Peroxidase- and Nitrite-Dependent Metabolism of the Anthracycline Anticancer Agents Daunorubicin and Doxorubicin†

Krzysztof J. Reszka,*,‡,§ Michael L. McCormick,‡,§,|| and Bradley E. Britigan‡,§,||,⊥

Research Service and Department of Internal Medicine, VA Medical Center, and Department of Internal Medicine and Free Radical and Radiation Biology Program of the Department of Radiology, University of Iowa, Iowa City, Iowa 52246

Received October 1, 2001

ABSTRACT: Oxidation of the anticancer anthracyclines doxorubicin (DXR) and daunorubicin (DNR) by lactoperoxidase(LPO)/H_2O_2 and horseradish peroxidase(HRP)/H_2O_2 systems in the presence and absence of nitrite (NO_2^-) has been investigated using spectrophotometric and EPR techniques. We report that LPO/H_2O_2/NO_2^- causes rapid and irreversible loss of anthracyclines' absorption bands, suggesting oxidative degradation of their chromophores. Both the initial rate and the extent of oxidation are dependent on both NO_2^- concentration and pH. The initial rate decreases when the pH is changed from 7 to 5, and the reaction virtually stops at pH 5. Oxidation of a model hydroquinone compound, 2,5-di-*tert*-butylhydroquinone, by LPO/H_2O_2 is also dependent on NO_2^-; however, in contrast to DNR and DXR, this oxidation is most efficient at pH 5, indicating that LPO/H_2O_2/NO_2^- is capable of efficiently oxidizing simple hydroquinones even in the neutral form. Oxidation of anthracyclines by HRP/H_2O_2/NO_2^- is substantially less efficient relative to that by LPO/H_2O_2/NO_2^- at either pH 5 or pH 7, most likely due to the lower rate of NO_2^- metabolism by HRP/H_2O_2. EPR measurements show that interaction of anthracyclines and 2,5-di-*tert*-butylhydroquinone with LPO/H_2O_2/NO_2^- generates the corresponding semiquinone radicals presumably via one-electron oxidation of their hydroquinone moieties. The possible role of the $^{\bullet}NO_2$ radical, a putative LPO metabolite of NO_2^-, in oxidation of these compounds is discussed. Because in vivo the anthracyclines may co-localize with peroxidases, H_2O_2, and NO_2^- in tissues, their oxidation via the proposed mechanism is likely. These observations reveal a novel, peroxidase- and nitrite-dependent mechanism for the oxidative transformation of the anticancer anthracyclines, which may be pertinent to their biological activities in vivo.

The anthracycline antibiotics doxorubicin (DXR, adriamycin)[1] and daunorubicin (DNR) are the most frequently prescribed anticancer agents. They are useful in the treatment of a number of malignancies including leukemia, non-Hodgkin's lymphoma, and breast cancer (*1*). The clinical efficacy of these agents is, however, severely limited by dose-dependent side effects, especially cardiotoxicity and development of drug resistance (*2*, *3*). Although several modes of action have been postulated, the exact molecular mechanisms responsible for the biological activities of the anthracyclines is (are) still not well understood. One potential mechanism is based on the ability of these agents to participate in electron-transfer processes, with the subsequent generation of free radicals. This property results from the presence in DNR and DXR of two very different types of redox-active groups, namely, the quinone and hydroquinone moieties (Figure 1, rings C and B, respectively). While these groups are both capable of generating free radicals, the mechanisms responsible are quite different.

Previous studies have shown that the quinone moiety of either DNR or DXR can undergo one-electron reduction to a semiquinone radical, which in the presence of oxygen gives rise to superoxide and other reactive oxygen species (ROS) (*4*−*9*). This reductive activation can be catalyzed by NADPH−cytochrome P450 reductase and mitochondrial NADH dehydrogenase (*4*−*9*). Recently it has been reported that the reductase domain of endothelial nitric oxide synthase is also able to reduce DXR and therefore stimulate superoxide production (*10*, *11*). The importance of this redox cycling stems from the fact that superoxide dismutates to H_2O_2 which, in the presence of iron, gives rise to hydroxyl radicals. Generation of ROS is considered to be especially important in development of anthracycline-dependent cardiomyopathy.

Generation of free radicals by the second redox-active group, the hydroquinone moiety (Figure 1, ring B), can be accomplished by its oxidation. In contrast to the reductive

† This work was supported by Merit Review research grants from the Research Service of the Department of Veterans Affairs (M.L.M., B.E.B.), and Research Grant AI34954 from the National Institutes of Health (B.E.B.).

* Address correspondence to this author at the VA Medical Center, Research-151, 601 Hwy 6 W., Iowa City, IA 52246. Phone: (319) 338-0581, ext.7548. Fax: (319) 339-7162. E-mail: reszka@icva.gov.

‡ Research Service, VA Medical Center.

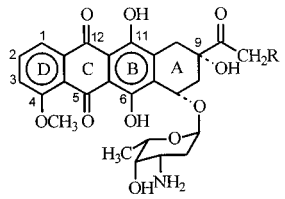
§ Free Radical and Radiation Biology Program of the Department of Radiology, University of Iowa.

|| Department of Internal Medicine, University of Iowa.

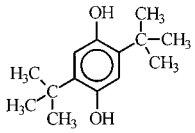
⊥ Department of Internal Medicine, VA Medical Center.

[1] Abbreviations: ABTS, 2,2′-diazinobis(3-ethylbenzothiazoline-6-sulfonic acid); 1,4-DHB, 1,4-dihydroxybenzene (*p*-hydroquinone); DNR, daunorubicin; DXR, doxorubicin (Adriamycin); 2,5-DTBHQ, 2,5-di-*tert*-butylhydroquinone; EPO, eosinophil peroxidase; EPR, electron paramagnetic resonance; HRP, HRP-I, HRP-II, horseradish peroxidase and its compounds I and II; hfsc's, hyperfine splitting constants; LPO, LPO-I, LPO-II, lactoperoxidase and its compounds I and II; MPO, myeloperoxidase; ROS, reactive oxygen species.

10.1021/bi011869c
Published on Web 11/20/2001



R: H Daunorubicin (DNR)
R: OH Doxorubicin (Adriamycin) (DXR)



2,5-Di-*tert*-butylhydroquinone (2,5-DTBHQ)

FIGURE 1: Structures of anticancer anthracyclines and 2,5-DTBHQ.

activation described above, oxidative metabolism of these compounds has rarely been considered. It has been reported that in alkaline solutions the hydroquinone moiety of DXR undergoes autoxidation, forming free radicals and leading to oxygen consumption (*12*, *13*). Another process that can lead to oxidation of anthracyclines is interaction with redox-active metal ions. It has been found that at physiological pH, both DNR and DXR form strong complexes with Fe^{3+} (*14*−*19*), but only the DXR−Fe^{3+} chelate leads to ROS formation (*16*, *17*, *19*). However, generation of ROS by DXR−Fe^{3+} chelates appeared not to involve the hydroquinone moiety. Rather, the hydroxyl group in the alkyl side chain at C9 in DXR appears to be responsible, since analogous DNR−Fe^{3+} chelates, in which the ligand (i.e., DNR) differs in only the structure of the side chain, were unable to generate ROS (*16*, *17*, *19*).

