

# Effect of Anthracycline Antibiotics on Oxygen Radical Formation in Rat Heart<sup>1</sup>

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## ABSTRACT

This investigation examined the effect of the anthracycline antitumor agents on reactive oxygen metabolism in rat heart. Oxygen radical production by doxorubicin, daunorubicin, and various anthracycline analogues was determined in heart homogenate, sarcoplasmic reticulum, mitochondria, and cytosol, the major sites of cardiac damage by the anthracycline drugs. Superoxide production in heart sarcosomes was significantly increased by anthracycline treatment; for doxorubicin, the reaction appeared to follow saturation kinetics with an apparent  $K_m$  of 112.62  $\mu\text{M}$ , required NADPH as cofactor, was accompanied by the accumulation of hydrogen peroxide, and probably resulted from the transfer of electrons to molecular oxygen by the doxorubicin semiquinone after reduction of the drug by sarcosomal NADPH:cytochrome P-450 reductase (NADPH:ferricytochrome oxidoreductase, EC 1.6.2.4). Superoxide formation was also significantly enhanced by the anthracycline antibiotics in the mitochondrial fraction. Doxorubicin stimulated mitochondrial superoxide formation in a dose-dependent manner that also appeared to follow saturation kinetics (apparent  $K_m$  of 454.55  $\mu\text{M}$ ); however, drug-related superoxide production by mitochondria required NADH rather than NADPH and was significantly increased in the presence of rotenone, which suggested that the proximal portion of the mitochondrial NADH dehydrogenase complex [NADH:(acceptor) oxidoreductase, EC 1.6.99.3] was responsible for the reduction of doxorubicin at this site. In heart cytosol, anthracycline-induced superoxide formation and oxygen consumption required NADH and were significantly reduced by allopurinol, a potent inhibitor of xanthine oxidase (xanthine:oxygen oxidoreductase, EC 1.2.3.2). Reactive oxygen production was detected in all of our studies despite the presence of both superoxide dismutase (superoxide:superoxide oxidoreductase, EC 1.15.1.1) and glutathione peroxidase (glutathione:hydrogen peroxide oxidoreductase, EC 1.11.1.9) in each cardiac fraction.

These results suggest that free radical formation by the anthracycline antitumor agents, which occurs in the same myocardial compartments that are subject to drug-induced tissue injury, may damage the heart by exceeding the oxygen radical detoxifying capacity of cardiac mitochondria and sarcoplasmic reticulum.

## INTRODUCTION

The anthracycline antitumor agents doxorubicin, daunorubi-

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cin, rubidazole, and others are among the most effective antineoplastic drugs currently available for the treatment of several different human cancers (7). Unfortunately, the clinical usefulness of these drugs has been diminished by their association with a potentially life-threatening form of cardiac toxicity (28) that in most circumstances limits cumulative drug dosage to the equivalent of 500 to 550 mg of doxorubicin per sq m (28).

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 drug intermediates *in vivo* (45). Handa and Sato (18), Goodman and Hochstein (17), and Bachur *et al.* (3-5) have established that hepatic microsomes support the transfer of electrons from NADPH to the quinone moiety of doxorubicin. This is probably due to an interaction between the anthracycline ring and the flavin component of microsomal NADPH:cytochrome P-450 reductase (24). Under anaerobic conditions, this interaction leads to the enzymatic cleavage of the anthracycline glycosidic bond (26). Aerobically, the anthracycline semiquinone may donate unpaired electrons to molecular oxygen, forming reactive oxygen radicals (such as superoxide anion, hydrogen peroxide, or others) that can critically disrupt a wide range of essential intracellular macromolecules (16, 49).

The significance of these observations is underscored by recent investigations suggesting that myocardial cells have a limited capacity to detoxify oxygen radicals enzymatically (12, 44, 49); hence, the heart may be particularly susceptible to injury from reactive oxygen species generated as a result of anthracycline administration. Conversely, enhancement of the endogenous defenses of the heart against oxygen radicals with free radical scavenging agents (such as *N*-acetylcysteine) may reduce the cardiac membrane damage that follows treatment with anthracycline drugs (11, 37, 41, 51).

To evaluate the role of oxygen radical formation in anthracycline cardiac toxicity further, we have attempted to quantitate the effect of these drugs on reactive oxygen metabolism in various subcellular fractions from rat heart. Our results indicate that drugs of the anthracycline class significantly increase both superoxide anion and hydrogen peroxide production in cardiac mitochondria, cytosol, and sarcoplasmic reticulum. Thus, the pathological picture of anthracycline cardiac toxicity characterized by disruption of heart mitochondrial and sarcoplasmic reticular membranes (15) may be explained by drug-induced free radical formation in specific myocardial compartments.

## MATERIALS AND METHODS

**Experimental Animals.** Male Sprague-Dawley rats weighing 180 to 200 g were obtained from Mission Laboratory Supply Co., Rosemead, Calif. From the time of weaning, these animals were maintained on a

diet of Wayne Lab-Blox rat pellets with water available *ad libitum*. Rat heart was chosen for study because the rat appears to develop both acute and chronic cardiac toxicity after anthracycline treatment that is similar in many respects to anthracycline cardiomyopathy in humans (35).

**Materials.** Doxorubicin hydrochloride of clinical grade, as well as chromatographically pure drug, was obtained from Adria Laboratories, Inc., Wilmington, Del. Daunorubicin, rubidazole, and 5-iminodaunorubicin were supplied by the Drug Synthesis and Chemistry Branch, Division of Cancer Treatment, National Cancer Institute, Bethesda, Md. Aclacinomycin A was generously supplied by Dr. W. T. Bradner, Bristol Laboratories, Syracuse, N. Y. Actinomycin D was obtained from Merck Sharp and Dohm, West Point, Pa. The drugs were reconstituted in sterile water unless indicated otherwise and protected from light until used. Glutathione (reduced form), glutathione reductase type III, sodium azide, ATP, MgCl<sub>2</sub>, bovine erythrocyte SOD,<sup>3</sup> [2900 units/mg as assayed by the method of McCord and Fridovich (33)], bovine albumin Fraction V, xanthine, xanthine oxidase (Grade 1), cytochrome *c* (type VI from horse heart), EDTA, NADPH type III, NADH Grade III, NADP<sup>+</sup>, NAD<sup>+</sup> Grade V, flavin adenine dinucleotide Grade III, flavin mononucleotide, sodium succinate, sucrose, D-mannitol, dimethyl sulfoxide Grade I, L-histidine, EGTA, sodium HEPES, DTNB, rotenone, dicumarol, D- $\alpha$ -tocopherol acid succinate type IV, D- $\alpha$ -tocopherol type I, AMP type III, and adenosine were purchased from Sigma Chemical Co., St. Louis, Mo. Deferoxamine mesylate was purchased from Ciba Pharmaceutical Co., Summit, N. J. Methanol (spectral grade), hydrogen peroxide (30% solution), ethyl alcohol (99% pure), potassium cyanide, sodium acetate, and acetic anhydride were obtained from Fisher Scientific Co., Fair Lawn, N. J. Chelex 100 resin (100 to 200 mesh, sodium salt) was purchased from Bio-Rad Laboratories, Richmond, Calif. Catalase (EC 1.11.1.6) of analytical grade (65,000 units/mg protein) was obtained from Boehringer Mannheim Biochemicals, Indianapolis, Ind., and was devoid of SOD activity when assayed by the method of McCord and Fridovich (33). Only glass-distilled, deionized water was used in these studies.

**Preparation of Rat Heart Subcellular Fractions.** Experimental animals were killed by cervical dislocation; the cardiac ventricles were excised, blotted dry, trimmed of connective tissue, and then minced into 20 to 30-mg replicates while being kept on melted ice. To prepare rat heart homogenate, the minced cardiac ventricles were vigorously washed free of erythrocytes with an iced solution of 225 mM mannitol and 75 mM sucrose, pH 7.4, containing 1 mM EGTA, 100  $\mu$ M D- $\alpha$ -tocopherol, and 1  $\mu$ g SOD per ml. The mannitol:sucrose:EGTA was treated with Chelex 100 resin before use to remove trace quantities of iron salts in the buffer. The ventricles were homogenized at 4° for 10 sec in 4 volumes of the mannitol:sucrose:EGTA buffer that contained tocopherol and SOD with a Brinkmann Model PCU-2-110 Polytron (Brinkmann Instruments, Inc., Westbury, N. Y.). We have shown previously that this procedure leads to minimal contamination of the homogenate by erythrocyte-derived proteins (12). The rat heart homogenate was centrifuged at 4° and 480  $\times$  *g* for 5 min to remove nuclear and myofibrillar debris, and the resulting supernatant was used without further modification as the crude homogenate fraction.

To prepare heart sarcosomes, the minced cardiac ventricles were vigorously washed with an iced solution of 100 mM KCl containing 5 mM histidine, pH 7.3, and were then homogenized with the Polytron at 4° for 2 min in 4 volumes of the KCl:histidine buffer. For certain studies, the homogenization was performed in KCl:histidine buffer that contained 100  $\mu$ M D- $\alpha$ -tocopherol and 1  $\mu$ g SOD per ml and that had been treated with Chelex to decrease the potential for membrane peroxidation during the homogenization process. The sarcosomal fraction was obtained by differential ultracentrifugation of the tissue homogenate as described by Martonosi (32) and was resuspended before use in 150

mM potassium phosphate buffer, pH 7.4, containing 100  $\mu$ M EDTA. Where indicated, heart sarcoplasmic reticulum was also prepared by the method described by Harigaya and Schwartz (19).

To produce the rat cardiac mitochondrial fraction, the minced heart muscle was vigorously washed 4 times with an iced solution of 225 mM mannitol and 75 mM sucrose containing 1 mM EGTA and then homogenized for 10 sec with the Polytron on ice in 10 volumes of iced mannitol:sucrose:EGTA. Where specified, the cardiac tissue was homogenized in iced mannitol:sucrose:EGTA that had been treated with Chelex and that contained 100  $\mu$ M D- $\alpha$ -tocopherol and 1  $\mu$ g SOD per ml. The heart mitochondrial fraction was prepared from the homogenate by a technique described previously (34) and was resuspended before use in 250 mM sucrose:20 mM HEPES buffer, pH 7.4. Where indicated, the mitochondrial fraction was frozen and thawed 3 times in a dry ice-methanol mixture to ensure membrane disruption prior to assays for oxygen radical formation.

To prepare rat heart cytosol, the minced ventricles were washed 4 times in 250 mM sucrose containing 1 mM EDTA and then homogenized on ice with the Polytron for 2 min in 4 volumes of iced sucrose:EDTA. The homogenate was centrifuged at 4° and 1000  $\times$  *g* for 20 min to eliminate nuclei and membranous debris; the resulting supernatant was centrifuged subsequently at 8000  $\times$  *g* for 20 min to remove cardiac mitochondria. The postmitochondrial supernatant was then centrifuged for 1 hr at 105,000  $\times$  *g* and 4° in a Beckman Model L5-50 ultracentrifuge (Beckman Instruments, Inc., Palo Alto, Calif). The final supernatant was decanted at the end of ultracentrifugation and was used directly as the cardiac cytosol fraction.

The experimental heart homogenate, as well as the sarcosomal, mitochondrial, or cytosolic fractions were used on the day of preparation.

**Superoxide Assay.** Superoxide anion production in experimental samples was determined by the rate of SOD-inhibitable acetylated cytochrome *c* reduction. The cytochrome *c* utilized was acetylated before use, as described by Azzi *et al.* (2), to eliminate interfering reactions by cytochrome *c* oxidases or reductases in the cardiac sarcosomal, mitochondrial, or cytosolic fractions. The initial, linear rate of acetylated cytochrome *c* reduction was determined spectrophotometrically at 550 nm and 37° in a Gilford Model 250 recording spectrophotometer (Gilford Instrument Laboratories, Inc., Oberlin, Ohio) equipped with a circulating water bath. Superoxide formation was calculated from the rate of acetylated cytochrome *c* reduction that was inhibited by SOD using an extinction coefficient for cytochrome *c* (reduced minus oxidized) of 19.6 mM<sup>-1</sup> cm<sup>-1</sup> (55). For experiments assessing the effect of DTNB on superoxide production, the sulfhydryl reagent was added to the paired reaction mixtures which were then preincubated for 2 min at 37° before the addition of NADPH. Preincubation was not performed in experiments examining the effect of other agents on the rate of superoxide formation.

