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HEPATIC REDOX HOMEOSTASIS FOLLOWING ACUTE ADRIAMYCIN INTOXICATION IN RATS

KENDALL B. WALLACE*
Department of Pharmacology, University of Minnesota-Duluth, Duluth, MN 55812, U.S.A.

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Abstract—Adriamycin toxicity is postulated to result from cytochrome P-450 reductase-catalyzed univalent reduction of the quinone to the semiquinone free radical intermediate. Oxygen radicals generated during the nonenzymatic reoxidation of the semiquinone have been implicated in the deleterious modification of a variety of tissue macromolecules. Detoxification of reactive products, such as hydroperoxides, is proposed to involve the consumption of vital cellular reducing equivalents which may, in itself, represent the primary causative event in toxic tissue damage. The present investigation demonstrates that hepatic tissue has sufficient glutathione (GSH) reductase to prevent a decrease in GSH following acute adriamycin administration to rats. Similarly, except for a transient decrease in NAD, adriamycin intoxication caused minimal changes in the hepatic pyridine nucleotide content *in vivo*. It is concluded that species- and tissue-specific differences in the distribution of antioxidant defense mechanisms may be primary determinants of the relative insensitivity of liver and, in contrast, the rather selective cardiomyopathy resulting from adriamycin administration *in vivo*.

Doxorubicin (adriamycin) is a broad spectrum antineoplastic agent effective in treating a variety of human cancers; however, the therapeutic utility is limited by the frequency of cumulative irreversible cardiomyopathy [1–3]. The mechanism by which adriamycin initiates tissue damage has been examined predominantly *in vitro*. Incubation of adriamycin with purified cytochrome P-450 reductase, hepatic microsomes, cardiac sarcosomes or submitochrondrial particles results in the utilization of NADPH, the consumption of molecular oxygen, the generation of semiquinone and oxygen radicals, and the initiation of peroxidation of membranous lipids [4–11]. Based on these observations, it has been proposed that the bioactivation of adriamycin and related anthracycline antibiotics involves enzyme-catalyzed univalent reduction of the quinone to form the semiquinone free radical intermediate [4–8]. In the presence of oxygen, the semiquinone is nonenzymatically reoxidized to form the parent quinone at the expense of generating reactive oxygen radicals. Finally, oxygen radicals thus formed during redox cycling of the quinone have been implicated in the initiation of tissue damage following adriamycin intoxication [9].

One of the ultimate consequences of adriamycin, as well as other free radical-mediated oxidants, is believed to be the peroxidation of membrane lipids leading to the disruption of subcellular functions vital to the viability of the cell and tissue [10, 11]. Once formed, lipid hydroperoxides serve as potent electrophilic substrates for cellular antioxidant enzymes, such as glutathione (GSH) peroxidase or pyridine nucleotide-dependent oxidases [12–16]. Cellular reducing equivalents thereby function as detoxifying agents and are consumed under conditions of excessive free radical fluxes and hydroperoxide formation.

Several correlates have been provided which suggest an important detoxifying or protective role for GSH and soluble sulfhydryl groups during adriamycin intoxication. *In vivo* administration of adriamycin to mice results in a significant decrease in heart, liver, and erythrocyte GSH concentration [17]. Pretreatment with the GSH-depleting agent diethylmaleate potentiates adriamycin lethality, whereas concomitant administration of sulfhydryl-containing agents, cysteamine or *N*-acetylcysteine, enhances survival following an acute intoxicating dose of adriamycin [17].

Despite the apparent central role of GSH in preventing adriamycin-induced tissue damage, cells are equipped with a variety of alternate antioxidants which may similarly influence tissue sensitivity. Examples include the reduced pyridine nucleotides and various metal complexes. The significance of pyridine necleotides in oxidative toxicity is demonstrated in the work of Brown *et al.* [18–20] who reported a depletion of pyridine nucleotide reducing equivalents following exposure to either hyperoxia or paraquat *in vivo*. Furthermore, supplementation of cellular NAD pools protects *Escherichia coli* from hyperoxic poisoning and diminishes the pulmonary toxicity of paraquat in rats.

