •OH production is mediated by Cu(II) (Dizdaroglu et al., 1991b; Aruoma et al., 1991). The high yield of FapyGua in the present work might result from enhanced reduction of the C-8 OH adduct radical of Gua, possibly via an NADH dehydrogenase-catalyzed electron transfer, considering that the ratio of FapyGua to 8-OH-Gua was markedly decreased when the enzyme was left out or heat-inactivated. The reduction and oxidation of the C-8 OH adduct radical of Gua leads to formation of FapyGua and 8-OH-Gua, respectively [for a review see Steenken (1989)].

Doxorubicin is a mutagen in bacterial and mammalian mutagenicity assays (Marquardt et al., 1976; Au et al., 1981). Some of the base modifications we observed in chromatin caused by doxorubicin/NADH/NADH dehydrogenase/Cu(II) or Fe(III), e.g., 8-OH-Gua (Wood et al., 1990; Moriya et al., 1991) and Thy glycol (Basu et al., 1989), are promutagenic when inserted site-specifically into DNA. It is possible that production of modified bases in vivo is one mechanism whereby doxorubicin exposure is mutagenic. Several observations suggest that doxorubicin may cause base modification in vivo. Enzyme-catalyzed one-electron reduction of doxorubicin occurs in intact isolated nuclei (Bachur et al., 1982; Mimnaugh et al., 1985). Also, the conditions we have used in these studies to demonstrate doxorubicin-mediated DNA base modification in vitro approach in vivo conditions in that doxorubicin was tested at physiologic concentrations and the DNA was bound to chromatin protein. Thus, reactive oxygen production by the redox cycling of the doxorubicin quinone moiety could have an important role in stimulating the mutagenicity of this agent.

## Acknowledgments

We thank Ms. Judith Stetzler, Ms. Marianne Metz, and Mr. Darrin Joy for excellent technical assistance.

## References

Aruoma, O. I., Halliwell, B., Gajewski, E., & Dizdaroglu, M. (1989) *J. Biol. Chem. 264*, 20509–20512.