Superoxide formation in the rat heart mitochondrial fraction was examined in a fashion similar to that for heart sarcosomes as described in Table 7. Chemotherapeutic agents were added, where indicated, before initiation with NADH. Superoxide production in rat cardiac cytosol was determined as described in Table 11.

**Measurement of NADPH Consumption.** The oxidation of NADPH by heart sarcosomes was determined in triplicate at 37° by the initial, linear change in optical density at 340 nm using the Gilford spectrophotometer. The 1-ml reaction mixture contained 150  $\mu$ mol potassium phosphate buffer, pH 7.4, 100 nmol EDTA, 200  $\mu$ g sarcosomal protein, 100 nmol NADPH, and the indicated amount of anthracycline. NADPH consumption was initiated by the addition of the sarcosomal protein and was calculated using an extinction coefficient of 6.22 mM<sup>-1</sup> cm<sup>-1</sup> (20).

**Oxygen Consumption Measurements in Cardiac Subcellular Fractions.** Oxygen consumption was measured at 37° with a YSI Model 53 oxygen-monitoring system (Yellow Springs Instrument Co., Yellow Springs, Ohio). Oxygen consumption by the crude rat heart homogenate was determined in a 3-ml reaction mixture that contained 750

<sup>3</sup> The abbreviations used are: SOD, superoxide dismutase; EGTA, ethyleneglycolbis( $\beta$ -aminoethylether)-*N,N'*-tetraacetic acid; HEPES, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; DTNB, 5,5'-dithiobis(2-nitrobenzoic acid).

$\mu\text{mol}$  sucrose,  $60 \mu\text{mol}$  HEPES, pH 7.4,  $300 \text{ nmol}$  EDTA,  $15 \mu\text{mol}$  KCN,  $10 \text{ mg}$  homogenate protein,  $3 \mu\text{mol}$  either NADH or NADPH, and, where indicated,  $600 \text{ nmol}$  doxorubicin. All reactants were bubbled with air for 30 min at  $37^\circ$  before use.

For the determination of oxygen consumption by cardiac sarcosomes, the 3-ml reaction system contained  $450 \mu\text{mol}$  potassium phosphate buffer (pH 7.4),  $300 \text{ nmol}$  EDTA, and  $1.5 \text{ mg}$  sarcosomal protein. After equilibration of buffers and sarcosomes for 4 min in the reaction vessel,  $3 \mu\text{mol}$  NADPH were added to initiate the reaction; the electrode was then inserted, and the linear control rate of oxygen consumption was determined for 10 min thereafter. In experiments with chemotherapeutic agents,  $405 \text{ nmol}$  of drug were added with the sarcosomes. In some studies, small volumes of specific reagents (typically 5 to  $10 \mu\text{l}$ ) were added to the reaction vessel using a Hamilton syringe introduced into the test chamber through the access slot of the oxygen electrode plunger. The rate of oxygen consumption was based on a value of  $597 \text{ nmol}$  for the total dissolved oxygen content of the reaction mixture (8).

Oxygen consumption in the rat heart mitochondrial fraction and rat heart cytosol was determined in a fashion similar to that for heart sarcosomes as described in Tables 10 and 12.

**Enzyme Assays.** The NADPH:cytochrome P-450 reductase activity of rat heart sarcosomes was measured by a technique described previously (48) using nonacetylated cytochrome *c* as the electron acceptor. To assess the effect of DTNB ( $100 \mu\text{M}$ ) and  $\text{NADP}^+$  ( $1 \text{ mM}$ ) on NADPH:cytochrome P-450 reductase activity, these reagents were preincubated with the sarcosomes for 2 min before the addition of NADPH.

Glutathione peroxidase activity was determined in rat heart subcellular fractions as described previously (12, 42), except that enzyme assays were initiated by  $440$  rather than  $220 \text{ nmol}$  hydrogen peroxide in these experiments. Glutathione peroxidase activity in heart cytosol was assayed using a protein concentration of  $80 \mu\text{g/ml}$  in the final reaction mixture. The heart sarcosomal fraction was resuspended after preparation in  $250 \text{ mM}$  sucrose:  $1 \text{ mM}$  EDTA, rather than potassium phosphate buffer, for the determination of glutathione peroxidase activity. Glutathione peroxidase activity was determined using  $400$  to  $500 \mu\text{g}$  sarcosomal protein per ml. Glutathione peroxidase activity in the mitochondrial fraction was measured using  $200 \mu\text{g}$  protein per ml; before the enzyme assay, rat heart mitochondria were disrupted by sonication at  $90 \text{ watts}$  with a Biosonik IV Sonifier equipped with a microtip. Sonication for 1 min was carried out in 4 cycles of 15 sec each with cooling of the mitochondria on melted ice between each cycle. Where indicated, the sonicated mitochondrial fraction was centrifuged for 60 min at  $105,000 \times g$  and  $4^\circ$ ; the resulting supernatant at a concentration of  $80 \mu\text{g}$  protein per ml and the pellet containing mitochondrial membranes ( $200 \mu\text{g}$  protein per ml) were also assayed for glutathione peroxidase activity. The data have been expressed as  $\text{nmol}$  NADPH oxidized to  $\text{NADP}^+$  per min per mg protein using the extinction coefficient for NADPH of  $6.22 \text{ mm}^{-1} \text{ cm}^{-1}$  (20).

Cardiac SOD levels were determined in the sarcosomal, mitochondrial, and cytosolic fractions using the xanthine:xanthine oxidase: cytochrome *c* assay as reported previously (12). In these experiments, acetylated cytochrome *c* ( $11.2 \mu\text{M}$ ) was utilized in addition to KCN ( $10 \mu\text{M}$ ) to eliminate interference from cytochrome oxidases in the experimental samples. The protein concentrations of the heart fractions assayed for SOD activity were adjusted to obtain approximately 50% inhibition of the rate of cytochrome *c* reduction produced by the xanthine:xanthine oxidase system. For heart cytosol, this required 15 to  $20 \mu\text{g}$  protein per ml. We used  $200 \mu\text{g}$  sarcosomal protein per ml and 40 to  $50 \mu\text{g}$  mitochondrial protein per ml to produce approximately half-maximal inhibition of the rate of cytochrome *c* reduction in this system. SOD activity in heart mitochondria was determined after sonication, as described for glutathione peroxidase, to remove permeability barriers for appropriate substrates in the assay. The SOD activity of sonicated mitochondria fractionated by ultracentrifugation was also examined. The effect of KCN ( $1 \text{ mM}$ ) on mitochondrial SOD levels was investigated after incubating mitochondrial preparations with this agent

for 15 min at  $25^\circ$ ; following incubation, these samples were appropriately diluted in buffer and assayed as described above.

**Protein Determination.** Protein concentrations in subcellular heart fractions were determined by the method of Lowry *et al.* (31) using crystalline bovine albumin as the standard.

**Statistical Methods.** Data were analyzed with the 2-tailed *t* test for independent means [not significant,  $p > 0.05$  (1)].

## RESULTS

### Rat Heart Crude Homogenate

**Effect of Doxorubicin on Oxygen Consumption by the Rat Heart Homogenate.** To examine the impact of doxorubicin on rat heart oxygen radical metabolism, we investigated the effect of the drug on oxygen consumption by a crude heart homogenate. In the presence of KCN to inhibit cytochrome oxidase, the control rate of oxygen consumption by the heart homogenate was  $0.99 \pm 0.13$  (S.E.)  $\text{nmol/min/mg}$  protein with NADH as cofactor ( $n = 4$ ) and  $1.65 \pm 0.01 \text{ nmol/min/mg}$  using NADPH ( $n = 3$ ). In the presence of chromatographically homogeneous doxorubicin ( $200 \mu\text{M}$ ), oxygen consumption by the homogenate fraction was significantly increased with either cofactor; the rate of oxygen consumption using NADH was  $4.51 \pm 0.71 \text{ nmol/min/mg}$  ( $n = 4$ ,  $p < 0.01$ ), and the rate with NADPH as cofactor was  $5.64 \pm 0.44 \text{ nmol/min/mg}$  ( $n = 4$ ,  $p < 0.01$ ). Because doxorubicin enhanced oxygen consumption by rat heart homogenate prepared with tocopherol and SOD, it is unlikely that membrane peroxidation during the homogenation process itself could account for these findings. We also found that denaturation of the homogenate by heating in a boiling water bath for 30 min decreased the rate of oxygen consumption in the doxorubicin-treated samples to control levels; the rate with NADH was  $1.41 \pm 0.11 \text{ nmol/min/mg}$  ( $n = 3$ ,  $p < 0.01$  compared to unheated samples), and the rate with NADPH was  $1.01 \pm 0.03 \text{ nmol/min/mg}$  ( $n = 3$ ,  $p < 0.01$  compared to unheated samples).

### Rat Heart Sarcoplasmic Reticulum

**Effect of Anthracycline Antibiotics on Superoxide Formation in Heart Sarcosomes.** To examine the hypothesis that free radical formation may play a role in anthracycline cardiac toxicity, we investigated the effect of anthracycline antitumor agents on oxygen radical formation by rat heart sarcoplasmic reticulum, a major intracellular site of drug-induced cardiac injury (15). In these experiments, we used a range of drug concentrations that approximated both the peak level of doxorubicin in rat plasma after i.v. drug administration (54) and the apparent  $K_m$  for the activation of doxorubicin to its free radical by hepatic microsomes (3). One of several representative experiments is shown in Chart 1. The addition of NADPH to rat cardiac sarcosomes produced a limited degree of acetylated cytochrome *c* reduction that was partially inhibited by SOD (Chart 1A). In the presence of doxorubicin, however, SOD-inhibitable acetylated cytochrome *c* reduction was substantially increased (Chart 1B). Doxorubicin enhanced superoxide production by heart sarcosomes in a concentration-related fashion that appeared to follow saturation kinetics (Chart 2). Kinetic constants were determined for doxorubicin from a Lineweaver-Burk plot of the data (Chart 2, *inset*); and the apparent  $K_m$  and  $V_{\text{max}}$  were found to be  $112.62 \mu\text{M}$  and  $13.25$

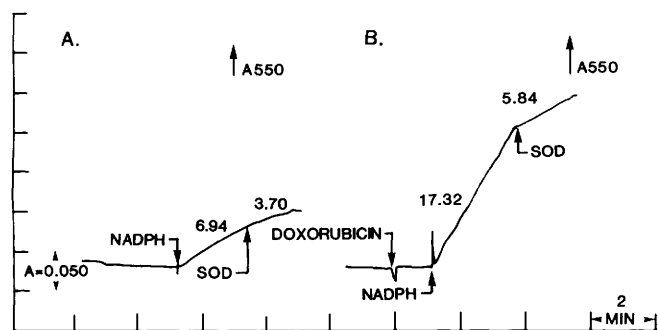


Chart 1. Effect of doxorubicin on acetylated cytochrome c reduction by cardiac sarcosomes. The sequential additions of NADPH (1  $\mu$ mol), doxorubicin (135 nmol), or SOD (10  $\mu$ g) have each been indicated by arrows. The numbers above the tracings represent the rate of acetylated cytochrome c reduction in nmol/min/mg protein. A, control reaction; B, cytochrome c reduction in the presence of doxorubicin.