One particular mechanism through which hydroquinones can give rise to reactive/cytotoxic products such as free radicals or electrophilic quinones is via oxidation by peroxidase/H_2O_2 systems. Such a mechanism may be particularly relevant in vivo (at sites of inflammation or cancer) where the anthracyclines can be exposed to various peroxidases, such as lactoperoxidase (LPO), myeloperoxidase (MPO), and eosinophil peroxidase (EPO). Phenolics and hydroquinones are classic examples of peroxidase substrates (*20*−*25*). Anticancer agents such as mitoxantrone (*26*, *27*), anthrapyrazoles (*28*), 5-iminodaunorubicin (*29*), and 4-hydroxytamoxifen (*30*), all of which contain phenolic and/or hydroquinone moieties, undergo oxidative activation by HRP/H_2O_2. In contrast, DXR and DNR appear to be resistant to this type of metabolism (*29*). While oxidation of highly concentrated DXR (10 mg/mL in DMSO) to a free radical was accomplished employing very large doses of HRP (10 mg/mL) (*13*), a more recent study showed that oxidation of DXR, but not DNR, can be accomplished employing micromolar concentrations of HRP in aqueous buffer (*31*).

Peroxidative metabolism involves several steps, the first of which is the interaction of peroxidase with H_2O_2 to form a reactive intermediate, compound I, which is 2 oxidizing equiv above the resting (ferric) state of the enzyme (*20*). In the presence of a suitable substrate, compound I is reduced back to the enzyme's resting state in two one-electron-transfer steps with the intermediacy of compound II, which is the product of the one-electron reduction of compound I. During this catalytic turnover, two molecules of the reducing substrate are oxidized to radicals. Equations 1−3 describe the typical peroxidative cycle for horseradish peroxidase (HRP) with hydroquinone (QH_2) as a reducing substrate:

$$\text{HRP } (P-Fe^{3+}) + H_2O_2 \rightarrow \text{HRP-I } (P^{\bullet+}-Fe^{IV}=O) \quad (1)$$

$$\text{HRP-I} + QH_2 \rightarrow \text{HRP-II } (P-Fe^{IV}=O) + Q^{\bullet-} + 2H^+ \quad (2)$$

$$\text{HRP-II} + QH_2 \rightarrow \text{HRP } (P-Fe^{3+}) + Q^{\bullet-} + 2H^+ \quad (3)$$

where HRP (P−Fe^{3+}), HRP-I ($P^{\bullet+}$−Fe^{IV}=O), HRP-II (P−Fe^{IV}=O), QH_2, and $Q^{\bullet-}$ represent HRP in the resting state, HRP compound I, HRP compound II, hydroquinone, and a semiquinone radical, respectively. P designates the porphyrin moiety of the heme.

Despite many structural and functional similarities, HRP, an enzyme of plant origin, differs markedly from the mammalian peroxidases (LPO, MPO, EPO), especially regarding substrate specificity and reactivity of their respective compounds I and II. We were therefore interested to examine whether anthracyclines could be oxidized by mammalian peroxidases, as this can be pertinent to their activities in vivo.

In the present work, we carried out detailed investigations of the interaction of DNR and DXR with LPO/H_2O_2, and examined the role of nitrite (NO_2^-) in these reactions. LPO has been identified in breast tissues (*32*, *33*), in airway mucous (*34*), and in saliva and tears (*35*). In the presence of H_2O_2, the enzyme forms a similar reactive intermediate, LPO compound I (LPO-I), and its reactions with QH_2 can be described by equations analogous to those for the HRP system (eqs 1−3). NO_2^- is included in this study because it is a recognized substrate for peroxidases (*36*−*39*) and because it is known to catalyze peroxidase-mediated oxidation of various biological targets, including other anticancer agents (*40*−*46*). In addition, NO_2^- is present in the body, being produced in tissues by aerobic oxidation of $^{\bullet}NO$, by anaerobic reduction of nitrate (NO_3^-), or delivered with the diet. Because NO_2^- may co-localize with peroxidases, peroxide, and anticancer agents in vivo, it is important to investigate its effects on oxidation of anthracyclines. Parallel experiments using HRP/H_2O_2 were also performed. Our results point to new and potentially important mechanisms of oxidative modification of anthracycline anticancer agents, which may be pertinent to their biological activities.

MATERIALS AND METHODS

Chemicals. DNR (hydrochloride form) was obtained from the Drug Synthesis and Chemistry Branch, Developmental Therapeutics Program, Division of Cancer Treatment and Diagnosis, National Cancer Institute, NIH (Bethesda, MD). Pharmaceutical preparation of DXR (hydrochloride form) solution for injection (2 mg/mL) (Ben Venue Laboratories, Inc., Bedford, OH) was purchased from The University of Iowa Hospitals and Clinics Pharmacy (Iowa City, IA). Stock solutions of DNR (10 mM) were prepared in deionized water and stored at 4 °C. 2,5-Di-*tert*-butylhydroquinone (2,5-DTBHQ, 99%) (Aldrich) was dissolved in methanol (0.1 M). LPO from bovine milk (EC 1.11.1.7) and HRP (Type VI, EC 1.11.1.7) were from Sigma (St. Louis, MO). H_2O_2 (30%) and sodium nitrite were from Fisher Scientific (Fair Lawn, NJ). 2,2′-Diazinobis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) was purchased from Aldrich (Milwaukee, WI). All chemicals were of the highest purity available. The concentrations of stock solutions of H_2O_2 ($\epsilon_{240} = 39.4\ M^{-1}\ cm^{-1}$), LPO ($\epsilon_{412} = 1.12 \times 10^5\ M^{-1}\ cm^{-1}$), HRP ($\epsilon_{403} = 1.02 \times 10^5\ M^{-1}\ cm^{-1}$), NO_2^- ($\epsilon_{355} = 23\ M^{-1}\ cm^{-1}$), and ABTS

($\epsilon_{340} = 3.6 \times 10^4$ M^{-1} cm^{-1}) were determined spectrophotometrically using appropriate extinction coefficients. Although anthracyclines tend to form dimers and higher aggregates in aqueous solutions, at concentrations used in this study, 50 μM or less, they are present mostly as monomers (*18*, *47*). The extent of aggregation of anthracyclines is even less at acidic pH, due to protonation of the amino group in the daunosamine moiety ($pK_a = 7.48$, *48*). Therefore, dimerization of these agents should have only a minimal effect on our results.