According to proposed mechanisms, both soluble sulfhydryl agents and pyridine nucleotide reducing equivalents appear to be important constituents of cellular defense mechanisms involved in limiting oxidative tissue damage resulting from exposure to free radical generating substances. The mechanism of detoxification may be either by directly reducing the organo-oxygen radicals to more stable intermediates or by reducing toxic hydroperoxides to the corresponding alcohols.

The present investigation was designed to evaluate

* Forward all correspondence to: Kendall B. Wallace, Ph. D., Department of Pharmacology, School of Medicine, University of Minnesota-Duluth, Duluth, MN 55812.

the effect of acute adriamycin intoxication *in vivo* on redox homeostasis using liver tissue as a convenient, well characterized model system capable of rapidly bioactivating the drug. A comprehensive description of redox status was accomplished by evaluating the effect of adriamycin on hepatic GSH and pyridine nucleotide concentrations as well as the effects on enzymes responsible for regulating the concentration and distribution of various cellular reducing equivalents.

METHODS

Male, Sprague–Dawley rats (Harlan Laboratories, Madison, WI) were given a single bolus of adriamycin by tail vein injection. Control rats received an equal volume of isotonic NaCl (i.v.). At various times thereafter, the animals were decapitated, and the livers were perfused with warm isotonic NaCl (saline) and excised into ice-cold isotonic saline. The livers were then minced and portioned into the appropriate buffer for the respective biochemical determinations.

A portion of the liver (1 g) was homogenized (Polytron, Brinkmann Instruments, Westbury, NY) in 4.0 ml of 6% trichloroacetic acid (TCA) and then centrifuged at 1000 *g* for 15 min. The concentration of nonprotein sulfhydryls in the supernatant fraction was determined by the method of Ellman [21]. An aliquot (25–50 μl) of the supernatant fraction was added to 0.3 M Na_2HPO_4 to a final volume of 2.0 ml. The increase in absorbance at 412 nm following addition of 0.04% 5,5′-dithiobis(2-nitro)benzoic acid (DTNB) was used as a measurement of sulfhydryl content ($E_{412\,nm} = 13.6\,mM^{-1}\,cm^{-1}$).

Oxidized and reduced pyridine nucleotides were selectively extracted from liver by homogenization of 1.0 g in 1.0 ml of 0.25 M glycylglycine buffer (pH 9.0) to which was added either 2.0 ml of hot 0.1 N HCl or 2.0 ml of hot 0.1 N NaOH, respectively. The samples were homogenized a second time and cooled on ice. The pH of the samples was then adjusted to 7.4–7.6 followed by centrifugation at 10,000 *g* for 10 min. The pellets were discarded, and the supernatant fraction was saved for polarographic determination of pyridine nucleotide concentration [22]. NAD and NADH concentrations were measured in an oxygen electrode (YSI model 53) chamber containing a total of 2.0 ml of 0.25 M glycylglycine (pH 7.6), 1 mM EDTA, 0.05% ethanol, 0.3 mg alcohol dehydrogenase (Sigma Chemical Co., St. Louis, MO), 0.2 mg phenazine methosulfate, and 10–300 μl of the acid or alkaline tissue extract, respectively. The concentration of phosphorylated pyridine nucleotides was estimated from the rate of oxygen consumption in a 2.0-ml reaction mixture containing 0.1 M Tris–1 mM EDTA (pH 8.0), 5 mM glucose-6-phosphate (G6P), 5 μg G6P dehydrogenase, 0.2 mg phenazine methosulfate, and 10–300 μl tissue extract. Polarographic molar extinction coefficients were calculated using commercial pyridine nucleotide standards (Sigma Chemical Co.).

All enzyme activities were determined using the 100,000 *g* supernatant fraction of 20% tissue homogenates in 0.2 M potassium phosphate buffer (pH 7.4). Glutathione peroxidase (GPx) was measured by a modification of the method of Paglia and Valentine [23]. The reaction mixture (2.5 ml) contained 1 mM GSH, 0.75 units exogenous GSH reductase, 0.1 mM NADPH, 0.5 mM *t*-butylhydroperoxide, and approximately 20 μg supernatant protein in 0.1 M 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid (HEPES; pH 7.5)–0.1 mM EDTA. GPx activity was estimated from the rate of oxidation of NADPH ($E_{340\,nm} = 58\,mM^{-1}\,cm^{-1}$).