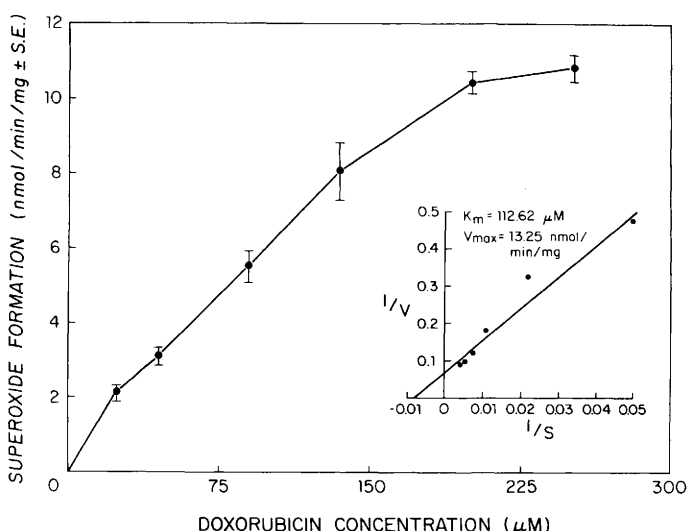


Chart 2. Effect of doxorubicin dose on superoxide anion production in rat heart sarcoplasmic reticulum and a Lineweaver-Burk plot for doxorubicin (inset). Points, mean of at least 3 experiments for each dose of doxorubicin; bars, S.E.

nmol/min/mg, respectively. In these experiments, superoxide production by doxorubicin varied with the amount of sarcosomal protein used; at a doxorubicin concentration of 135  $\mu$ M, superoxide formation increased from ( $n = 3$ )  $1.03 \pm 0.21$  nmol/min to  $1.49 \pm 0.26$ ,  $1.70 \pm 0.29$ , and  $2.35 \pm 0.39$  nmol/min when 100, 200, 300, or 500  $\mu$ g of sarcosomal protein per ml were used in the assay. Superoxide production was abolished after the heart sarcosomes were denatured by heat (Table 1); and as shown in Table 1, all components of this experimental system, including acetylated cytochrome c, NADPH, heart sarcosomes, and anthracycline drug, were necessary to demonstrate a significant enhancement of superoxide formation by doxorubicin. As demonstrated in Table 1, we found that sarcosomal superoxide production was not diminished by the potent iron chelating agent deferoxamine or by the use of chromatographically pure doxorubicin. These results strongly suggest that the superoxide production measured in this system is not a byproduct of iron contamination or of homogenization-related membrane damage. Furthermore, superoxide production was not enhanced by an energy source in the form of ATP and  $MgCl_2$  (Table 1). The cofactor requirement for oxygen radical production in our sarcosomal preparation

was specific; only NADPH could support drug-related cytochrome c reduction in these studies (Table 2).

We also performed experiments to assess the specificity of our acetylated cytochrome c assay for the quantitation of drug-induced superoxide formation. We found that SOD-inhibitable cytochrome c reduction was not affected by dimethyl sulfoxide, a potent hydroxyl radical scavenger, or by catalase, used in a concentration sufficient to eliminate hydrogen peroxide from the reaction mixture (Table 1). Furthermore, the addition of heat-denatured SOD to the experimental system produced no significant change in the rate of drug-stimulated superoxide production (Table 1), strongly suggesting that superoxide was measured in our studies. We found that at a constant drug dose, the apparent rate of superoxide production increased with the amount of cytochrome c used in the assay system; however, the rate of drug-related superoxide production by heart sarcosomes was maximal at an acetylated cytochrome c concentration of 56  $\mu$ M and did not increase further when

Table 1  
Requirements for doxorubicin-stimulated superoxide formation in the rat heart sarcosomal fraction

Superoxide production in heart sarcosomes was determined in paired, 1-ml reaction mixtures containing 150  $\mu$ M of potassium phosphate buffer, pH 7.4, 100 nmol of EDTA, 56 nmol of acetylated cytochrome c, 200  $\mu$ g of sarcosomal protein, and either 0 or 10  $\mu$ g of SOD. The chemotherapeutic agent was added to the paired reaction mixtures, where specified, before the initiation of the reaction by addition of 1  $\mu$ mol of NADPH.

Experimental system	Superoxide production (nmol cytochrome c reduced/min/mg)	n <sup>a</sup>
Control	1.39 $\pm$ 0.15 <sup>b</sup>	12
- cytochrome c	0.00 $\pm$ 0.00	3
- NADPH	0.00 $\pm$ 0.00	3
- sarcosomes	0.22 $\pm$ 0.04	3
Using heat-denatured sarcosomes <sup>c</sup>	0.00 $\pm$ 0.00	3
Using heat-denatured SOD <sup>c</sup>	1.07 $\pm$ 0.25	3
+ catalase (3000 units)	1.53 $\pm$ 0.51	3
+ dimethyl sulfoxide (13 $\mu$ mol)	1.66 $\pm$ 0.08	3
Using sarcosomes prepared with tocopherol and SOD	1.48 $\pm$ 0.31	4
Doxorubicin (135 nmol)	9.03 $\pm$ 0.68 <sup>d</sup>	8
- cytochrome c	0.00 $\pm$ 0.00	3
- NADPH	0.00 $\pm$ 0.00	3
- sarcosomes	0.50 $\pm$ 0.04	3
Using heat-denatured sarcosomes	0.00 $\pm$ 0.00	3
Using heat-denatured SOD	7.60 $\pm$ 0.33 <sup>e</sup>	3
+ catalase (3000 units)	8.26 $\pm$ 0.41 <sup>e</sup>	3
+ dimethyl sulfoxide (13 $\mu$ mol)	7.85 $\pm$ 0.69 <sup>e</sup>	3
Doxorubicin (135 nmol) <sup>f</sup>	13.97 $\pm$ 0.97 <sup>g</sup>	6
+ deferoxamine (100 nmol) <sup>h</sup>	12.62 $\pm$ 0.13 <sup>g</sup>	3
- EDTA	13.77 $\pm$ 0.26 <sup>g</sup>	3
- EDTA + deferoxamine (100 nmol)	13.18 $\pm$ 0.33 <sup>g</sup>	3
- EDTA + deferoxamine (100 nmol) + $MgCl_2$ (4 $\mu$ mol) + ATP (1 $\mu$ mol)	13.01 $\pm$ 0.26 <sup>g</sup>	3

<sup>a</sup> Number of experiments.  
<sup>b</sup> Mean  $\pm$  S.E. of the rate of superoxide production in the rat heart sarcosomal fraction.  
<sup>c</sup> Sarcosomes or SOD heated for 60 min in a boiling water bath; samples containing heat-denatured SOD were paired against identical mixtures with native dismutase.  
<sup>d</sup> Significantly different from control ( $p < 0.001$ ).  
<sup>e</sup> No significant difference between results from samples containing heat-denatured SOD, catalase, or dimethyl sulfoxide and those obtained using doxorubicin alone.  
<sup>f</sup> Doxorubicin used in these experiments was preservative free and chromatographically homogeneous.  
<sup>g</sup> Sarcosomes for these experiments were prepared with tocopherol and SOD; and the potassium phosphate buffer used had undergone ion-exchange chromatography to remove trace amounts of iron.  
<sup>h</sup> Deferoxamine was added before the EDTA.

Table 2

Cofactor requirements for doxorubicin-stimulated superoxide production in rat cardiac sarcosomes

Superoxide production in the rat heart sarcosomal fraction was determined as described in "Materials and Methods." For these experiments, doxorubicin was included in the reaction mixtures (135 nmol), and superoxide formation was initiated by the addition of the cofactor. For these studies, the cofactor concentration was 1 mM.

Cofactor	Superoxide formation (nmol cytochrome c reduced/min/mg)	n <sup>a</sup>
NADPH	8.85 ± 0.82 <sup>b</sup>	3
NADP <sup>+</sup>	0.43 ± 0.05	3
NADH	0.82 ± 0.36	8
NAD <sup>+</sup>	0.33 ± 0.09	3
FMN <sup>c</sup>	0.69 ± 0.13	3
FAD	0.26 ± 0.11	3
Adenosine	0.49 ± 0.08	3

<sup>a</sup> Number of experiments.

<sup>b</sup> Mean ± S.E. of the rate of doxorubicin-enhanced superoxide formation in the sarcosomal fraction.

<sup>c</sup> FMN, flavin mononucleotide; FAD, flavin adenine dinucleotide.

higher cytochrome c concentrations were examined (data not shown). Furthermore, we determined that the inhibition of cytochrome c reduction by SOD did not increase above a dismutase concentration of 10 μg/ml; thus, this enzyme concentration was chosen for all experimental studies (data not shown). Finally, we examined the rate of drug-induced superoxide production in rat heart sarcoplasmic reticulum prepared by an alternate technique described previously (19). Using sarcosomes prepared by this method, the addition of doxorubicin (135 μM) significantly increased superoxide formation from the control rate (n = 4) of 1.56 ± 0.38 nmol/min/mg to 5.20 ± 0.33 nmol/min/mg (n = 4, p < 0.001). These studies suggested that rat heart sarcosomes, which are known to contain sarcoplasmic reticular membranes (19), are capable of supporting oxygen radical formation that is significantly increased in the presence of doxorubicin.

Because various anthracycline antibiotics in addition to doxorubicin are known to produce cardiac toxicity in humans (28) and in experimental animals (9), superoxide production by a series of anthracycline drugs was investigated in heart sarcosomes (Table 3). At equimolar concentrations daunorubicin, rubidazole, and aclacinomycin A increased superoxide production 5 to 9.5 times above control levels, p < 0.01. On the other hand, 5-iminodaunorubicin, a daunorubicin analogue in which a nitrogen has been substituted for one of the quinone oxygen atoms, did not stimulate superoxide production in heart sarcosomes. Finally, actinomycin D, a nonanthracycline antitumor antibiotic that may enhance the cardiac toxicity of doxorubicin (27), produced a small but statistically significant increase in the rate of cardiac superoxide formation.

In an attempt to determine the mechanism of doxorubicin-enhanced oxygen radical formation in the heart, we measured the rate of NADPH consumption in cardiac sarcosomes after treatment with the anthracycline drug. Doxorubicin stimulated NADPH oxidation in a dose-related manner; NADPH consumption (n = 4) increased from the 2.29 ± 0.28 nmol/min/mg control level to 4.28 ± 0.10, 7.02 ± 0.31, 9.26 ± 0.63, and 13.02 ± 0.71 nmol/min/mg after treatment of the sarcosomes with 20, 45, 90, or 135 μM doxorubicin, respectively. At each drug concentration tested, the rate of NADPH consumption was significantly greater than control levels (p < 0.001). For comparison, we also examined the ability of 5-iminodaunorubicin to enhance sarcosomal NADPH consumption. At concen-

trations of 45, 90, and 135 μM, this drug produced no significant increase over control levels in the rate of NADPH oxidation by heart sarcosomes. We also found that rat heart sarcosomes contained substantial NADPH:cytochrome P-450 reductase activity (Table 4). When the sarcosomal preparation was treated with agents known to inhibit the activity of NADPH:cytochrome P-450 reductase [NADP<sup>+</sup> and DTNB (48)], enzyme activity decreased to 38.8% of control levels with DTNB, p < 0.001, and to 46.2% of control activity in presence of excess NADP<sup>+</sup> (p < 0.001) (Table 4). The effect of the cytochrome P-450 reductase inhibitors on superoxide formation paralleled their action on sarcosomal P-450 reductase activity; doxorubicin-enhanced superoxide production was 39% of control levels after treatment of the sarcosomes with the sulfhydryl reagent DTNB, p < 0.05, and 38.4% of control levels when excess NADP<sup>+</sup> was added to the experimental system, p < 0.01 (Table 4). Taken together, these experiments suggested that doxorubicin significantly increased the transfer of electrons from NADPH to molecular oxygen by the cytochrome P-450 reductase of cardiac sarcoplasmic reticulum.

It has been reported recently that 2 chemicals, adenosine

Table 3

Effect of anticancer agents on superoxide production by the rat cardiac sarcosomal fraction

Superoxide production in rat heart sarcosomes was determined as described in "Materials and Methods." For these studies, all drugs were present at a concentration of 135 μM.

Drug	Superoxide formation (nmol cytochrome c reduced/min/mg)	n <sup>a</sup>
Daunorubicin	9.06 ± 0.39 <sup>b, c</sup>	3
Rubidazole	7.22 ± 0.94 <sup>c</sup>	3
Aclacinomycin A	13.18 ± 0.46 <sup>c</sup>	3
5-Iminodaunorubicin	1.96 ± 0.43 <sup>d</sup>	3
Actinomycin D	2.47 ± 0.31 <sup>c</sup>	3

<sup>a</sup> Number of experiments.

<sup>b</sup> Mean ± S.E.

<sup>c</sup> Significantly higher than control rate of superoxide formation in heart sarcosomes (p < 0.01; Table 1).

<sup>d</sup> Significantly different from doxorubicin-enhanced rate of superoxide formation in heart sarcosomes (p < 0.001; Table 1).