Peroxidative Activity of LPO and HRP. The peroxidative activity of LPO and HRP was determined using an ABTS assay (*49*). In this assay, ABTS undergoes oxidation to a relatively stable radical cation ($ABTS^{\bullet+}$) which is characterized by two intense absorption bands at 414 nm ($\epsilon_{414} = 3.6 \times 10^4$ M^{-1} cm^{-1}) and 660 nm ($\epsilon_{660} = 1.2 \times 10^4$ M^{-1} cm^{-1}). Briefly, to ABTS (50 μM) in pH 7.0 buffer (50 mM, phosphate) containing H_2O_2 (50 μM) was added an aliquot of LPO (or HRP), and the time course of absorption changes at 660 nm was recorded. The initial rate of ABTS oxidation, measured as the initial slope of the A_{660} versus time traces, was taken as a measure of enzyme activity.

Spectrophotometric Measurements. Absorption spectra of aqueous solutions of DNR show a broad band centered around 480 nm (Figure 2A, curve *a*). The position of this absorption maximum does not change appreciably in the pH range used in this study, although its intensity increases slightly upon acidification as a result of increased content of monomers at acid pH. Spectra of DXR are very similar to those of DNR. Because of this intense characteristic absorption in the visible region, oxidation of DNR and DXR was studied by following changes in their absorption spectra using a Hewlett-Packard diode array spectrophotometer model 8453. Measurements were performed in phosphate or acetate buffers (50 mM) in the pH range 8.0−4.7 at room temperature. Solutions of anthracycline in buffer of a given pH contained NO_2^- and H_2O_2 at the desired concentrations, and, unless otherwise stated, the reaction was initiated by addition of LPO (or HRP) as the last component. Time course measurements were carried out following changes in absorbance at 480 nm. Data were collected in 2 or 5 s intervals during continuous stirring of the sample in the cuvette (1 cm light path). The initial rates of oxidation, V_i, were determined from initial slopes of absorbance versus time traces, $\Delta A_{480}/\Delta t$ (using a linear regression fitting procedure), and converted into M s^{-1} units by dividing by 11 500 M^{-1} cm^{-1}, the molar absorptivity of DNR and DXR at 480 nm (*18*, *47*). Plots were prepared using V_i determined from fits with $r > 0.99$. In preliminary experiments, it was established that at pH 7.0 and at constant [LPO], [H_2O_2], and [NO_2^-], V_i increased continuously when [DNR] changed from 0 to 50 μM. At [DNR] >50 μM, V_i changed insignificantly (not shown). Therefore, all experiments were carried out using [DNR] not exceeding 50 μM, as this concentration seems to be a good compromise between the requirement that the compound be present predominantly in the monomeric form and that V_i should be invariant with respect to small changes in [DNR]. The same conditions were applied to DXR samples.

Oxidation of 2,5-DTBHQ was studied using procedures similar to those applied for DNR. The absorption spectrum of 2,5-DTBHQ shows a maximum at 288 nm, which is virtually insensitive to changes in pH in the investigated pH range 7.48−5.08 (Figure 7A,B). Upon reaction with LPO/H_2O_2/NO_2^-, 2,5-DTBHQ undergoes oxidation to a respective quinone, 2,5-DTB quinone, with an absorption maximum at 260 nm. At acid pH, the product aggregates/polymerizes as evidenced by increased noncharacteristic absorption extending into the visible region of the spectrum (Figure 7B). For this reason, at all pHs the time course of the reaction was monitored at 260 nm (product accumulation).

Reaction of DNR with $^{\bullet}NO_2$. Two milliliters of DNR (ca. 50 μM), in either pH 7.4 (0.1 M, phosphate) or pH 5.0 (0.1 M, acetate) buffer, was transferred to a 10 mL plastic tube, that was sealed with a rubber septum. With a gastight syringe, a desired volume of air-diluted dinitrogen tetroxide (N_2O_4, 99.5%, Aldrich) was injected into the headspace of the DNR solution through the septum. To facilitate the reaction, the samples were vigorously mixed by vortexing. Dilution was necessary to shift the equilibrium $N_2O_4 \leftrightarrow 2^{\bullet}NO_2$ to the right to expose the drug primarily to monomeric $^{\bullet}NO_2$. Dilution also avoids complications due to the fact that N_2O_4 reacts rapidly with water to form NO_2^- and NO_3^-, and causes acidification, which affects the redox property of DNR. The pH of the samples checked before and after the reaction showed only a small decrease, from 7.4 (5.0) for a nontreated sample to 7.22 (4.88) for sample to which the largest amount of $^{\bullet}NO_2$ was added. We consider this drop in pH to affect redox properties of DNR only minimally. Oxidation of DNR was measured by following changes in the absorption spectrum of the drug. The amount of $^{\bullet}NO_2$ added to samples was determined by measuring [NO_2^-] and [NO_3^-] in samples by using a chemiluminescence technique using a Sievers nitric oxide analyzer as described (*50*).

EPR Measurements. EPR measurements were conducted using a Bruker EMX EPR spectrometer with 100 kHz modulation and equipped with a TM_{110} cavity. Because of limited solubility in aqueous solutions, samples of anthracyclines for EPR analysis (~1 mM) were prepared in pH 7.0 buffer/MeOH (2:1 v/v) mixtures. For the same reason, samples of 2,5-DTBHQ were prepared in pH 6.0/MeOH (260:200 v/v) mixture. The reaction was initiated by addition of an aliquot of H_2O_2 as the last component. Next the sample was transferred to an aqueous flat EPR cell, bubbled briefly with N_2 gas, and positioned in the cavity, and scans were executed promptly. All experiments were carried out at room temperature.