Glutathione reductase (GRed) activity was measured spectrophotometrically (340 nm) from the rate of oxidation of NADPH in the presence of exogenous oxidized glutathione (GSSG) according to the method of Racker [24]. The reaction mixture (2.5 ml) consisted of 0.1 mM NADPH, 3 mM GSSG, and approximately 0.3 mg supernatant protein in 0.02 M potassium phosphate (pH 7.6)–0.1 mM EDTA.

Glucose-6-phosphate dehydrogenase (G6P-d) activity was determined by the spectrophotometric procedure described by Langdon [25]. Approximately 1 mg of supernatant protein was incubated in a total of 2.5 ml of 25 mM Tris (pH 7.5)–10 mM $MgCl_2$ containing 1 mM G6P and 0.1 mM NADP. G6P dehydrogenase activity was estimated from the rate of NADP reduction as monitored by the increase in absorbance at 340 nm. Malate dehydrogenase (Mal-d) activity was measured in a similar fashion after substituting the reaction with 1 mM malic acid and 1 mM NAD.

Statistical comparison of treatment groups was performed by randomized analysis of variance [26]. Significant differences between means were detected by the Student's paired *t*-test. A probability of $P < 0.05$ was used as the criterion for significance.

Statistical comparisons were performed for each treatment group with time-adjusted control animals, which received isotonic saline and were killed at the same times as the corresponding adriamycin-treated rats. All animals were killed between 8:00 a.m. and noon, thereby avoiding significant differences between control groups due to circadium rhythms. Since there were no differences, all control data were pooled and averaged to comprise the values depicted at zero hours of treatment. Preliminary experiments demonstrated no direct effect of adriamycin on the *in vitro* assay for hepatic sulfhydryl or pyridine nucleotide content as well as no effect on GPx, GRed, G6P-d or Mal-d enzyme activities.

RESULTS

In a preliminary experiment (data not shown), all eight adult male rats given a single intravenous injection of 12 mg/kg adiamycin survived for 6 days. However, 25% of the animals succumbed by day 7 and only three rats survived until day 8 after treatment. The percent initial (pretreatment) body weight decreased gradually from 98% on day 4 to 85% 7 days following adriamycin treatment. Neither the liver to body weight ratio nor the hepatic protein concentration were altered significantly during the first 4 days following i.v. administration of 12 mg/kg adriamycin.

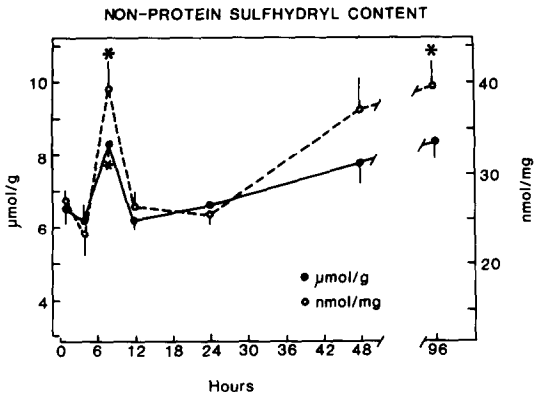

Fig. 1. Hepatic non-protein sulfhydryl (NPSH) content following intravenous administration of 12 mg/kg adiamycin to rats. Each point represents the mean ± S. E. of four animals. Values are expressed per g of liver or per mg protein. Asterisks (*) indicate a statistically significant difference compared to saline-injected control animals ($P < 0.05$).

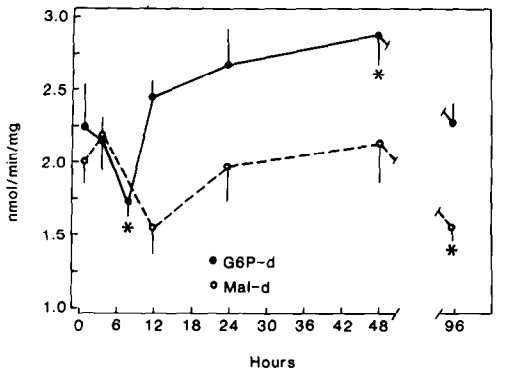

Fig. 3. Effect of 12 mg/kg adriamycin (i.v.) on the activities of glucose-6-phosphate dehydrogenase (G6P-d) and malate dehydrogenase (Mal-d) in 100,000 *g* supernatant fractions of adult rat liver homogenates. Each point represents the mean ± S.E. of four animals. Asterisks (*) indicate a statistically significant difference compared to saline-injected control animals ($P < 0.05$).