Table 4

Effect of inhibitors of NADPH-cytochrome P-450 reductase on superoxide formation in heart sarcosomes

NADPH:cytochrome P-450 reductase was assayed at 30° as described in "Materials and Methods" using nonacetylated cytochrome c and 200 μg sarcosomal protein per ml. Reactions were initiated with 100 nmol NADPH. Where indicated, the reaction mixtures were preincubated with DTNB or NADP<sup>+</sup> for 2 min prior to the initiation of cytochrome c reduction. In these experiments, superoxide production was assessed as described in "Materials and Methods" except that 100 nmol rather than 1 μmol of NADPH were used.

Experimental system	NADPH:cytochrome P-450 reductase activity (nmol cytochrome c reduced/min/mg)	Superoxide formation (nmol SOD-inhibitable acetylated cytochrome c reduced/min/mg)
Control	15.12 ± 0.61 <sup>a</sup> (4) <sup>b</sup>	0.56 ± 0.13 (4)
+ DTNB (100 nmol)	5.87 ± 0.31 (4) <sup>c</sup>	0.38 ± 0.13 (3)
+ NADP <sup>+</sup> (1 μmol)	6.99 ± 0.66 (4) <sup>c</sup>	0.26 ± 0.08 (3)
Doxorubicin (135 nmol)		5.23 ± 0.56 (4)
+ DTNB (100 nmol)		2.04 ± 0.76 (3) <sup>d</sup>
+ NADP <sup>+</sup> (1 μmol)		2.01 ± 0.01 (3) <sup>e</sup>

<sup>a</sup> Mean ± S.E.

<sup>b</sup> Numbers in parentheses, number of experiments.

<sup>c</sup> Significantly different from control (p < 0.001).

<sup>d</sup> Significantly different from experiments using doxorubicin alone (p < 0.05).

<sup>e</sup> Significantly different from experiments using doxorubicin alone (p < 0.01).

Table 5

Effect of tocopherol on superoxide formation and NADPH oxidation in the rat heart sarcosomal fraction  
Superoxide formation and NADPH oxidation were determined spectrophotometrically at 37° as described in "Materials and Methods."

Tocopherol used	Reaction system	Superoxide formation (nmol cytochrome c reduced/min/mg)	n <sup>a</sup>	NADPH oxidation (nmol/min/mg)	n
D- $\alpha$ -Tocopherol acid succinate <sup>b</sup>	Control	1.15 $\pm$ 0.13 <sup>c</sup>	6	4.29 $\pm$ 0.23 <sup>d</sup>	6
	+ tocopherol (100 $\mu$ M)			3.42 $\pm$ 0.31 <sup>d</sup>	4
	+ tocopherol (200 $\mu$ M)	0.66 $\pm$ 0.46 <sup>c</sup>	3		
	+ SOD (10 $\mu$ g/ml)			5.91 $\pm$ 0.39 <sup>d</sup>	3
	Doxorubicin (135 $\mu$ M)	9.26 $\pm$ 0.28 <sup>c</sup>	6	10.16 $\pm$ 0.47 <sup>d</sup>	6
	+ tocopherol (100 $\mu$ M)			4.23 $\pm$ 0.31 <sup>d, e</sup>	5
D- $\alpha$ -Tocopherol <sup>f</sup>	+ tocopherol (200 $\mu$ M)	4.36 $\pm$ 0.66 <sup>c, e</sup>	6		
	+ SOD (10 $\mu$ g/ml)			8.79 $\pm$ 0.71 <sup>d</sup>	3
	Control <sup>g</sup>	1.73 $\pm$ 0.69	3		
	+ tocopherol <sup>g</sup> (200 $\mu$ M)	1.56 $\pm$ 0.36	3		
	Doxorubicin (135 $\mu$ M) <sup>g</sup>	7.32 $\pm$ 0.31	3		
	+ tocopherol <sup>g</sup> (200 $\mu$ M)	3.88 $\pm$ 0.41 <sup>e</sup>	7		

<sup>a</sup> Number of experiments.

<sup>b</sup> D- $\alpha$ -Tocopherol succinate in absolute ethanol hydrolyzed with 2 molar equivalents of KOH.

<sup>c</sup> Mean  $\pm$  S.E. of the rate of superoxide formation; absolute ethanol concentration 2%.

<sup>d</sup> Mean  $\pm$  S.E. of the rate of NADPH oxidation; absolute ethanol concentration 1%.

<sup>e</sup> Significantly different from the rate of drug-treated samples without tocopherol ( $p < 0.01$ ).

<sup>f</sup> D- $\alpha$ -Tocopherol prepared in dimethyl sulfoxide.

<sup>g</sup> Dimethyl sulfoxide concentration, 280 mM in all experiments.

and  $\alpha$ -tocopherol, are capable of reducing the cardiac toxicity of doxorubicin under certain experimental conditions (39, 51). Thus, we investigated the effect of these agents on superoxide production in heart sarcosomes. Sarcosomal superoxide production in the presence of 135  $\mu$ M doxorubicin and 100  $\mu$ M NADPH was 5.71  $\pm$  0.23 nmol/min/mg,  $n = 3$ . After the addition of 1 or 10  $\mu$ mol AMP (the intracellular product of exogenous adenosine administration) to this experimental system, superoxide production was 6.38  $\pm$  0.18 nmol/min/mg ( $n = 5$ , not significant) or 1.63  $\pm$  0.31 nmol/min/mg ( $n = 3$ ,  $p < 0.001$ ), respectively. Thus, the cardioprotective role of adenosine might be mediated by the enhancement of intracellular AMP content, perhaps because AMP is a known inhibitor of NADPH:cytochrome P-450 reductase (53).

As shown in Table 5,  $\alpha$ -tocopherol also significantly reduced drug-related superoxide formation in heart sarcoplasmic reticulum. Using either natural D- $\alpha$ -tocopherol or D- $\alpha$ -tocopherol succinate, doxorubicin-stimulated superoxide formation in heart sarcosomes was reduced by 50% in the presence of 200  $\mu$ M tocopherol. A reduction in anthracycline-enhanced oxygen radical formation by tocopherol has been suggested to underlie the mechanism of cardioprotective activity of tocopherol in experimental animal models of doxorubicin cardiac toxicity (37, 51). However, in our sarcosomal system,  $\alpha$ -tocopherol also reduced the rate of NADPH oxidation by more than 58%,  $p < 0.01$ , whereas SOD, the most potent scavenger of superoxide anion known (16), had no significant effect on the rate of drug-induced NADPH consumption (Table 5). Finally, tocopherol (1 mM) had no effect on the base-line absorbance of the experimental concentrations of cytochrome *c* or NADPH used in these studies when examined at 550 and 340 nm, respectively. Thus, the direct oxygen radical scavenging actions of tocopherol which can be demonstrated under specific experimental conditions (40) may not explain the inhibitory effect of tocoph-

erol on sarcosomal superoxide formation that we found in these investigations.

**Effect of Anthracycline Antibiotics on Oxygen Consumption in Heart Sarcosomes.** To confirm that the anthracycline antibiotics stimulated reactive oxygen metabolism by cardiac sarcoplasmic reticulum, we examined the effect of these drugs on the rate of oxygen consumption by heart sarcosomes. We also investigated the effect of a variety of chemical modifiers on drug-enhanced free radical formation. The addition of doxorubicin (135  $\mu$ M) to heart sarcosomes increased the rate of oxygen consumption by more than 5 nmol/min/mg ( $p < 0.01$ ) (Table 6). It is probable that a substantial part of this increase in sarcosomal oxygen consumption was due to the stimulation of superoxide formation demonstrated previously. We found that treatment of the sarcosomes with sodium azide (a potent inhibitor of catalase) or potassium cyanide (which inhibits both catalase and SOD) significantly enhanced the apparent rate of drug-related oxygen consumption in the sarcosomal preparation (Table 6). This suggested that oxygen radical metabolism in heart sarcosomes may, at least partially, be diminished by the antioxidant enzyme activities associated with the subcellular fraction used for study. Conversely, it would also appear that the cardiac free radical formation that is stimulated by doxorubicin can exceed the detoxifying capacity of the sarcosomal antioxidant enzyme system.

As shown in Table 6, dicumarol had no effect on sarcosomal oxygen consumption; hence, the enzyme DT diaphorase [NAD(P)H:(quinone-acceptor) oxidoreductase, EC 1.6.99.2] which is exquisitely sensitive to inhibition by dicumarol (14) may not be involved in the activation of anthracycline drugs to free radicals in rat heart sarcosomes. On the other hand, when cytochrome *c* was present in the reaction mixture before initiation with NADPH, the apparent rate of sarcosomal oxygen consumption was significantly reduced (Table 6); this may be

Table 6

Effect of doxorubicin on oxygen consumption by the rat heart sarcosomal fraction

Oxygen consumption in rat heart sarcosomes was examined as described in "Materials and Methods." For these experiments, doxorubicin and the other reactants were equilibrated in the 3-ml reaction vessel for 4 min, and then oxygen consumption was initiated by the addition of 3 μmol of NADPH.

Reaction system	Oxygen consumption (nmol O <sub>2</sub> /min/mg)	n <sup>a</sup>
Control	10.31 ± 0.48 <sup>b</sup>	19
+ sodium azide (3 μmol)	9.75 ± 0.20	3
+ KCN (3 μmol)	7.56 ± 0.40	3
Doxorubicin (405 nmol)	15.40 ± 0.60 <sup>c</sup>	25
+ sodium azide (3 μmol)	17.23 ± 0.27 <sup>d</sup>	3
+ KCN (3 μmol)	20.98 ± 0.28 <sup>e</sup>	3
+ dicumarol (30 nmol)	16.91 ± 0.60	3
+ adenosine (3 μmol)	16.95 ± 0.48	3
+ acetylated cytochrome c (168 nmol)	12.61 ± 0.67 <sup>f</sup>	3
+ tocopherol <sup>g</sup>		
600 nmol	11.94 ± 0.24 <sup>f</sup>	3
3 μmol	8.76 ± 1.59 <sup>f</sup>	3
5-Iminodaunorubicin (405 nmol)	10.43 ± 0.44 <sup>h</sup>	9

<sup>a</sup> Number of experiments.

<sup>b</sup> Mean ± S.E. of the rate of oxygen consumption in the rat heart sarcosomal fraction.

<sup>c</sup> Significantly higher than control, *p* < 0.01.

<sup>d</sup> Significantly higher than samples treated with doxorubicin alone (*p* < 0.02).

<sup>e</sup> Significantly higher than samples treated with doxorubicin alone (*p* < 0.01).

<sup>f</sup> Significantly lower than samples treated with doxorubicin alone (*p* < 0.01).

<sup>g</sup> α-Tocopherol succinate prepared as described in Table 5.

<sup>h</sup> Significantly different from samples treated with doxorubicin (*p* < 0.01).

explained by the direct reduction of ferricytochrome *c* by the superoxide anion to yield ferrocyanochrome *c* and molecular oxygen which is continuously regenerated in the reaction vessel.

In further experiments, we found that adenosine had no effect on sarcosomal oxygen consumption but that tocopherol was strongly inhibitory (Table 6). In order to examine the possibility that buffer conditions might have contributed to our results with tocopherol, we repeated these experiments exactly as described in "Materials and Methods" and in Table 6, except that oxygen consumption was measured using a 0.25 M sucrose:20 mM HEPES buffer, pH 7.4, rather than the 0.15 M potassium phosphate buffer. Under these conditions, the addition of tocopherol (1 mM) reduced doxorubicin-enhanced oxygen consumption from 26.07 ± 1.39 nmol/min/mg (*n* = 3) to 5.37 ± 0.36 nmol/min/mg (*n* = 3, *p* < 0.01). Finally, we have confirmed that the alteration of the anthracycline quinone ring in 5-iminodaunorubicin produces a drug with a significantly diminished capacity to stimulate reactive oxygen metabolism (Table 6).

Because several recent studies have suggested that oxygen radical cytotoxicity may result from intracellular hydrogen peroxide accumulation (12, 44, 49), the drug-stimulated heart sarcosomes were examined for evidence of hydrogen peroxide formation. As seen in Chart 3A, oxygen was released by the addition of excess catalase to rat heart sarcosomes treated with doxorubicin, indicating that hydrogen peroxide as well as superoxide anion had been produced in these experiments. Catalase-induced oxygen release increased from 1.53 ± 0.30% (*n* = 3) of the total sarcosomal oxygen consumption in control preparations to 6.36 ± 0.94% (*n* = 4) in sarcosomes exposed to 135 μM doxorubicin (*p* < 0.01). When excess acetylated cytochrome *c* was added to the drug-treated cardiac sarcosomes after free radical formation had commenced, sub-

stantial oxygen release was detected (Chart 3B). This may have been due to the reduction of ferricytochrome *c* by superoxide but could also reflect the peroxidatic activity of cytochrome *c* that has been shown to occur in the presence of NAD(P)H under well-defined experimental conditions (36).