RESULTS

(A) Oxidation of Anthracyclines by LPO/H_2O_2/NO_2^-

Requirement of Nitrite. When DNR (50 μM) was incubated with H_2O_2 (~1 mM) in the presence of nanomolar concentrations of LPO, very slow changes in absorption spectra were observed, suggesting that the compound is a poor peroxidase substrate (not shown). However, addition of NO_2^- (1 mM) to a reaction mixture containing LPO (62 nM), H_2O_2 (1 mM), and DNR (50 μM) in pH 7.0 buffer caused a gradual decrease in the visible absorption band of the anthracycline, suggesting oxidation of the drug (Figure 2A). The loss of the 480 nm absorption was associated with the formation of a new weak absorption maximum centered around 348 nm (Figure 2A), which can be attributed to an oxidation product. Isosbestic points were identified at 316

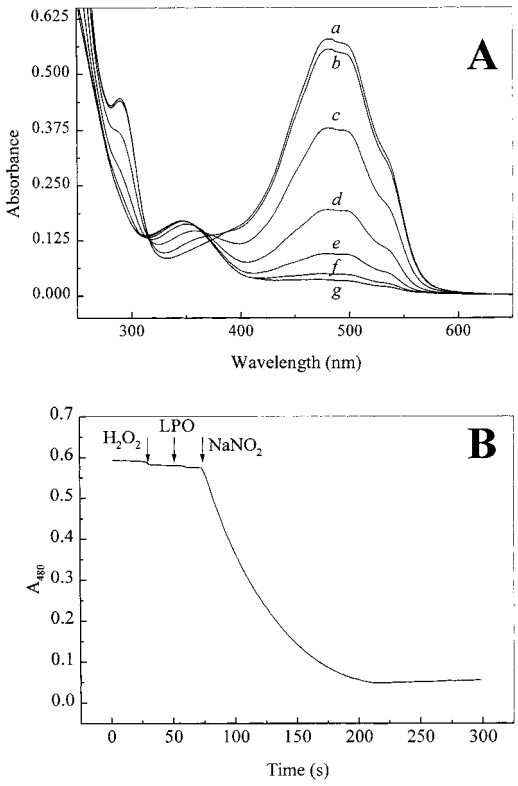


FIGURE 2: (A) Absorption spectra observed during oxidation of DNR by LPO/H_2O_2/NO_2^- at pH 7.0 (50 mM phosphate buffer). *a*, DNR (50 μM); *b*, 2 min after addition of NO_2^- (1 mM) and H_2O_2 (1 mM) to sample *a*; *c*−*g*, 15, 45, 75, 105, and 135 s after addition of LPO (62 nM) to sample *b*. (B) Time course of absorption changes at 480 nm induced by sequential addition of H_2O_2 (1 mM), LPO (62 nM), and NO_2^- (1mM) to DNR (50 μM) in pH 7.0 buffer.

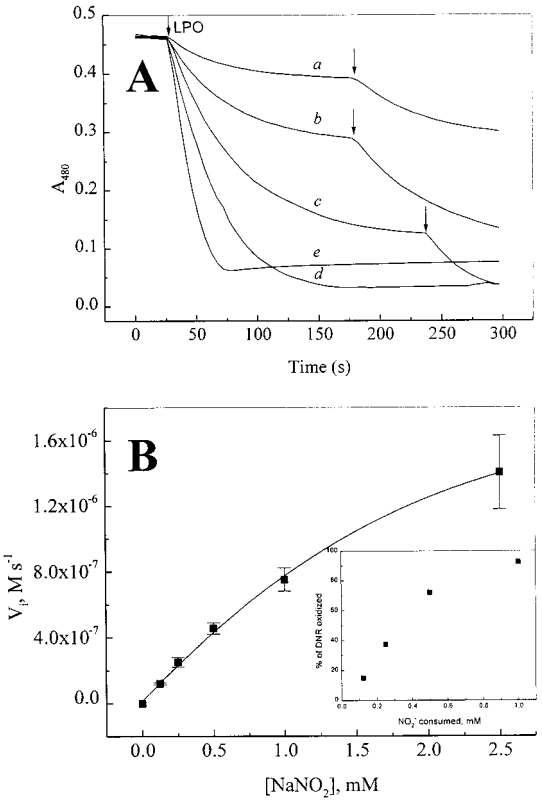


FIGURE 3: Oxidation of DNR by LPO/H_2O_2/NO_2^- at pH 7.0: Dependence on $[NO_2^-]$. (A) Time course of absorption changes at 480 nm during oxidation of DNR (43 μM) by LPO (62 nM) and H_2O_2 (1 mM) at various $[NO_2^-]$: 0.125, 0.25, 0.50, 1.0, and 2.5 mM for traces *a*−*e*, respectively. Additional doses of NO_2^- (equimolar) were added as indicated by arrows (traces *a*, *b*, and *c*). (B) Initial rate of DNR oxidation, V_i, plotted versus $[NO_2^-]$. Inset shows the percentage of DNR oxidized versus NO_2^- consumed.

and ~365 nm indicating the presence of only two light-absorbing chromophores.

Figure 2B shows the time course of absorption changes at 480 nm recorded during sequential additions of H_2O_2, LPO, and NO_2^- to a DNR sample. It shows that a rapid decrease in absorption of DNR is observed only after the addition of NO_2^-. The requirement for NO_2^- suggests that an LPO metabolite of NO_2^-, presumably $^{\bullet}NO_2$, may also be involved. When DXR was incubated with the same LPO/H_2O_2/NO_2^- system, changes in absorption spectra identical to those recorded for DNR were observed (not shown). This is understandable because both these agents possess identical chromophores (Figure 1) and should exhibit the same peroxidative chemistry. No noticeable precipitate formed as a result of the oxidation even when more concentrated solutions of DNR (DXR) were used (~364 μM). This is in contrast to previous studies which showed that oxidation with concentrated H_2O_2 and sodium periodate leads to formation of insoluble materials, consisting primarily of aglycons (from DNR and DXR) and, in the case of DXR, of a carboxylic acid (at C9) derivative of the compound (*51*, *52*). In all these oxidation products, the anthraquinone chromophore remained intact (*51*, *52*).

The oxidation of DXR and DNR was irreversible as addition of reducing agents such as ascorbate or cysteine did not cause any recovery of the 480 nm absorption (not shown). However, addition of ascorbate or cysteine, prior to the addition of LPO (or H_2O_2), inhibited oxidation of the drugs in a concentration-dependent manner (not shown), consistent with an earlier report that showed that ascorbate and cysteine are rapidly oxidized by LPO/H_2O_2/NO_2^- (*42*).

Effect of Nitrite Concentration. To further examine the role of NO_2^- in the oxidation of DNR and DXR, the time course of absorption changes at 480 nm was recorded at various $[NO_2^-]$ and constant [LPO] and $[H_2O_2]$ (Figure 3A). The reaction was carried out at pH 7.0, because at this pH oxidation of DNR (and DXR) is most efficient (see below). It was found that the initial rate of DNR oxidation, V_i, shows a hyperbolic-type dependence on $[NO_2^-]$ (Figure 3B). The rate of DXR oxidation showed a dependence on $[NO_2^-]$ similar to that determined for DNR (data not shown).