In contrast to that reported in mice [17], *in vivo* administration of adriamycin to rats did not lead to a depletion of hepatic non-protein sulfhydryl (NPSH) groups (Fig. 1). The concentration of NPSH 4 hr following i.v. injection of adriamycin was slightly, yet not significantly, diminished compared to control animals. This was followed by a 30–70% increase to values significantly greater than that of time-adjusted controls, whether expressed on the basis of liver weight or protein concentration. Hepatic NPSH concentration subsequently diminished to control values by 12 hr and remained relatively unchanged throughout 24 hr of treatment. Finally, NPSH concentration increased gradually until by 4 days hepatic NPSH content of treated animals was significantly greater than that of controls when expressed on a per mg protein basis.

Time-dependent changes in hepatic NPSH were not paralleled by adriamycin-induced changes in the activities of enzymes regulating the glutathione redox potential (Fig. 2). The activities of both GPx and GRed did not change significantly until 12 hr after i.v. injection at which time both enzyme activities were diminished to approximaely 75% that of time-adjusted control rats. Both GPx and GRed activities subsequently increased to control values by 24 hr post-treatment and remained unchanged at 2 days. On day 4, however, the activity of both enzymes was only half that of control liver. Hepatic G6P-d activity was decreased significantly (77% of control) 8 hr after adriamycin administration and then returned to control values by 12 hr (Fig. 3). Adriamycin-induced changes in hepatic Mal-d activity tended to parallel that of G6P-d (Fig. 3). Mal-d activity was diminished, but not significantly, at 12 hr followed by an increase to control values by 24 hr following drug treatment. At 2 days, hepatic G6P-d activity was significantly greater than that of control liver. In contrast, hepatic Mal-d activity remained relatively unchanged between 24 and 48 hr

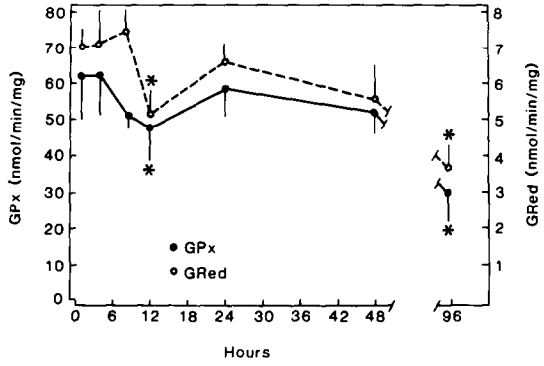

Fig. 2. Effect of 12 mg/kg adriamycin (i.v.) on the activities of glutathione peroxidase (GPx) and glutathione reductase (GRed) in 100,000 *g* supernatants fractions of adult rat liver homogenates. Each point represents the mean ± S.E. of four animals. Asterisks (*) indicate a significant difference compared to saline-injected controls when expressed on a per mg protein basis ($P < 0.05$).

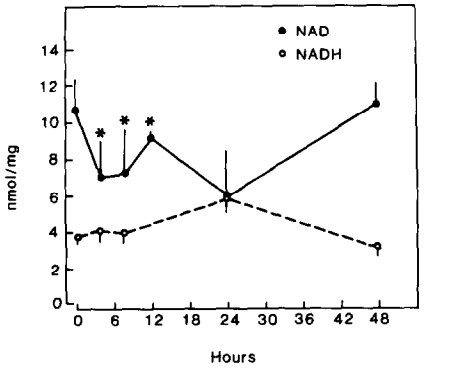

Fig. 4. Effect of 12 mg/kg (i.v.) adriamycin on hepatic NAD and NADH concentrations. Each point represents the mean ± S.E. of the pyridine nucleotide concentration in extracts from crude liver homogenates from four animals. Asterisks (*) indicate a significant difference from controls ($P < 0.05$).