### Rat Heart Mitochondrial Fraction

**Effect of Anthracycline Antibiotics on Superoxide Formation in the Rat Heart Mitochondrial Fraction.** We examined the effect of anthracycline antibiotics on superoxide formation by a rat heart mitochondrial fraction because ultrastructural damage to the heart after anthracycline treatment is characterized by disruption of cardiac mitochondria as well as sarcoplasmic reticular membranes (15). A representative experiment, shown in Chart 4, revealed that acetylated cytochrome *c* reduction by heart mitochondria was initiated by the addition of NADH and was partially inhibited by SOD. In the presence of doxorubicin (135 μM), mitochondrial cytochrome *c* reduction was markedly increased (Chart 4B). For the mitochondrial fraction, doxorubicin stimulated superoxide production in a concentration-dependent manner that appeared to follow saturation kinetics (Chart 5). The kinetic constants for doxorubicin in this mitochondrial system were determined from a Lineweaver-Burk plot of the data (Chart 5, inset); the apparent *K<sub>m</sub>*

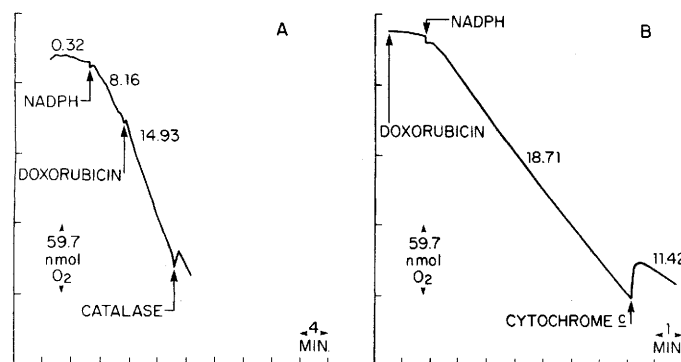


Chart 3. Stimulation of the rate of sarcosomal oxygen consumption by doxorubicin. Oxygen consumption by heart sarcosomes is shown in representative examples from multiple experiments. The addition of NADPH (3 μmol), doxorubicin (405 nmol), catalase (4500 units), or acetylated cytochrome *c* (168 nmol) to the 3-ml reaction vessel was accomplished through the access slot of the oxygen electrode plunger and has been indicated by an arrow. The numbers above each experiment indicate the rate of oxygen consumption in nmol/min/mg protein. A, doxorubicin-enhanced oxygen consumption and the effect of catalase; B, effect of cytochrome *c* on doxorubicin-stimulated oxygen consumption.

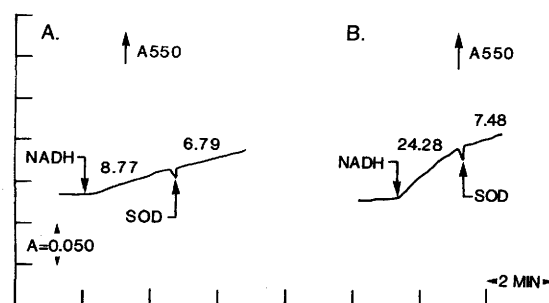


Chart 4. Effect of doxorubicin on acetylated cytochrome *c* reduction by the heart mitochondrial fraction. The sequential addition of NADH (1 μmol) or SOD (10 μg) to the 1-ml reaction system has been indicated by an arrow. Experiment A (A) is the mitochondrial control sample; the sample in Experiment B (B) contained doxorubicin (135 μM). The numbers above each experiment represent the rate of acetylated cytochrome *c* reduction in nmol/min/mg protein.

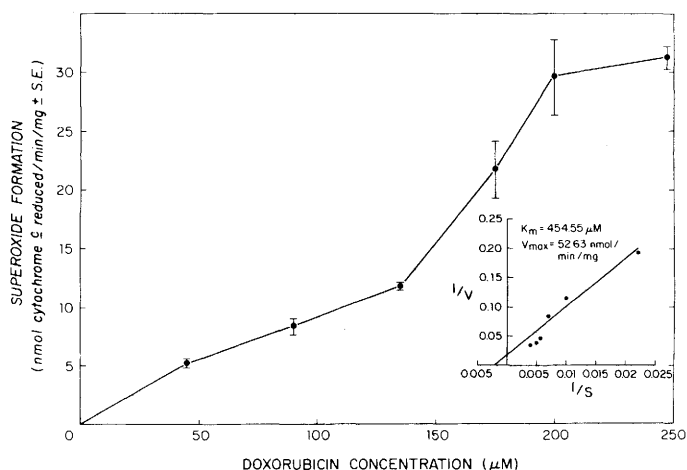


Chart 5. Effect of doxorubicin concentration on superoxide anion production in the rat heart mitochondrial fraction and a Lineweaver-Burk plot for doxorubicin (inset). Points, mean of at least 3 experiments for each dose of doxorubicin; bars, S.E.

Table 7

Requirements for doxorubicin-enhanced superoxide production in rat heart mitochondria

Superoxide formation in the mitochondrial fraction was examined using paired, 1-ml reaction mixtures containing 250  $\mu$ mol of sucrose, 20  $\mu$ mol of HEPES, pH 7.4, 100 nmol of EDTA, 56 nmol of acetylated cytochrome *c*, 50  $\mu$ g of mitochondrial protein, and either 0 or 10  $\mu$ g of SOD. The reaction mixture was preincubated for 4 min at 37° with 4 nmol of rotenone before initiation with 1  $\mu$ mol of NADH.

Reaction mixture	Superoxide formation (nmol cytochrome <i>c</i> reduced/min/mg)	<i>n</i> <sup>a</sup>
Control	2.04 ± 0.41 <sup>b</sup>	16
– rotenone	3.57 ± 0.51	6
– NADH	0.00 ± 0.00	6
– NADH plus NADPH (1 $\mu$ mol)	0.51 ± 0.20	3
Using freeze-thawed mitochondria	2.86 ± 0.71	3
Using mitochondria prepared with tocopherol and SOD	1.02 ± 0.71	4
Doxorubicin (135 nmol)	12.75 ± 0.82 <sup>c</sup>	24
– NADH	0.51 ± 0.20 <sup>d</sup>	6
– rotenone	8.57 ± 0.82 <sup>d</sup>	14
Using heat-denatured mitochondria <sup>e</sup>	0.00 ± 0.00	3
Using heat-denatured SOD <sup>e</sup>	8.87 ± 1.12	3
+ NAD <sup>+</sup> (10 $\mu$ mol)	3.32 ± 0.26 <sup>f</sup>	3
Using freeze-thawed mitochondria	15.40 ± 1.53	5
Doxorubicin (135 nmol) <sup>g</sup>	18.77 ± 0.92 <sup>h</sup>	4
+ deferoxamine (100 nmol) <sup>i</sup>	1.38 ± 1.02 <sup>h</sup>	3
– EDTA	17.95 ± 1.84 <sup>h</sup>	3
– EDTA plus deferoxamine (100 nmol)	13.46 ± 1.33 <sup>h</sup>	3
– EDTA + deferoxamine (100 nmol) + MgCl <sub>2</sub> (4 $\mu$ mol) + ATP (1 $\mu$ mol)	22.34 ± 2.45 <sup>h</sup>	3

<sup>a</sup> Number of experiments.

<sup>b</sup> Mean ± S.E. of the rate of superoxide production in the rat heart mitochondrial fraction.

<sup>c</sup> Significantly different from control ( $p < 0.001$ ).

<sup>d</sup> Rate of superoxide formation significantly lower than in reaction mixture containing rotenone ( $p < 0.01$ ).

<sup>e</sup> Mitochondria or SOD was heated for 60 min in a boiling water bath; samples containing heat-denatured SOD were paired against identical mixtures with native dismutase.

<sup>f</sup> Rate of superoxide formation significantly lower than in reaction mixture containing NADH alone ( $p < 0.001$ ).

<sup>g</sup> Doxorubicin used in these experiments was preservative free and chromatographically homogeneous.

<sup>h</sup> Mitochondria for these experiments were prepared with tocopherol and SOD; experiments were performed in buffer that had undergone ion-exchange chromatography with Chelex to remove trace amounts of iron.

<sup>i</sup> Deferoxamine was added before the EDTA.

and  $V_{max}$  were found to be 454.55  $\mu$ M and 52.63 nmol/min/mg, respectively. In these experiments, drug-stimulated superoxide formation was NADH dependent, was abolished after the heart mitochondria were denatured by heat, and was not significantly altered by disruption of mitochondrial membrane integrity with 3 cycles of freezing and thawing (Table 7). Furthermore, drug-induced oxygen radical production was not decreased by the iron-chelating agent deferoxamine or by preparation of the mitochondria in a tocopherol- and SOD-containing buffer (Table 7). The cofactor requirement for mitochondrial free radical production was specific; only NADH was capable of supporting drug-induced superoxide formation in both intact mitochondria and those exposed to 3 cycles of freezing and thawing (Table 8). Furthermore, doxorubicin-enhanced superoxide formation was significantly reduced when mitochondrial electron transport was not blocked at the NADH dehydrogenase site by rotenone or when excess NAD<sup>+</sup> was added to the reaction system (Table 7). This suggested that the activity of NADH dehydrogenase that can be inhibited by excess NAD<sup>+</sup> (25) may be responsible for the activation of anthracycline antibiotics to free radicals in heart mitochondria. Finally, we found that the addition of SOD that had been denatured by heat to the mitochondrial system produced no significant change in the rate of drug-enhanced superoxide formation, suggesting that, as in the experiments with heart sarcoplasmic reticulum, superoxide anion production had been measured in these studies with mitochondria (Table 7).

We also investigated the ability of several other anthracycline antibiotics to increase superoxide formation in the heart mitochondrial fraction. At equimolar concentrations (135  $\mu$ M), daunorubicin, rubidazole, and aclacinomycin A all significantly enhanced the rate of mitochondrial superoxide production over control levels,  $p < 0.001$  (Table 9). Furthermore, a comparison of Table 3 with Table 9 reveals that for both the heart mitochondrial fraction and heart sarcosomes, aclacinomycin A increased superoxide formation most and rubidazole least. In the mitochondrial system, 5-iminodaunorubicin did not stimu-

Table 8

Cofactor requirements for doxorubicin-enhanced superoxide formation in rat heart mitochondria

Superoxide production in the rat heart mitochondrial fraction was determined as described in "Materials and Methods." For these experiments, doxorubicin was included in the reaction mixtures (135 nmol), and superoxide production was initiated by the addition of the cofactor. The cofactor concentration was 1 mM except for sodium succinate which was used at a concentration of 5 mM in the reaction system.

Cofactor	Superoxide formation (nmol/min/mg)		
	Intact mitochondria	<i>n</i> <sup>a</sup>	Freeze-thawed mitochondria <sup>b</sup>
NADH	12.85 ± 1.84 <sup>c</sup>	4	20.40 ± 3.34 <sup>d</sup>
NAD <sup>+</sup>	0.24 ± 0.15	3	0.00 ± 0.00
NADPH	3.16 ± 0.51	3	0.00 ± 0.00
NADP <sup>+</sup>	0.00 ± 0.00	3	0.00 ± 0.00
FMN <sup>e</sup>	0.68 ± 0.36	3	0.00 ± 0.00
FAD	0.00 ± 0.00	3	0.00 ± 0.00
Succinate	0.68 ± 0.34	3	0.00 ± 0.00

<sup>a</sup> Number of experiments.

<sup>b</sup> Mitochondria used for these experiments were prepared with tocopherol and SOD and were exposed to 3 cycles of freezing and thawing before use as described in "Materials and Methods."

<sup>c</sup> Mean ± S.E. of the rate of doxorubicin-enhanced superoxide formation in the mitochondrial fraction.

<sup>d</sup> Doxorubicin used in these experiments was preservative free and chromatographically homogeneous.

<sup>e</sup> FMN, flavin mononucleotide; FAD, flavin adenine dinucleotide.