Figure 3A also shows that at low $[NO_2^-]$ after an initial decrease, the absorbance versus time traces level (traces *a*−*c*) before oxidation of DNR goes to completion, indicating that only a certain percentage of the DNR molecules were oxidized. That this effect was due to depletion of NO_2^- was illustrated by showing that when a second dose of NO_2^- was added the reaction resumed (Figure 3A, traces *a*−*c*). This rapid depletion of NO_2^- in the investigated system suggested that either NO_2^- does not redox cycle, or the process is relatively inefficient. A relationship between the percentage of DNR oxidized versus NO_2^- consumed is shown in Figure 3B (inset).

Effect of H_2O_2 Concentration. Oxidation of DNR was investigated at various $[H_2O_2]$ at pH 7.0 (Figure 4A). The initial rate of oxidation increased sharply when $[H_2O_2]$ was

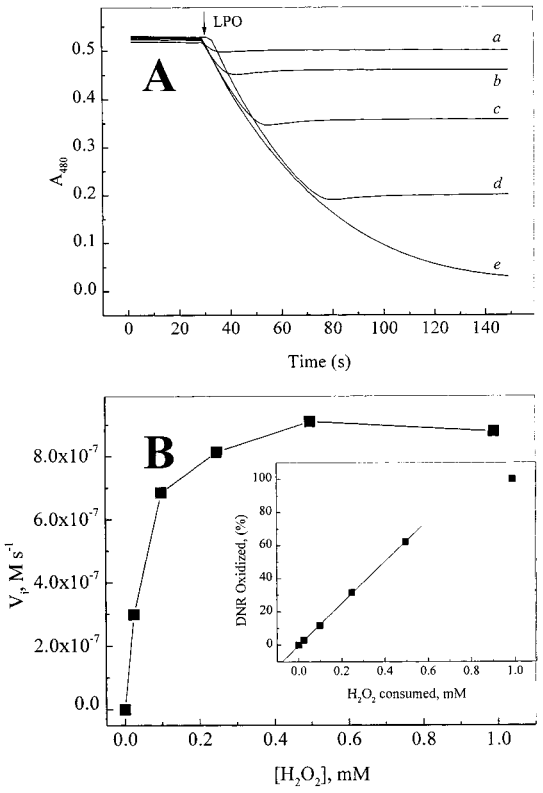


FIGURE 4: Oxidation of DNR by LPO/H_2O_2/NO_2^-: Dependence on $[H_2O_2]$. (A) Time course of absorption changes at 480 nm observed during oxidation of DNR by LPO/H_2O_2/NO_2^- at various $[H_2O_2]$ at pH 7.0. $[H_2O_2]$ = 25, 99, 248, 497, and 990 μM for traces *a*−*e*, respectively. (B) Initial rate, V_i, plotted versus $[H_2O_2]$. Inset: percentage of DNR oxidation versus $[H_2O_2]$ consumed. [DNR] = 45 μM, [LPO] = 47 nM, $[NO_2^-]$ = 1 mM.

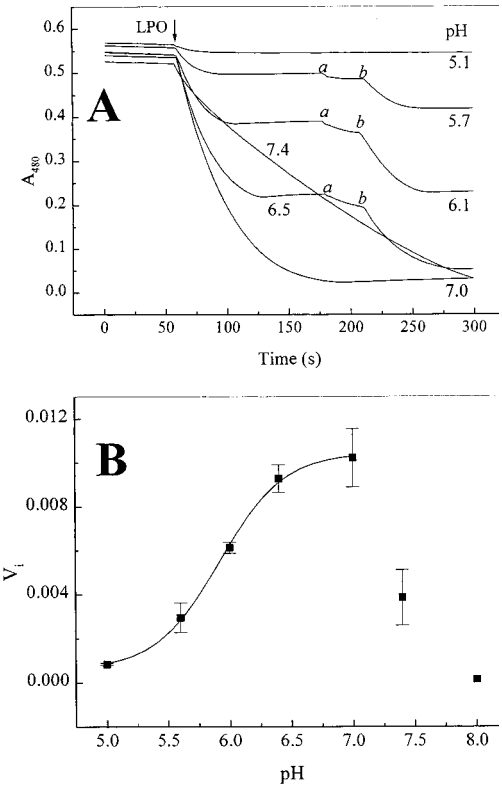


FIGURE 5: Oxidation of DNR by LPO/H_2O_2/NO_2^-: Dependence on pH. (A) Time course of absorption changes at 480 nm during interaction of DNR (50 μM) with LPO (46 nM), H_2O_2 (1 mM), and NO_2^- (1 mM) at various pHs. The reaction was initiated by addition of LPO 60 s after the start of recording. Note that at pH 7.4 and 7.0 single doses of H_2O_2 and $NaNO_2$ sufficed to bring oxidation of DNR almost to completion. Additional doses of H_2O_2 and $NaNO_2$ were added at points "a" and "b", respectively, in samples at pH 6.5, 6.1, and 5.7. (B) Initial rates of DNR oxidation, V_i, versus pH. V_i is expressed as ΔA_{480}/1 s and represents the mean of four measurements.

increased from 0 to ~0.3 mM, and at higher $[H_2O_2]$ it remained at an almost constant level (Figure 4B). The extent of DNR oxidation also depended on $[H_2O_2]$, as the oxidation ceased when H_2O_2 was consumed (Figure 4A, traces *a*−*d*). Based on data in Figure 4A, the difference between the initial and final A_{480} levels was used to calculate the percentage of oxidized DNR and was plotted versus H_2O_2 consumed (Figure 4B, inset). It shows that under applied conditions the percentage of oxidized DNR increases linearly with respect to $[H_2O_2]$ up to ~0.5 mM, reaching ~100% at 1 mM H_2O_2.

Effect of pH. It has been shown that phenols and 1,4-DHB can be oxidized directly by peroxidases/H_2O_2 (in the absence of NO_2^-) with rates that increase on going from acidic to alkaline pH (*20*, *22*, *23*, *25*). In contrast, peroxidases (HRP, LPO, MPO) metabolize NO_2^- with rates that increase when the pH is changed from neutral to acidic values (*36*, *37*, *39*, *40*, *45*). It was therefore of interest to examine the effect of pH on oxidation of anthracyclines. Figure 5A shows the time course of absorption changes at 480 nm observed when DNR reacted with LPO/H_2O_2/NO_2^- in buffers of various pH. The results show that both the rate of DNR oxidation and the extent of this process are strongly influenced by pH. First, on changing the pH from 7.0 to 5.0, V_i decreases (Figure 5B). But when the pH was increased from 7.0 to 8.0, V_i decreased (Figure 5A,B); V_i was maximal at neutral pH. From the plot in Figure 5B, the apparent pK_a = 5.9 has been determined. This pK_a is close to that reported earlier for an amino acid residue in LPO, probably the distal histidine (*22*).