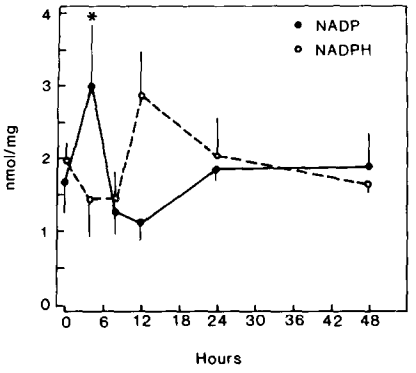

Fig. 5. Effect of 12 mg/kg (i.v.) adriamycin on hepatic NADP and NADPH concentrations in rats. Each point represents the mean ± S.E. of determinations of phosphorylated pyridine nucleotides in crude liver extracts from four animals. Asterisks (*) indicate a significant difference compared to saline-injected control rats ($P < 0.05$).

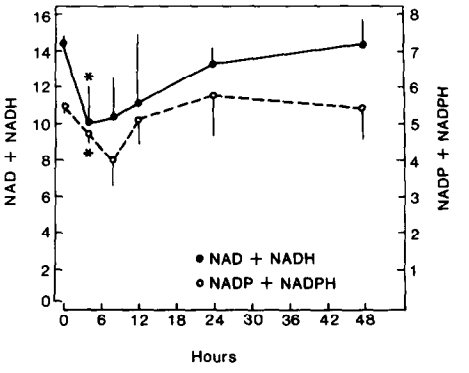

Fig. 7. Effect of adriamycin (12 mg/kg, i.v.) on total hepatic pyridine nucleotide content expressed on a per mg protein basis. Each point represents the mean ± S.E. of four determinations. Asterisks (*) indicate a significant difference compared to controls ($P < 0.05$).

after adriamycin treatment. Both the hepatic G6P-d and Mal-d activities decreased between day 2 and day 4 following adriamycin administration.

Intravenous injection of adriamycin resulted in a sustained decrease in hepatic NAD concentration (*ca* 65% of control) throughout the first 12 hr following drug treatment (Fig. 4). The concentration of NAD in adriamycin-treated livers subsequently returned to control values by 48 hr. In contrast, *in vivo* administration of adriamycin caused negligible changes in the concentration of NADH in liver.

The time-dependent changes in hepatic phosphorylated pyridine nucleotide concentration were highly variable, especially during the first 12 hr following adriamycin administration (Fig. 5). The only significant drug effect detected was an increase in NADP at 4 hr. However, hepatic NADP content returned to control values by 8 hr after drug treatment. No significant difference in the hepatic concentration of either NADP or NADPH compared to control animals was detected 4 days following adriamycin administration.

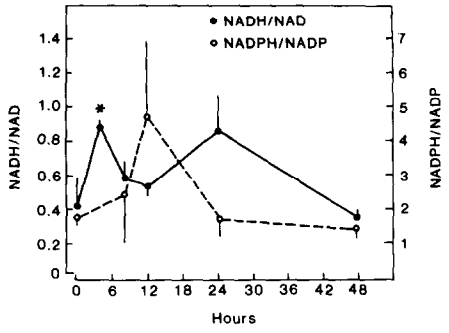

Fig. 6. Effect of adriamycin 12 mg/kg (i.v.) on the ratios of reduced to oxidized pyridine nucleotides in crude liver homogenates. Each point represents the mean ± S.E. of determinations from four animals. Asterisks (*) indicate a significant difference compared to saline-injected control animals when expressed on a per mg protein basis ($P < 0.05$).

The drug-induced decrease in hepatic NAD concentration was reflected by a 2-fold increase in the ratio of NADH/NAD 4 hr after adriamycin injection (Fig. 6). The ratio of NADH/NAD subsequently decreased to control values by 8 hr. In contrast, the measurement of NADPH/NADP was highly variable, and no significant difference was observed for the redox ratio of phosphorylated pyridine nucleotides at any of the times examined.

Finally, the adriamycin-induced depression of hepatic NAD concentration was also reflected by a significant (30%) decrease in the total nonphosphorylated pyridine nucleotide content 4 hr after drug treatment (Fig. 7). Similarly, despite no significant decrease in NADP or NADPH concentration (Fig. 5), a significant (15%) decrease was observed for the total hepatic phosphorylated pyridine nucleotide content 4 hr following intravenous injection of adriamycin (Fig. 7). The total hepatic content of both phosphorylated and nonphosphorylated pyridine nucleotides subsequently returned to control values by 12 hr and remained relatively unchanged throughout the remainder of the 48-hr observation period.