Table 9

Effect of chemotherapeutic agents on superoxide formation by rat heart mitochondria

Superoxide production in the rat heart mitochondrial fraction was determined as described in "Materials and Methods." For these experiments, all drugs were present at a concentration of 135  $\mu\text{M}$ .

Drug	Superoxide formation (nmol cytochrome <i>c</i> reduced/min/mg)	<i>n</i> <sup>a</sup>
Daunorubicin	17.03 $\pm$ 2.35 <sup>b, c</sup>	3
Rubidazole	9.38 $\pm$ 2.14 <sup>c</sup>	6
Acclacinomycin A	29.89 $\pm$ 3.16 <sup>c</sup>	3
5-Iminodaunorubicin	0.00 $\pm$ 0.00	3
Actinomycin D	3.26 $\pm$ 0.41 <sup>d</sup>	3

<sup>a</sup> Number of experiments.

<sup>b</sup> Mean  $\pm$  S.E. of the rate of drug-enhanced superoxide formation in the rat heart mitochondrial fraction.

<sup>c</sup> Significantly higher than control rate of superoxide formation in the heart mitochondrial fraction ( $p < 0.001$ , Table 7).

<sup>d</sup> Significantly different from control rate of superoxide formation in the heart mitochondrial fraction ( $p < 0.01$ , Table 7).

Table 10

Effect of doxorubicin on oxygen consumption by rat heart mitochondria

Oxygen consumption in the rat heart mitochondrial fraction was determined in a 3-ml reaction vessel containing 750  $\mu\text{mol}$  of sucrose, 60  $\mu\text{mol}$  of HEPES, pH 7.4, 300 nmol of EDTA, 12 nmol of rotenone, 600  $\mu\text{g}$  of mitochondrial protein, 3  $\mu\text{mol}$  of NADH, and, where specified, doxorubicin. For these experiments, doxorubicin and the other reagents were equilibrated in the reaction vessel for 4 min; oxygen consumption was then initiated by the addition of NADH.

Reaction system	Oxygen consumption (nmol O <sub>2</sub> /min/mg)	<i>n</i> <sup>a</sup>
Control	4.28 $\pm$ 0.30 <sup>b</sup>	15
+ sodium azide (3 $\mu\text{mol}$ )	4.48 $\pm$ 0.40	5
Doxorubicin (405 nmol)	7.32 $\pm$ 0.34 <sup>c</sup>	19
+ sodium azide (3 $\mu\text{mol}$ )	9.45 $\pm$ 0.30 <sup>d</sup>	8
+ KCN (3 $\mu\text{mol}$ )	8.76 $\pm$ 0.30 <sup>e</sup>	7
+ dicumarol (30 nmol)	7.73 $\pm$ 0.43	3
+ tocopherol <sup>f</sup>		
300 nmol	6.66 $\pm$ 0.19	3
3 $\mu\text{mol}$	7.26 $\pm$ 1.29	3
Doxorubicin (750 nmol)	9.65 $\pm$ 0.60 <sup>g</sup>	3

<sup>a</sup> Number of experiments.

<sup>b</sup> Mean  $\pm$  S.E. of the rate of oxygen consumption in the rat heart mitochondrial fraction.

<sup>c</sup> Significantly higher than control,  $p < 0.001$ .

<sup>d</sup> Significantly higher than samples treated with doxorubicin alone,  $p < 0.001$ .

<sup>e</sup> Significantly higher than samples treated with doxorubicin alone,  $p < 0.05$ .

<sup>f</sup>  $\alpha$ -Tocopherol succinate hydrolyzed with 2 molar equivalents of KOH as described previously (3).

<sup>g</sup> Significantly higher than samples treated with the lower doxorubicin dose,  $p < 0.01$ .

late free radical production, and actinomycin D treatment led to a small, but significant, increase in the rate of superoxide formation (Table 9).

**Effect of Anthracycline Antibiotics on Oxygen Consumption by the Rat Heart Mitochondrial Fraction.** To investigate further the effect of the anthracycline antibiotics on mitochondrial reactive oxygen metabolism, the rate of oxygen consumption by rat heart mitochondria exposed to doxorubicin was examined. Doxorubicin significantly increased mitochondrial oxygen consumption over control levels in a dose-dependent manner (Table 10). In these experiments, sodium azide and KCN [which inhibit catalase but not mitochondrial SOD (52)] both significantly enhanced mitochondrial oxygen consumption in the presence of doxorubicin (Table 10); however, neither dicumarol nor  $\alpha$ -tocopherol produced any statistically significant effect on the rate of oxygen consumption by this preparation (Table 10). Oxygen consumption in both the control and

drug-treated samples varied with the concentration of mitochondrial protein used; at mitochondrial protein concentrations of 50 and 200  $\mu\text{g}/\text{ml}$ , oxygen consumption increased from 0.38  $\pm$  0.03 to 0.82  $\pm$  0.12 nmol/min ( $n = 4$ ,  $p < 0.01$ ) in the control samples and from 0.56  $\pm$  0.06 nmol/min to 1.67  $\pm$  0.08 nmol/min ( $n = 4$ ,  $p < 0.001$ ) in samples treated with 135  $\mu\text{M}$  doxorubicin. Much of this increase in oxygen consumption produced by doxorubicin probably reflects the drug-induced superoxide formation demonstrated previously.

In addition to superoxide anion production, doxorubicin also stimulated hydrogen peroxide formation by the rat heart mitochondrial fraction. As shown in Chart 6, A and C, oxygen release observed after the addition of excess catalase to the mitochondrial fraction was more substantial in the drug-treated sample. However, oxygen release was much more apparent in mitochondria preincubated with sodium azide (Chart 6, B and D); in these preparations, catalase-induced oxygen release increased from 3.9  $\pm$  1.1% ( $n = 3$ ) of the total mitochondrial oxygen consumption in the control samples to 15.9  $\pm$  3.1% ( $n = 3$ ) in mitochondria exposed to doxorubicin (135  $\mu\text{M}$ ) ( $p < 0.02$ ).

## Rat Heart Cytosol

**Effect of Doxorubicin on Reactive Oxygen Metabolism in Rat Heart Cytosol.** To complete our investigation of the site(s) of cardiac free radical formation by the anthracycline antibiotics, heart cytosol was examined for its ability to support drug-induced superoxide anion formation. We found that doxorubicin increased superoxide production more than 10-fold over control levels in cardiac cytosol ( $p < 0.001$ , Table 11). Cytosolic reactive oxygen metabolism required NADH rather than NADPH, was ablated when the heart cytosol was denatured by heat, and was reduced by more than 35% after treatment with

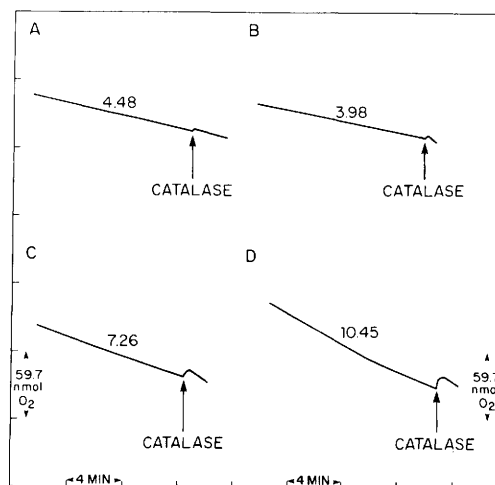


Chart 6. Effect of sodium azide on the rate of oxygen consumption in the rat heart mitochondrial fraction. Oxygen consumption by the rat heart mitochondrial fraction is shown in representative examples from multiple experiments. In A, catalase (4500 units) has been added to the 3-ml control mitochondrial fraction after oxygen consumption was begun with NADH (3  $\mu\text{mol}$ ); in B, sodium azide (3  $\mu\text{mol}$ ) had been added to the mitochondrial sample before the addition of NADH. For the experiment shown in C, the mitochondrial fraction was treated with doxorubicin (405 nmol) before initiation with NADH; in D, the 3-ml doxorubicin-treated mitochondrial sample contained sodium azide (3  $\mu\text{mol}$ ). Catalase was added with a Hamilton syringe through the access slot of the oxygen electrode plunger. The numbers above each experiment represent the rate of oxygen consumption in nmol/min/mg protein.

the xanthine oxidase inhibitor allopurinol (100  $\mu\text{M}$ ) ( $p < 0.02$ ; Table 11).

Free radical production by doxorubicin in the cytosol fraction was confirmed by measurement of drug-stimulated oxygen consumption. However, because the specific activities of the antioxidant enzymes in cardiac cytosol are the highest of any cellular compartment in the heart (see below), it was necessary to inhibit cytosolic SOD and catalase with KCN to measure the effect of doxorubicin on cytosolic oxygen radical metabolism accurately. As shown in Table 12, doxorubicin significantly increased the rate of oxygen consumption in heart cytosol by a process that required NADH. This drug-related increase was not altered by dicumarol but was significantly reduced by both acetylated cytochrome *c* and allopurinol (Table 12). Taken together, the results of these experiments suggested that doxorubicin stimulated reactive oxygen metabolism (principally superoxide anion formation) in heart cytosol as well as cardiac sarcosomes and mitochondria.

### Antioxidant Enzyme Levels

**Glutathione Peroxidase.** We have examined previously the enzymatic defenses of mouse heart against oxidant challenge; the results of that investigation indicated that SOD and selenium-dependent glutathione peroxidase played a major role in the detoxification of cardiac reactive oxygen metabolites (12). In the present work, we have extended those studies by determining the specific activities of SOD and glutathione peroxidase in each of the cellular compartments of rat heart examined for the ability to support free radical production by doxorubicin. As seen in Table 13, the activity of cardiac glutathione peroxidase was greatest in rat heart cytosol,  $p < 0.001$ . The glutathione peroxidase level of the preparation of heart sarcoplasmic reticulum used for our free radical experiments was less than 15% of that in the cytosol fraction (Table 13). The glutathione peroxidase activity of heart mitochondria exposed to ultrasonic disruption was less than 10% of the corresponding level in cardiac cytosol. However, ultracentrifugation of the sonicated

Table 11

#### Effect of doxorubicin on superoxide production in rat heart cytosol

Superoxide production in rat cardiac cytosol was determined with paired, 1-ml reaction mixtures that contained 150  $\mu\text{mol}$  potassium phosphate buffer, pH 7.4, 100 nmol EDTA, 56 nmol acetylated cytochrome *c*, 100  $\mu\text{g}$  cytosolic protein, and either 0 or 10  $\mu\text{g}$  SOD. Reactions were begun by the addition of 1  $\mu\text{mol}$  NAD(P)H in the presence or absence of doxorubicin.

Reaction system	Superoxide production (nmol cytochrome <i>c</i> reduced/min/mg)	<i>n</i> <sup>a</sup>
Control		
Using NADPH	0.71 $\pm$ 0.31 <sup>b</sup>	6
Using NADH	0.56 $\pm$ 0.20	6
Doxorubicin (135 nmol)		
Using NADPH	0.84 $\pm$ 0.36	6
Using NADH	6.22 $\pm$ 0.67 <sup>c</sup>	15
- NADH	0.00 $\pm$ 0.00 <sup>d</sup>	3
Using NADH with heat-denatured cytosol <sup>e</sup>	0.56 $\pm$ 0.51 <sup>d</sup>	3
Using NADH plus allopurinol (100 nmol)	4.03 $\pm$ 0.26 <sup>f</sup>	4

<sup>a</sup> Number of experiments.

<sup>b</sup> Mean  $\pm$  S.E. of the rate of superoxide formation in rat heart cytosol.

<sup>c</sup> Significantly different from control levels ( $p < 0.001$ ).

<sup>d</sup> Significantly different from complete drug-treated sample utilizing NADH ( $p < 0.001$ ).

<sup>e</sup> Cytosol heated for 60 min in a boiling water bath.

<sup>f</sup> Significantly different from sample treated with doxorubicin alone ( $p < 0.02$ ).

Table 12

#### Effect of doxorubicin on oxygen consumption by rat heart cytosol

Oxygen consumption in the cytosol fraction was examined in a 3-ml volume that contained 750  $\mu\text{mol}$  sucrose, 60  $\mu\text{mol}$  HEPES, pH 7.4, 300 nmol EDTA, 300  $\mu\text{g}$  cytosolic protein, 3  $\mu\text{mol}$  either NADH or NADPH, and 405 nmol doxorubicin where specified. For these experiments, doxorubicin and the other reactants were equilibrated in the 3-ml reaction vessel for 4 min; oxygen consumption was then initiated by the addition of 3  $\mu\text{mol}$  NAD(P)H through the access slot of the electrode plunger. Sodium azide and KCN were used in a concentration of 1 mM for all experiments.