Second, the decrease in pH was associated with a dramatic decrease in the extent of DNR oxidation (Figure 5A). Specifically, while oxidation at pH 7.4 and 7.0 proceeded almost to completion (~97% at 300 and 200 s, respectively), the corresponding yields of oxidation at pH 6.5, 6.1, 5.7, and 5.1 were, approximately, 60%, 28%, 11%, and 3.5% after 150 s, respectively (Figure 5A). The complete cessation of the oxidation at acid pH, when only a small percentage of DNR has reacted, was unexpected considering that both H_2O_2 and NO_2^- were present in large excess over DNR (1 versus 0.05 mM), and that LPO was still enzymatically active. When additional doses of H_2O_2 and NO_2^- were added, the oxidation resumed (Figure 5A, points "a" and "b", respectively), suggesting that H_2O_2 and NO_2^- are consumed faster at acid pH and without formation of sufficiently reactive intermediates. Essentially identical results were seen with DXR.

Reaction of DNR with $^{\bullet}NO_2$. Because one possible LPO metabolite of NO_2^- is the $^{\bullet}NO_2$ radical, it was necessary to examine whether the species can oxidize the anthracyclines. For this purpose, DNR was exposed to various amounts of $^{\bullet}NO_2$ gas at pH 7.4 and 5.0, after which absorption spectra were measured. The spectra recorded at pH 7.4 (Figure 6) are similar to those induced by LPO/H_2O_2/NO_2^-. Figure 6 (inset) shows that the loss of absorption at 480 nm correlates well with the amount of $^{\bullet}NO_2$ added (measured as NO_2^-).

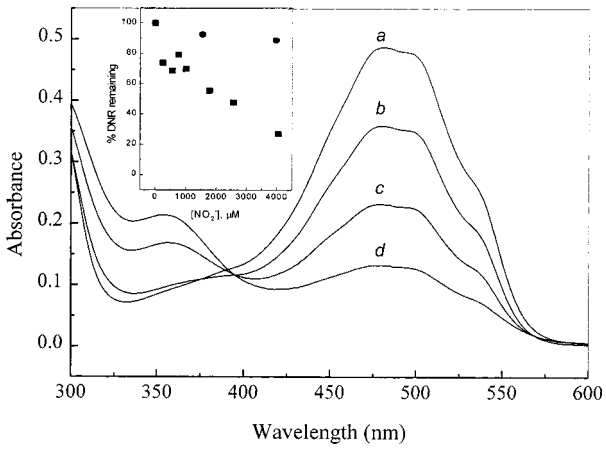


FIGURE 6: Oxidation of DNR by $^{\bullet}NO_2$. Typical spectra observed following addition of gaseous $^{\bullet}NO_2$ to DNR in pH 7.4 buffer. Spectral lines *a*−*d* are from DNR samples in which the level of NO_2^- resulting from $^{\bullet}NO_2$ addition was 0 (control), 0.254, 2.56, and 4.0 mM, respectively. The inset shows loss of the 480 nm absorption measured at pH 7.4 (■) and 5.0 (●).

This result suggests that if LPO/H_2O_2/NO_2^- generates $^{\bullet}NO_2$, it could contribute to DNR oxidation. Oxidation of DNR by $^{\bullet}NO_2$ at pH 5.0 was substantially less efficient (Figure 6, inset), which is similar to the reaction carried out by the enzymatic system.

(B) Oxidation of 2,5-DTBHQ by LPO/H_2O_2/NO_2−

Results from experiments described above show that oxidation of DNR and DXR by LPO/H_2O_2/NO_2^- is strongly pH-dependent. One possible explanation of this pH effect is that ionization of the hydroquinone group in DNR and DXR changes in the pH range 5.0−7.4. Ionization of hydroquinone groups might play a role because reduction potentials of semiquinone/hydroquinone couples decrease on going from acidic to alkaline pH (*53*). It has also been reported that $^{\bullet}NO_2$ reacts readily with a monoanion of 1,4-DHB, while it does not react, or reacts very slowly, with nonionized 1,4-DHB (*54*). Thus, if $^{\bullet}NO_2$ is involved, its role in oxidation of DNR at acid pH would decrease. To find out whether the observed dependence on pH is unique to DNR (DXR), we studied oxidation of another hydroquinone compound, 2,5-DTBHQ,[2] by LPO/H_2O_2/NO_2^- (Figure 1). This compound was chosen because we found that its oxidation by LPO/H_2O_2 alone is inefficient, presumably due to the presence of two bulky *tert*-butyl groups that hinder access of the hydroquinone moiety to the LPO heme. In this respect, the compound mimics the behavior of the anthracyclines. 2,5-DTBHQ autoxidizes slowly at pH 7.0, but the reaction is stopped at acidic pH. In the absence of NO_2^-, 2,5-DTBHQ undergoes a slow metabolism by LPO/H_2O_2, but addition of NO_2^- greatly enhances the process (Figure 7A,B, insets). Figure 7A shows spectra recorded in intervals at pH 7.0. Three isosbestic points (237, 278, and 306 nm) indicate that oxidation of 2,5-DTBHQ gives rise only to one product, most likely the respective quinone, 2,5-DTBQuinone, identified by its strong absorption at 260 nm.

[2] 1,4-DHB could not be used in this experiment because the compound is an excellent substrate for peroxidases and is rapidly oxidized by LPO/H_2O_2 in the absence of NO_2^-, and, therefore, the catalytic role of NO_2^- could not be unequivocally demonstrated.