DISCUSSION

According to the proposed mechanism of adriamycin toxicity, oxygen radicals generated during the nonenzymatic reoxidation of semiquinone intermediates are primarily responsible for eliciting the irreversible organopathy. Such a frec radical mechanism is similar to that implicated in the actions of a variety of other toxicants including hyperoxia, oxidant gasses, paraquat, bleomycin, and perhaps carbon tetrachloride. Oxygen radicals, regardless of the source, are highly electrochemically unstable and react rapidly and nonselectively with various tissue macromolecules. The interaction may be covalent ionic binding or univalent electron transfer reactions. The products of such redox reactions are predominantly unstable ionic intermediates, and ultimately undergo further reactions such as hydrolysis, desatu-

ration or subsequent univalent reduction-oxidation to form more stable secondary products. The ultimate effect of such reactions may be manifest as the covalent modification of biomolecules or interference with vital cellular regulatory processes.

Cells are equipped with a variety of defense mechanisms to combat such oxidative stresses. Constituents include superoxide dismutase, catalase, soluble sulfhydryl agents coupled with the glutathione peroxidase-reductase system, pyridine nucleotide reducing equivalents, as well as glucose-6-phosphate dehydrogenase which replenishes NADPH through activation of the hexose-monophosphate shunt. Accordingly, tissue damage or cytotoxicity is likely to be indicative of oxidative stress which overwhelms the proficiency of endogenous antioxidant defense mechanisms. As a consequence, cellular reducing equivalents are depleted and intermediates, such as peroxides, accumulate within the tissue leading to irreversible loss of cell function. Replenishing cellular reducing equivalents may retard the progression of oxidative damage either by directly reducing oxygen free radical intermediates or by alkylating toxic intermediates of cellular metabolism. In the case of lipid peroxidation, reactive lipid hydroperoxides are postulated to be reduced to the corresponding alcohols in the presence of sulfhydryl agents.

The participation of oxygen free radical-mediated lipid peroxidation in adriamycin-induced tissue damage is supported by several experimental observations. Numerous investigators have demonstrated the generation of semiquinone and oxygen free radicals during adriamycin metabolism *in vitro* [4–11]. Goodman and Hochstein [27] and Thayer [9] reported the production of lipid peroxides in adriamycin-exposed hepatic and cardiac tissues and proposed that free radical-mediated lipid peroxidation may be the major mechanism of adriamycin toxicity. Addition of superoxide dismutase, GSH, EDTA, alpha-tocopherol, or dimethylurea to incubations of liver or heart microsomal fractions inhibited the adriamycin-induced production of lipid peroxides [28]. Furthermore, treatment of mice with alpha-tocopherol delays or diminishes the extent of lipid peroxidation as well as lethality following acute adriamycin intoxication [10, 11, 29, 30]. Finally, dietary selenium restriction decreases cardiac glutathione peroxidase activity and potentiates adriamycin lethality in mice [31]. These data provide strong support for the proposal that the primary mechanism of adriamycin toxicity involves stimulation of lipid peroxidation associated with the depletion of vital stores of cellular reducing equivalents.

In contrast to the suppression of hepatic GSH content in mice demonstrated by Olson and coworkers [17], no significant depletion of hepatic NPSH was detected after intravenous injection of adriamycin to rats. Explanations for such a discrepancy include that due to possible genetic differences between species, or to the differences in the route of administration. Although adriamycin has repeatedly been shown to deplete GSH content in murine tissues [17, 31], this effect is far less remarkable in rats. Incubation of rat hepatocytes with 0.1 mM adriamycin causes a slight, but insignificant, decrease in cellular GSH concentration [32]. Not unlike that demonstrated with other toxicants, this species-difference probably reflects a greater capacity of rat liver to maintain GSH homeostasis and thus to better withstand oxidative stress compared to the hepatotoxic susceptibility of mice.