Experimental system	Oxygen consumption (nmol O <sub>2</sub> /min/mg)	<i>n</i> <sup>a</sup>
Control		
+ sodium azide and NADPH	1.89 $\pm$ 0.16 <sup>b</sup>	3
+ sodium azide and NADH	2.19 $\pm$ 0.20	6
+ KCN and NADH	1.79 $\pm$ 0.40	3
+ KCN, NADH, and allopurinol (300 nmol)	0.00 $\pm$ 0.00	3
Doxorubicin (405 nmol)		
+ sodium azide and NADPH	3.18 $\pm$ 0.40	3
+ sodium azide and NADH	10.94 $\pm$ 0.99 <sup>c</sup>	5
+ KCN and NADH	11.74 $\pm$ 0.60 <sup>d</sup>	5
+ KCN, NADH, and dicumarol (30 nmol)	10.35 $\pm$ 1.39	3
+ KCN, NADH, and acetylated cytochrome <i>c</i> (168 nmol)	8.56 $\pm$ 0.40 <sup>e</sup>	3
+ KCN, NADH, and allopurinol (300 nmol)	7.16 $\pm$ 1.39 <sup>f</sup>	3

<sup>a</sup> Number of experiments.

<sup>b</sup> Mean  $\pm$  S.E. of the rate of oxygen consumption in heart cytosol.

<sup>c</sup> Significantly different from azide-treated control ( $p < 0.001$ ).

<sup>d</sup> Significantly different from KCN-treated control ( $p < 0.001$ ).

<sup>e</sup> Significantly different from KCN-treated sample containing doxorubicin alone ( $p < 0.01$ ).

<sup>f</sup> Significantly different from KCN-treated sample containing doxorubicin alone ( $p < 0.02$ ).

Table 13

#### Antioxidant enzyme levels in rat heart subcellular fractions

Rat heart cytosol, sarcosomes, and mitochondria were prepared and assayed for glutathione peroxidase and SOD activity as described in "Materials and Methods." Before determination of enzyme activity, the mitochondrial fraction was exposed to ultrasonic disruption as detailed in "Materials and Methods" in order to eliminate permeability barriers to appropriate substrates in these assays; the supernatant and pellet resulting from centrifugation of the sonicated mitochondria at 105,000  $\times g$  and 4 $^{\circ}$  for 60 min were also assayed for glutathione peroxidase and SOD activity.

Heart fraction	Glutathione peroxidase (nmol NADPH oxidized/min/mg)	SOD ( $\mu\text{g}$ SOD/mg)
Cytosol	704.2 $\pm$ 33.4 <sup>a, b</sup>	13.20 $\pm$ 0.88 <sup>c</sup>
Sarcosomes	100.8 $\pm$ 0.27	2.78 $\pm$ 0.10
Mitochondria	64.3 $\pm$ 11.3	7.60 $\pm$ 0.10
		7.95 $\pm$ 0.90 <sup>d, e</sup>
105,000 $\times g$ supernatant	307.0 $\pm$ 5.8	9.96 $\pm$ 0.62
		8.80 $\pm$ 0.24 <sup>d, e</sup>
105,000 $\times g$ pellet	53.6 $\pm$ 2.2	7.30 $\pm$ 1.60
		6.40 $\pm$ 0.30 <sup>d, e</sup>

<sup>a</sup> Mean  $\pm$  S.E. of triplicate determinations of enzyme activity from at least 3 experimental samples.

<sup>b</sup> Glutathione peroxidase activity in cytosol was significantly higher than enzyme levels in sarcosomes or sonicated mitochondria ( $p < 0.001$ ).

<sup>c</sup> SOD activity in cytosol was significantly higher than enzyme levels in sarcosomes or sonicated mitochondria ( $p < 0.01$ ).

<sup>d</sup> SOD activity in these samples was assayed after treatment with 1 mM KCN for 15 min at 25 $^{\circ}$ .

<sup>e</sup> No significant difference in the SOD activity of corresponding mitochondrial fractions with or without KCN treatment.

mitochondria produced a supernatant with nearly 45% of the specific activity of the heart cytosol fraction (Table 13). Thus, it seemed probable that the glutathione peroxidase activity of rat heart mitochondria [in contrast to that in the mouse (23)] was located primarily in the mitochondrial matrix.

**Superoxide Dismutase.** The distribution of SOD within the rat heart was similar to that described for glutathione peroxi-

dase. SOD activity in cytosol was significantly greater than the level measured in either heart sarcosomes or mitochondria,  $p < 0.01$  (Table 13). Furthermore, a major part of the SOD activity of the mitochondrial fraction was contained in the mitochondrial matrix (Table 13). Preincubation with KCN did not significantly change the specific activity of SOD measured in any mitochondrial preparation; this suggested that the manganese-dependent enzyme comprised a major portion of the SOD in rat heart mitochondria (52). However, the presence of a SOD activity from the intermembranous space and its cyanide sensitivity were not addressed in these experiments; and thus, the possibility that rat heart mitochondria also contain a copper and zinc-dependent dismutase similar to that in liver cannot be excluded (52).

## DISCUSSION

If drug-induced oxygen radical formation is a major determinant of the cardiac toxicity of the anthracycline antibiotics, it should help to explain specific morphological features of the heart damage that characteristically accompanies treatment with these drugs. Furthermore, it should also provide some insight into the apparently selective nature of this particular form of myocardial insult. In this study, we have attempted to evaluate the role of reactive oxygen metabolism in the cardiac toxicity of the anthracycline antitumor agents along these lines by investigating both the extent and the mechanism(s) of anthracycline-related free radical production in rat heart.

We have shown that treatment of heart sarcosomes, mitochondria, and cytosol with anthracycline drugs produces a concentration-related increase in superoxide anion and hydrogen peroxide production. This occurs despite the presence of both superoxide dismutase and glutathione peroxidase (2 major cardiac enzymatic defenses against oxygen radicals) in each heart fraction. Although drug-stimulated oxygen radical metabolism was enhanced further when these enzymes, as well as cardiac catalase, were inhibited by treatment with cyanide or azide, our findings suggest that oxygen radical production by the anthracycline antibiotics may exceed the detoxifying capacity of the individual heart fractions. Because anthracycline cardiac toxicity is characterized by degeneration of heart mitochondria and sarcoplasmic reticulum (15), reactive oxygen accumulation may explain drug-induced membrane damage at these specific intracellular sites.

Enhancement of oxygen radical metabolism by the anthracycline drugs was demonstrated for each myocardial fraction examined; however, the mechanism(s) of free radical formation in these compartments differed. Superoxide production by rat heart sarcosomes required NADPH, was not inhibited by cyanide or dicumarol, and was accompanied by the oxidation of NADPH. Furthermore, inhibition of sarcosomal cytochrome P-450 reductase activity by treatment with excess  $\text{NADP}^+$  or a sulfhydryl reagent produced a parallel decrease in superoxide formation by doxorubicin. These experiments strongly suggest that the NADPH: cytochrome P-450 reductase activity of heart sarcosomes was responsible for the reduction of the anthracycline antibiotics to their respective semiquinones.

On the other hand, superoxide formation in the mitochondrial fraction was dependent on NADH, and not succinate, was rotenone and dicumarol insensitive, and was significantly inhibited by addition of excess  $\text{NAD}^+$ . These experiments indi-

cate that the NADH dehydrogenase complex was the site of anthracycline reduction in our experiments with intact mitochondria. Thayer (49) obtained similar results recently for doxorubicin using a preparation of cardiac submitochondrial particles. However, in the absence of specific mitochondrial fractionation studies, one cannot exclude the possibility that the NADH:cytochrome  $b_5$  reductase (NADH:cytochrome  $b_5$  oxidoreductase, EC 1.6.2.2) of the outer mitochondrial membrane (6), in addition to a component of the electron transport chain, contributed to these findings.

Finally, we found that reactive oxygen metabolism in heart cytosol was heat labile, dicumarol insensitive, and dependent on NADH as cofactor; treatment of the cytosol fraction with allopurinol resulted in a significant reduction in drug-related superoxide formation and oxygen consumption. Thus, part of the free radical formation observed in heart cytosol might be related to the activation of doxorubicin to its semiquinone intermediate by xanthine oxidase, a reaction demonstrated previously with a purified preparation of this enzyme (43). Although our heart cytosol was carefully prepared in an attempt to eliminate any substantial contamination by the mitochondrial fraction, it is still possible that mitochondrial components could have interfered with these experiments and might be responsible for supporting at least part of the drug-related superoxide formation in the cytosol fraction.

From these studies, it may be suggested that 3 different flavin-containing enzymes (NADPH:cytochrome P-450 reductase, NADH dehydrogenase, and xanthine oxidase), which have been shown previously to be capable of catalyzing the one-electron reduction of a wide variety of quinone drugs (21, 38), are responsible for the activation of the anthracycline antibiotics to free radical species in heart sarcoplasmic reticulum, mitochondria, and cytosol. Furthermore, because inhibition of cardiac DT diaphorase by dicumarol had no effect on free radical production in any heart fraction, it appears that this enzyme has no significant impact on the activation of anthracycline drugs in rat heart.

In an aerobic environment, such as the myocardial cell, anthracycline semiquinone radicals rapidly donate their unpaired electrons to molecular oxygen, forming superoxide anion and/or hydrogen peroxide (22). We have shown that treatment of heart sarcosomes with anthracycline drugs leads to the production of both of these reactive oxygen metabolites. A comparison of the rates of oxygen consumption and superoxide formation in sarcosomes treated with doxorubicin suggests that under identical experimental conditions, the production of superoxide anion, at least initially, accounts for most of the oxygen consumed. In that case, the hydrogen peroxide formed must result from the spontaneous or SOD-catalyzed dismutation of superoxide anion rather than from the direct, 2-electron reduction of oxygen.

If the anthracycline antitumor agents augment the production of reactive oxygen metabolites *in vivo*, the limited capacity of cardiac muscle to detoxify free radicals could be overwhelmed (10), resulting in an extensive alteration in myocardial function at the sites of oxygen radical accumulation. Drug-induced peroxidation of sarcoplasmic reticular membrane could interfere with tubular calcium transport and muscular contractility (30) and might explain the sarcoplasmic vacuolation that appears to be the earliest morphological concomitant of anthracycline cardiac toxicity in humans (50). Reduction of the an-

tracycline quinone at a proximal portion of the mitochondrial electron transport chain could divert electron flow from energy production, thereby reducing myocardial ATP content; furthermore, mitochondrial membrane integrity and the regulation of the level of intracellular reducing equivalents might be altered in this manner (46). Free radical production in the soluble compartment could lead to the oxidation of critical sulfhydryl-containing enzymes (such as glyceraldehyde:3-phosphate dehydrogenase) with an additional, further decrease in cardiac energy metabolism. As we have suggested recently (10), because heart cells are so rich in heme-containing proteins that may undergo autoxidation, it is probable that the presence of excess intracellular peroxide in the anthracycline-treated heart would establish an extraordinarily favorable milieu for the formation of highly reactive hydroxyl and peroxy radicals with a proven capacity to produce membrane peroxidation.

Finally, we evaluated the effect of several anthracycline and nonanthracycline analogues on reactive oxygen metabolism in the rat heart fractions. Daunorubicin, rubidazole, and aclacinomycin A all effectively stimulated superoxide production in cardiac sarcosomes and mitochondria. Actinomycin D, which has been reported to increase the cardiac toxicity of doxorubicin (27), produced a small, but significant, increase in superoxide production in the heart fractions; this is in agreement with a recent report indicating that actinomycin D augments superoxide formation by hepatic microsomes (47). However, the daunorubicin analogue 5-iminodaunorubicin did not produce any significant effect on either sarcosomal or mitochondrial free radical formation, which supports a study by Lown *et al.* (29), suggesting that oxidation-reduction cycling after chemical reduction of daunorubicin is essentially eliminated by the imino substitution on the quinone ring. These results may explain reports of lesser cardiac toxicity for this agent (29).