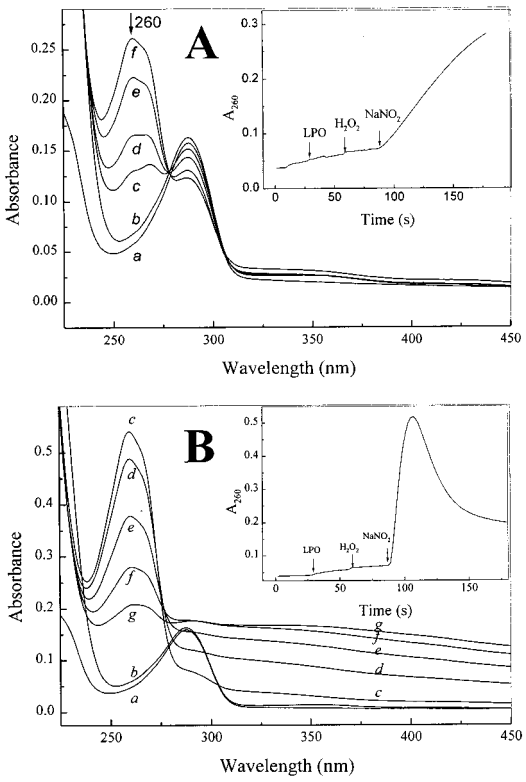


FIGURE 7: Oxidation of 2,5-DTBHQ by LPO/H_2O_2/NO_2^-. (A) Absorption spectra observed at pH 7.0 (50 mM phosphate buffer). *a*, 2,5-DTBHQ (50 μM); *b*, after addition of NO_2^- (250 μM) and LPO (62 nM) to sample *a*; *c*−*f*, 15, 30, 60, and 90 s after addition of H_2O_2 (250 μM) to sample *b*. (B) Absorption spectra observed at pH 5.1. *a*, *b*, same as in panel A but at pH 5.1; *c*−*g*, 10, 20, 30, 45, and 90 s after addition of H_2O_2 (250 μM) to sample *b*. Note that spectra *d*−*g* represent those recorded after reaching the maximum. Insets in panels A and B: Time course of absorption at 260 nm at pH 7.0 and 5.1, respectively, showing that the rapid oxidation of 2,5-DTBHQ by LPO/H_2O_2 is dependent on the presence of NO_2^-.

Measurements of the time course of absorption at 260 nm as a function of pH revealed that the initial rate of 2,5-DTBHQ oxidation increases when the pH is changed from 7.48 to 5.08 (Figure 8A,B); i.e. it parallels the rate of the metabolism of NO_2^-. From the sigmoid plot of V_i versus pH, the apparent pK_a was determined to be 5.9 (Figure 8B). Data in Figure 8 clearly show that LPO/H_2O_2/NO_2^- readily oxidizes 2,5-DTBHQ in the entire pH range studied with the highest rate at pH 5. This result is opposite to that observed with DNR and DXR. It shows that LPO/H_2O_2/NO_2^- can oxidize hydroquinones efficiently even at acid pH, which suggests that factors other than ionization of the hydroquinone moiety play a role in oxidation of the anthracyclines.

The dependence on pH of the oxidation of 2,5-DTBHQ by LPO/H_2O_2/NO_2^- is also opposite to the pH dependence of peroxidation of 1,4-DHB by MPO/H_2O_2 (*25*) and by LPO/H_2O_2 (*23*). Kettle and Winterbourn (*25*) observed that rate of the oxidation of 1,4-DHB increases when the pH increases from 5 to 7.4, and is characterized by a distinct lag period. They found that the lag phase was caused by reduction of ferric MPO to ferrous MPO by 1,4-DHB benzosemiquinone (formed in a reaction analogous to that given by eq 2), which then reacted with O_2 to form MPO compound III (oxyperoxidase). Compound III of peroxidases is considered to be outside the normal peroxidase cycle, and its formation retards

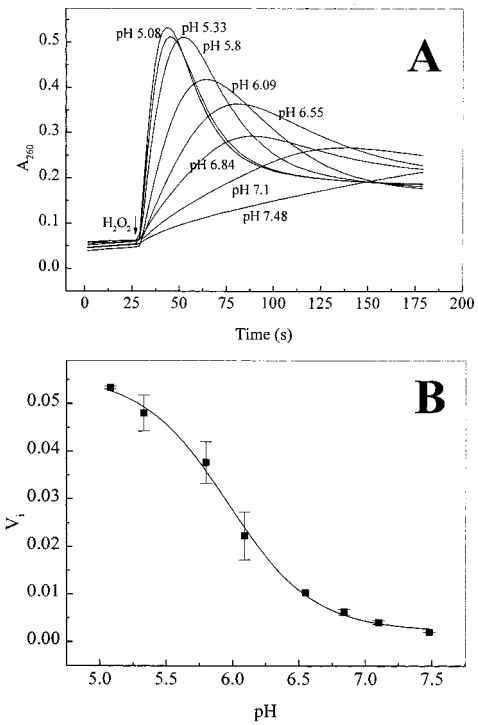


FIGURE 8: Oxidation of 2,5-DTBHQ by LPO/H_2O_2/NO_2^-: Dependence on pH. (A) Time course of absorption changes at 260 nm during interaction of 2,5-DTBHQ (50 μM) with LPO (46 nM), H_2O_2 (250 μM), and NO_2^- (250 μM) at various pHs (as indicated). The reaction was initiated by addition of H_2O_2 30 s after the start of recording. (B) Initial rates of the reaction, V_i, plotted versus pH.

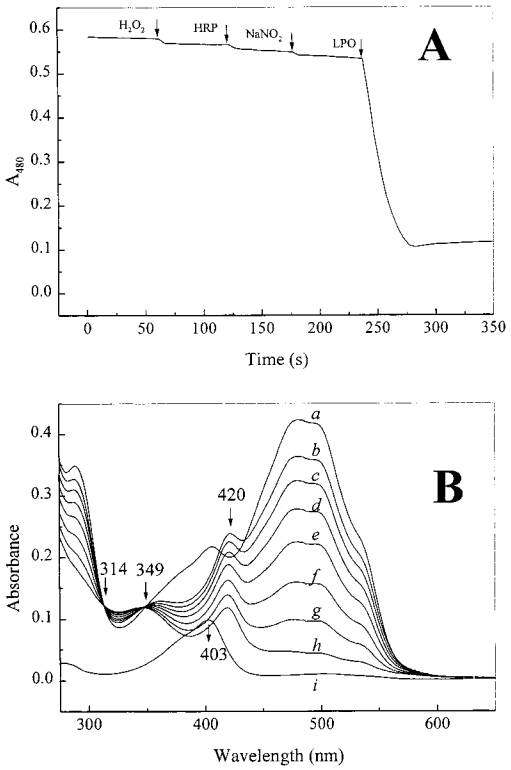


FIGURE 9: Oxidation of DNR by HRP/H_2O_2 at pH 7.0. (A) Time course of absorption changes at 480 nm during sequential addition of H_2O_2 (1 mM), HRP (0.18 μM), NO_2^- (1 mM), and LPO (0.14 μM) to DNR (50 μM) in pH 7.0 buffer. Peroxidative activities of HRP and LPO were equalized based on the initial rate of ABTS oxidation (1.6 μM/1 s), determined in separate experiments. (B) Spectra of DNR (36.4 μM) at pH 7.0 in the presence of HRP (1 μM), H_2O_2 (1 mM), $NaNO_2$ (1 mM) recorded (from *a* to *h*) 0, 1, 3, 6, 10, 15, 20, and 25 min after H_2O_2 addition, respectively. The spectrum "*i*" is HRP (1 μM) only. The absorption peak at 420 nm represents HRP-II.

recovery of ferric peroxidase and, consequently, slows down oxidation of substrates. We did not observe the lag phase during oxidation of 2,5-DTBHQ by LPO/H_2O_2/NO_2^- (Figure 8). Our results suggests that NO_2^- can markedly modify peroxidation of hydroquinones, possibly because NO_2^- is an excellent LPO substrate, and reacts rapidly with the less reactive forms of the enzyme LPO-II and LPO-III (*38*, *55*). In contrast, 2,5-DTBHQ is unable to reduce LPO-II (data not shown).