Aruoma, O. I., Halliwell, B., Gajewski, E., & Dizdaroglu, M. (1991) *Biochem. J. 273*, 601–604.
Au, W. W., Butler, M. A., Matney, T. S., & Loo, T. L. (1981) *Cancer Res. 41*, 376–379.
Bachur, N. R., Gordon, S. L., & Gee, M. V. (1978) *Cancer Res. 38*, 1745–1750.
Bachur, N. R., Gordon, S. L., Gee, M. V., & Kon, H. (1979) *Proc. Natl. Acad. Sci. U.S.A. 76*, 954–957.
Bachur, N. R., Gee, M. V., & Friedman, R. D. (1982) *Cancer Res. 42*, 1078–1081.
Basu, A. K., Loechler, E. L., Leadon, S. A., & Essigmann, J. M. (1989) *Proc. Natl. Acad. Sci. U.S.A. 86*, 7677–7681.
Blakely, W. F., Fuciarelli, A. F., Wegher, B. J., & Dizdaroglu, M. (1990) *Radiat. Res. 121*, 338–343.
Bonner, J., Chalkley, G. R., Dahmus, M., Fambrough, D., Fujimura, F., Huang, R. C., Huberman, J., Jensen, R., Marushige, K., Ohlenbusch, H., Olivera, B., & Widholm, J. (1968) *Methods Enzymol. 12*, 3–65.
Chaires, J. B. (1990) *Biophys. Chem. 35*, 191–202.
Dizdaroglu, M. (1991) *Free Radical Biol. Med. 10*, 225–242.
Dizdaroglu, M., Nackerdien, Z., Chao, B.-C., Gajewski, E., & Rao, G. (1991a) *Arch. Biochem. Biophys. 285*, 388–390.
Dizdaroglu, M., Rao, G., Halliwell, B., & Gajewski, E. (1991b) *Arch. Biochem. Biophys. 285*, 317–324.
Doroshow, J. H. (1983) *Cancer Res. 43*, 4543–4551.
Eliot, H., Gianni, L., & Myers, C. (1984) *Biochemistry 23*, 928–936.
Gajewski, E., Rao, G., Nackerdien, Z., & Dizdaroglu, M. (1990) *Biochemistry 29*, 7876–7882.
Gianni, L., Zweier, J. L., Levy, A., & Myers, C. E. (1985) *J. Biol. Chem. 260*, 6820–6826.
Goldstein, S., & Czapski, G. (1986) *J. Free Radicals Biol. Med. 2*, 3–11.
Goodman, J., & Hochstein, P. (1977) *Biochem. Biophys. Res. Commun. 77*, 797–803.
Halliwell, B., & Gutteridge, J. M. C. (1989) *Free Radicals in Biology and Medicine*, Clarendon Press, Oxford.
Halliwell, B., & Aruoma, O. I. (1991) *FEBS Lett. 281*, 9–19.
Handa, K., & Sato, S. (1975) *Gann 66*, 43–47.
Houee-Levin, C., Gardes-Albert, M., Rouscilles, A., Ferradini, C., & Hickel, B. (1991) *Biochemistry 30*, 8216–8222.
Laemmli, U. K. (1970) *Nature 227*, 680–685.
Lown, J. W., Sim, S. K., & Chen, H. H. (1978) *Can. J. Biochem. 56*, 1042–1047.
Lown, J. W., Chen, H. H., Plambeck, J. A., & Acton, E. M. (1979) *Biochem. Pharmacol. 28*, 2563–2568.
MacGregor, C. R., & Caskey, C. T. (1989) *Nucleic Acids Res. 17*, 2365.
Mahler, H. R. (1955) *Methods Enzymol. 2*, 689–693.
Marquardt, H., Phillips, F. S., & Steinberg, S. S. (1976) *Cancer Res. 36*, 2065–2069.
Mimnaugh, E. G., Kennedy, K. A., Trush, M. A., & Sinha, B. K. (1985) *Cancer Res. 45*, 3296–3304.
Moriya, M., Ou, C., Bodepudi, V., Johnson, F., Takeshita, M., & Grollman, A. P. (1991) *Mutat. Res. 254*, 281–288.
Nackerdien, Z., Kasprzak, K. S., Rao, G., Halliwell, B., & Dizdaroglu, M. (1991) *Cancer Res. 51*, 5837–5842.
Pan, S. S., Pedersen, L., & Bachur, N. R. (1981) *Mol. Pharmacol. 19*, 184–186.
Pezzano, H., & Podo, F. (1980) *Chem. Rev. 80*, 366–401.
Pietronigro, D. D., McGinness, J. E., Koren, M. J., Crippa, R., Seligman, M. L., & Demopoulos, H. B. (1979) *Physiol. Chem. Phys. 11*, 405–414.
Repine, J. E., Eaton, J. W., Anders, M. W., Hoidal, J. R., & Fox, R. B. (1979) *J. Clin. Invest. 64*, 1642–1651.
Rowley, D. A., & Halliwell, B. (1983) *Biochim. Biophys. Acta 761*, 86–93.
Sagripanti, J.-L., & Kraemer, K. H. (1989) *J. Biol. Chem. 264*, 1729–1734.
Samuni, A., Aronovitch, J., Godinger, D., Chevion, M., & Czapski, G. (1983) *Eur. J. Biochem. 137*, 119–124.
Steenken, S. (1989) *Chem. Rev. 89*, 503–520.
Sugioka, K., & Nakano, M. (1982) *Biochim. Biophys. Acta 713*, 333–343.
Wood, M. L., Dizdaroglu, M., Gajewski, E., & Essigmann, J. M. (1990) *Biochemistry 29*, 7024–7032.
Yamamoto, K., & Kawanishi, S. (1989) *J. Biol. Chem. 264*, 15435–15440.
Young, R. C., Ozols, R. F., & Myers, C. E. (1981) *N. Engl. J. Med. 305*, 139–153.
Ziegenhorn, J., Senn, M., & Bucher, T. (1976) *Clin. Chem. 22*, 151–160.
Zweier, J. L. (1983) *J. Biol. Chem. 259*, 6056–6058.