In summary, we propose that the anthracycline antibiotics selectively injure the heart because their administration may lead to oxygen radical accumulation in the specific myocardial compartments capable of reductively activating the drugs to semiquinone free radical intermediates.

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## REFERENCES

- Armitage, P. *Statistical Methods in Medical Research*, pp. 104–126. Oxford: Blackwell Scientific Publications, Ltd., 1971.
- Azzi, A., Montecucco, C., and Richter, C. The use of acetylated ferricytochrome *c* for the detection of superoxide radicals produced in biological membranes. *Biochem. Biophys. Res. Commun.*, **65**: 597–603, 1975.
- Bachur, N. R., Gordon, S. L., and Gee, M. V. Anthracycline antibiotic augmentation of microsomal electron transport and free radical formation. *Mol. Pharmacol.*, **13**: 901–910, 1977.
- Bachur, N. R., Gordon, S. L., and Gee, M. V. A general mechanism for microsomal activation of quinone anticancer agents to free radicals. *Cancer Res.*, **38**: 1745–1750, 1978.
- Bachur, N. R., Gordon, S. L., Gee, M. V., and Kon, H. NADPH cytochrome P-450 reductase activation of quinone anticancer agents to free radicals. *Proc. Natl. Acad. Sci. U. S. A.*, **76**: 954–957, 1979.
- Bernardi, P., and Azzone, G. F. Cytochrome *c* as an electron shuttle between the outer and inner mitochondrial membranes. *J. Biol. Chem.*, **256**: 7189–7192, 1981.
- Carter, S. K. Adriamycin—a review. *J. Natl. Cancer Inst.*, **55**: 1265–1274, 1975.
- Chappel, J. B. The oxidation of citrate, isocitrate, and *cis*-aconitate by

- isolated mitochondria. *Biochem. J.*, **90**: 225–237, 1964.
- Dantchev, D., Slioussartchouk, V., Paintrand, M., Hayat, M., Bourut, C., and Mathé, G. Electron microscopic studies of the heart and light microscopic studies of the skin after treatment of golden hamsters with Adriamycin, detorubicin, AD-32, and aclacinomycin. *Cancer Treat. Rep.*, **63**: 875–888, 1979.
- Doroshov, J. H., and Locker, G. Y. Role of oxygen radical formation in anthracycline cardiac toxicity. *In*: F. Muggia, C. W. Young, and S. K. Carter (eds.), *Anthracycline Antibiotics in Cancer Therapy*, pp. 317–330. Boston: Martinus Nijhoff Publishers, 1982.
- Doroshov, J. H., Locker, G. Y., Ifrim, I., and Myers, C. E. Prevention of doxorubicin cardiac toxicity in the mouse by *n*-acetylcysteine. *J. Clin. Invest.*, **68**: 1053–1064, 1981.
- Doroshov, J. H., Locker, G. Y., and Myers, C. E. Enzymatic defenses of the mouse heart against reactive oxygen metabolites: alterations produced by doxorubicin. *J. Clin. Invest.*, **65**: 128–135, 1980.
- Doroshov, J. H., and Reeves, J. Anthracycline-enhanced oxygen radical formation in the heart. *Proc. Am. Assoc. Cancer Res.*, **21**: 266, 1980.
- Ernster, L. DT diaphorase. *Methods Enzymol.*, **10**: 309–317, 1967.
- Ferrans, V. J. Overview of cardiac pathology in relation to anthracycline cardiotoxicity. *Cancer Treat. Rep.*, **62**: 955–961, 1978.
- Fridovich, I. The biology of oxygen radicals. *Science (Wash. D. C.)*, **201**: 875–880, 1978.
- Goodman, J., and Hochstein, P. Generation of free radicals and lipid peroxidation by redox cycling of Adriamycin and daunomycin. *Biochem. Biophys. Res. Commun.*, **77**: 797–803, 1977.
- Handa, K., and Sato, S. Stimulation of microsomal NADPH oxidation by quinone group-containing anticancer chemicals. *Gann*, **67**: 523–528, 1976.
- Harigaya, S., and Schwartz, A. Rate of calcium binding and uptake in normal animal and failing human cardiac muscle: membrane vesicles (relaxing system) and mitochondria. *Circ. Res.*, **25**: 781–794, 1969.
- Horcker, B. L., and Kornberg, A. The extinction coefficients of the reduced band of pyridine nucleotides. *J. Biol. Chem.*, **175**: 385–390, 1948.
- Iyanagi, T., and Yamazaki, I. One-electron-transfer reactions in biochemical systems. V. Difference in the mechanism of quinone reduction by the NADH dehydrogenase and the NAD(P)H dehydrogenase (DT-diaphorase). *Biochim. Biophys. Acta*, **216**: 282–294, 1970.
- Kalyanaraman, B., Perez-Reyes, E., and Mason, R. P. Spin-trapping and direct electron spin resonance investigations of the redox metabolism of quinone anticancer drugs. *Biochim. Biophys. Acta*, **630**: 119–130, 1980.
- Katki, A., and Myers, C. E. Membrane-bound glutathione peroxidase-like activity in mitochondria. *Biochem. Biophys. Res. Commun.*, **96**: 85–91, 1980.
- Kharasch, E. D., and Novak, R. F. Ring current effects in Adriamycin-flavin mononucleotide complexation as observed by <sup>1</sup>H FT NMR spectroscopy. *Biochem. Biophys. Res. Commun.*, **92**: 1320–1326, 1980.
- King, T. E., and Howard, R. L. Preparations and properties of soluble NADH dehydrogenases from cardiac muscle. *Methods Enzymol.*, **10**: 275–294, 1967.
- Komiyama, T., Oki, T., and Inui, T. A proposed reaction mechanism for the enzymatic reductive cleavage of glycosidic bond in anthracycline antibiotics. *J. Antibiot. (Tokyo)*, **32**: 1219–1222, 1979.
- Kushner, J. P., Hansen, V. L., and Hammar, S. P. Cardiomyopathy after widely separated courses of Adriamycin exacerbated by actinomycin-D and mithramycin. *Cancer (Phila.)*, **36**: 1577–1584, 1975.
- Lenaz, L., and Page, J. Cardiotoxicity of Adriamycin and related anthracyclines. *Cancer Treat. Rev.*, **3**: 111–120, 1976.
- Lown, J. W., Chen, H.-H., Plambeck, J. A., and Acton, E. M. Diminished superoxide anion generation by reduced 5-iminodaunorubicin relative to daunorubicin and the relationship to cardiotoxicity of the anthracycline antitumor agents. *Biochem. Pharmacol.*, **28**: 2563–2568, 1979.
- Lowrey, K., Glende, E. A., and Recknagel, R. O. Destruction of liver microsomal calcium pump activity by carbon tetrachloride and bromotrichloromethane. *Biochem. Pharmacol.*, **30**: 135–140, 1981.
- Lowry, O., Rosebrough, N. J., Farr, A. L., and Randall, R. J. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.*, **193**: 265–275, 1951.
- Martonosi, A. Sarcoplasmic reticulum. IV. Solubilization of microsomal adenosine triphosphatase. *J. Biol. Chem.*, **243**: 71–81, 1968.
- McCord, J. M., and Fridovich, I. Superoxide dismutase—an enzymatic function of erythrocyte hemocoupein. *J. Biol. Chem.*, **244**: 6049–6055, 1969.
- Mela, L., and Seitz, S. Isolation of mitochondria with emphasis on heart mitochondria from small amounts of tissue. *Methods Enzymol.*, **55**: 39–46, 1979.
- Mettler, F. P., Young, D. M., and Ward, J. M. Adriamycin-induced cardiotoxicity (cardiomyopathy and congestive heart failure) in rats. *Cancer Res.*, **37**: 2705–2713, 1977.
- Misra, H. P., and Fridovich, I. A peroxide-dependent reduction of cytochrome *c* by NADH. *Biochim. Biophys. Acta*, **292**: 815–824, 1973.
- Myers, C. E., McGuire, W. P., Liss, R. H., Ifrim, I., Grotzinger, K., and Young, R. C. Adriamycin: the role of lipid peroxidation in cardiac toxicity and tumor response. *Science (Wash. D. C.)*, **197**: 165–167, 1977.

38. Nakamura, S., and Yamazaki, I. One-electron transfer reactions in biochemical systems. IV. A mixed mechanism in the reaction of milk xanthine oxidase with electron acceptors. *Biochim. Biophys. Acta*, **189**: 29-37, 1969.
39. Newman, R. A., Hacker, M. P., and Krakoff, I. H. Amelioration of Adriamycin and daunorubicin myocardial toxicity by adenosine. *Cancer Res.*, **41**: 3483-3488, 1981.
40. Nishikimi, M., Yamada, H., and Yagi, K. Oxidation by superoxide of tocopherols dispersed in aqueous media with deoxycholate. *Biochim. Biophys. Acta*, **627**: 101-108, 1980.
41. Olson, R. D., MacDonald, J. S., Van Bostel, C. J., Boerth, R. C., Harbison, R. D., Slonim, A. E., Freeman, R. W., and Oates, J. A. Regulatory role of glutathione and soluble sulfhydryl groups in the toxicity of Adriamycin. *J. Pharmacol. Exp. Ther.*, **215**: 450-454, 1980.
42. Paglia, D. E., and Valentine, W. N. Studies on the quantitative and qualitative characterization of erythrocyte glutathione peroxidase. *J. Lab. Clin. Med.*, **70**: 158-169, 1967.
43. Pan, S.-S., and Bachur, N. R. Xanthine oxidase catalyzed reductive cleavage of anthracycline antibiotics and free radical formation. *Mol. Pharmacol.*, **17**: 95-99, 1980.
44. Revis, N. W., and Marusic, N. Glutathione peroxidase activity and selenium concentration in the hearts of doxorubicin-treated rabbits. *J. Mol. Cell. Cardiol.*, **10**: 945-951, 1978.
45. Sato, S., Iwaizumi, M., Handa, K., and Tamura, Y. Electron spin resonance study on the mode of generation of free radicals of daunomycin, Adriamycin, and carboquone in NAD(P)H-microsome system. *Gann*, **68**: 603-608, 1977.
46. Sies, H., Graf, P., and Estrela, J. Hepatic calcium efflux during cytochrome P-450-dependent drug oxidations at the endoplasmic reticulum in intact liver. *Proc. Natl. Acad. Sci. U. S. A.*, **78**: 3358-3362, 1981.
47. Sinha, B. K., and Cox, M. G. Stimulation of superoxide formation by actinomycin D and its *N*<sup>2</sup>-substituted spin-labeled derivatives. *Mol. Pharmacol.*, **17**: 432-434, 1980.
48. Strobel, H. W., and Dignam, J. D. Purification and properties of NADPH-cytochrome P-450 reductase. *Methods Enzymol.*, **52**: 89-96, 1978.
49. Thayer, W. S. Adriamycin stimulated superoxide formation in submitochondrial particles. *Chem.-Biol. Interact.*, **19**: 265-278, 1977.
50. Unverferth, D. V., Magorien, R. D., Unverferth, B. P., Talley, R. L., Balcerzak, S. P., and Baba, N. Human myocardial morphologic and functional changes in the first 24 hours after doxorubicin administration. *Cancer Treat. Rep.*, **65**: 1093-1097, 1981.
51. Wang, Y.-M., Madanat, F. F., Kimball, J. C., Gleiser, C. A., Ali, M. K., Kaufman, M. W., and Van Eys, J. Effect of vitamin e against Adriamycin-induced toxicity in rabbits. *Cancer Res.*, **40**: 1022-1027, 1980.
52. Weisiger, R. A., and Fridovich, I. Mitochondrial superoxide dismutase: site of synthesis and intramitochondrial localization. *J. Biol. Chem.*, **248**: 4793-4796, 1973.
53. Williams, C. H. Flavin-containing dehydrogenases. *In*: P. D. Boyer (ed.), *The Enzymes*, Vol. 13, pp. 165-173. New York: Academic Press, Inc., 1976.
54. Yesair, D. W., Schwartzbach, E., Shuck, D., Denine, E. P., and Asbell, M. A. Comparative pharmacokinetics of daunomycin and Adriamycin in several animal species. *Cancer Res.*, **32**: 1177-1183, 1972.
55. Yonetani, T., and Ray, G. S. Studies on cytochrome c peroxidase: purification and some properties. *J. Biol. Chem.*, **240**: 4503-4508, 1965.