Hepatic GSH content represents a dynamic homeostasis, the steady-state concentration being under direct regulation by the rate of utilization through both redox (GPx) and alkylating (GSH *S*-aryl transferase) reactions and by the rate of synthesis, catalyzed either by GRed or by GSH synthetase. In view of the univalent free radical mechanism of toxicity, it would be suspect that GSH participates in adriamycin metabolism by providing reducing equivalents to stabilize highly reactive peroxide intermediates. Consequently, GSH is likely oxidized to GSSG, with the steady-state concentration of GSH being determined primarily by the relative efficiency of GPx and GRed. The failure to detect a significant depletion of GSH following acute adriamycin intoxication may therefore reflect a sufficiently adequate battery of hepatic GRed to accommodate the drug-induced oxidation of GSH. It is interesting that hepatic GSH was maintained within control limits despite a significant drug-induced suppression of both GPx and GRed activities. This, in turn, provides additional support for a well facilitated metabolic capability of the liver to regulate an adequate GSH redox homeostasis in the face of considerable oxidative stress. The similar pattern of suppression of all four enzyme activities examined suggests a common effect of adriamycin, perhaps on protein synthesis. It may be that the early (4–12 hr) suppression of enzyme activity reflects the antineoplastic effect of the drug, whereas the delayed decrease in enzyme activity at 4 days coincides with a general deteriorization of the health of the animal.

Doroshow and associates [31] suggest that tissue-specific differences in the distribution of antioxidant defense mechanisms may be a primary determinant of the rather selective cardiotoxicity of adriamycin. These investigators reported that mouse cardiac tissue contains 25% as much superoxide dismutase and only a fraction of the catalase activity compared to liver [27]. Such a relative deficiency in antioxidant defense mechanisms may limit the ability of cardiac tissue to dispose of peroxides and thereby withstand toxic concentrations of adriamycin-induced free radical fluxes. Although perhaps the primary source, GSH is not the sole substrate for reductive detoxification of reactive intermediates of free radical reactions. Certainly, either NADH or NADPH could feasibly serve as cofactors to various reductive enzymes contributing to the ability of the cell to withstand accelerated rates of production of toxic intermediates. Both pyridine nucleotides have previously been implicated as beneficial redox reactants in free radical mechanisms of toxicity [18–20].

Similar to the lack of a depletion of GSH, we observed no decrease in either hepatic NADH or NADPH concentration following acute adriamycin intoxication. This occurred despite the fact that NADPH is apparently a required substrate for the bioactivation of adriamycin [4–8]. The only significant effect on hepatic pyridine nucleotide con-

centration observed was a sustained decrement in NAD content between 4 and 12 hr following a single intravenous dose of adriamycin. The depletion of NAD was reflected by an increase in the redox ratio of NADH/NAD and by a decrease in total pyridine nucleotide concentration shortly after adriamycin administration. The exact explanation for this curious result remains tentative, but may involve rather selective enhancement of the rate of degradation of the nucleotide. On the other hand, Brown and Song [19] attribute the decrease in cellular NAD following hyperbaric hyperoxia to an inhibition in coenzyme synthesis. Regardless, liver tissue was demonstrated to be adequately equipped with sufficient regulatory processes to sustain near normal redox homeostasis of pyridine nucleotides throughout the experimental period, despite adriamycin-induced suppression of both glucose-6-phosphate dehydrogenase and malate dehydrogenase activities.

In conclusion, although the proposed mechanism of bioactivation and toxicity of adriamycin suggests an important role of cellular reducing equivalents to detoxify reactive intermediates, liver tissue appears to be equipped with sufficient antioxidant enzymes to sustain adequate intracellular redox homeostasis. Accordingly, rat liver appears to be capable of metabolically accommodating the flux of reactive intermediates associated with adriamycin bioactivation, thereby preventing irreversible tissue damage in response to toxic concentrations of the drug *in vivo*. On the other hand, as suggested by Doroshow and coworkers [31], the deficiency of cardiac antioxidants may be the primary determinant of tissue selectivity in adriamycin-induced cardiomyopathy. This selective tissue toxicity occurs despite the greater rate of bioactivation of adriamycin and stimulation of *in vitro* lipid peroxidation by hepatic microsomes compared to that of cardiac tissue [28]. Accordingly, the primary determinant of adriamycin organopathy appears to be the proficiency of antioxidant defense mechanisms in selected tissues.

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