(C) Oxidation of Anthracyclines by HRP/H_2O_2

Because HRP is frequently employed to study peroxidative metabolism of drugs and other xenobiotics, and since 1,4-DHB, phenols, and compounds possessing phenolic groups are typical HRP substrates, it was of interest to investigate the ability of HRP to oxidize the anthracyclines. In agreement with an earlier report (*29*), we found that at nanomolar concentrations HRP was unable to efficiently catalyze oxidation of DNR (or DXR) by H_2O_2 either in the absence or in the presence of NO_2^-. Figure 9A shows the time course of DNR oxidation recorded during sequential addition of H_2O_2, HRP, NO_2^-, and LPO. Only after the addition of LPO (last component) does the 480 nm absorbance decrease. It should be emphasized that in this experiment, the concentrations of peroxidases were adjusted so that peroxidative activities of both enzymes were the same based on ABTS assay.

In contrast, at high HRP concentrations (~1 μM), HRP/H_2O_2 slowly oxidized DNR, and although NO_2^- (1 mM) increased the rate, it was still markedly less than that observed in the presence of a LPO/H_2O_2/NO_2^- system. Figure 9B shows absorption spectra from a system consisting of DNR (36.4 μM), HRP (1 μM), H_2O_2 (1 mM), and NO_2^- (1 mM) recorded over the period of 25 min. Two isosbestic points were identified at 316 and 349 nm. The peak at 420 nm (Figure 9B) formed after H_2O_2 addition corresponds to HRP-II (*20*). It persisted even in the presence of NO_2^-, indicating that reduction of HRP-II by NO_2^- must be a very slow process. Indeed, the second-order rate constant determined for this reaction at near neutral pH is quite low, 25 (*37*) and 13.3 M^{-1} s^{-1} (*38*). When a similarly high concentration of LPO was employed, oxidation of DNR proceeded at a very low rate. The simultaneous presence of LPO-II was evidenced by the characteristic absorption maximum at 430 nm, suggesting that interaction of LPO-II with DNR may be the rate-limiting step. However, in contrast to the HRP system discussed above, addition of NO_2^- caused a rapid conversion of LPO-II to native LPO, and was concomitant with a substantial decrease in the 480 nm absorption due to DNR oxidation (not shown). The NO_2^--dependent transformation of LPO-II to ferric LPO has been observed in other systems (*55*−*57*). The second-order rate constant for the reduction of LPO-II by NO_2^- has been determined to be 3.5 $\times$ 10^5 M^{-1} s^{-1} (*55*). These values are substantially higher than that for the reduction of HRP-II by NO_2^-. Results similar to those with DNR were also obtained with DXR (not shown).

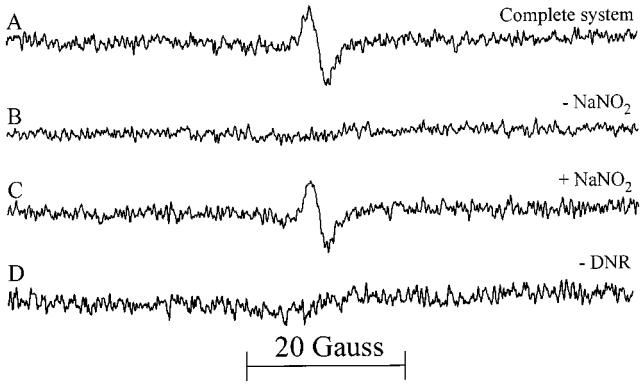


FIGURE 10: EPR spectra generated by oxidation of DNR (1.6 mM) by LPO (~0.2 μM), H_2O_2 (8.3 mM), and NO_2^- (8.5 mM) in pH 7.0/MeOH (2:1 v/v) mixture. (A) Complete system; (B) same as (A) but with nitrite omitted; (C) after addition of nitrite to sample (B); (D) same as (A) but with DNR omitted. Instrumental settings: microwave power, 40 mW; modulation amplitude, 2 G; time constant, 82 ms; scan rate, 80 G/41.94 s.

(D) EPR Investigations

Free Radicals Formed by Oxidation of Anthracyclines. To examine whether oxidation of the anthracyclines by LPO/H_2O_2/NO_2^- gives rise to free radical products, EPR measurements were performed. Samples were prepared in pH 7.0 buffer/MeOH (2:1 v/v) mixture to prevent aggregation of anthracyclines in concentrated solutions (~1 mM).

When DNR reacted with LPO/H_2O_2 in the presence of NO_2^-, the EPR signal shown in Figure 10A, was detected. When either NO_2^- or DNR was omitted, no signal could be observed (Figure 10, spectra B and D, respectively); and addition of NO_2^- to a control sample initially without nitrite (Figure 10B) restored the signal (Figure 10C). Also no signal was observed when LPO or H_2O_2 were omitted (not shown), consistent with the requirement that the complete enzymatic system, including DNR and NO_2^-, is required to generate radicals from DNR. A spectrum similar to that in Figure 10A was detected when DXR reacted with LPO/H_2O_2/NO_2^- (not shown). These anthracycline-derived radicals decay most likely by disproportionation to form the parent compound and a di-quinone-type product(s). It has been reported that semiquinone radicals generated by pulse radiolytic oxidation of structurally related compounds such as 1,4-dihydroxyanthraquinones and naphthazarin (1,4-dihydroxy-5,8-naphthoquinone) decay, forming respective di-quinones (*58*, *59*).

Free Radicals Formed by Oxidation of 2,5-DTBHQ. To compare the oxidative generation of radicals from anthracyclines to that from other simple hydroquinones, we exposed 2,5-DTBHQ to LPO/H_2O_2/NO_2^-. Figure 11A shows an EPR spectrum of the 2,5-DTBHQ semiquinone observed from a such system in a pH 6.0/MeOH (260:200 v/v) mixture. The spectrum is a triplet with the intensity ratio of 1:2:1, due to interaction of the unpaired electron with two equivalent ring hydrogens, and the hyperfine coupling of 2.08 G (2H). No further resolution due to interaction of the unpaired electron with hydrogens of the *tert*-butyl groups could be detected. The generation of this radical was fully dependent on the simultaneous presence of the hydroquinone, LPO, H_2O_2, and nitrite, as confirmed by control measurements in which each of these components was omitted (Figure 11C−E).

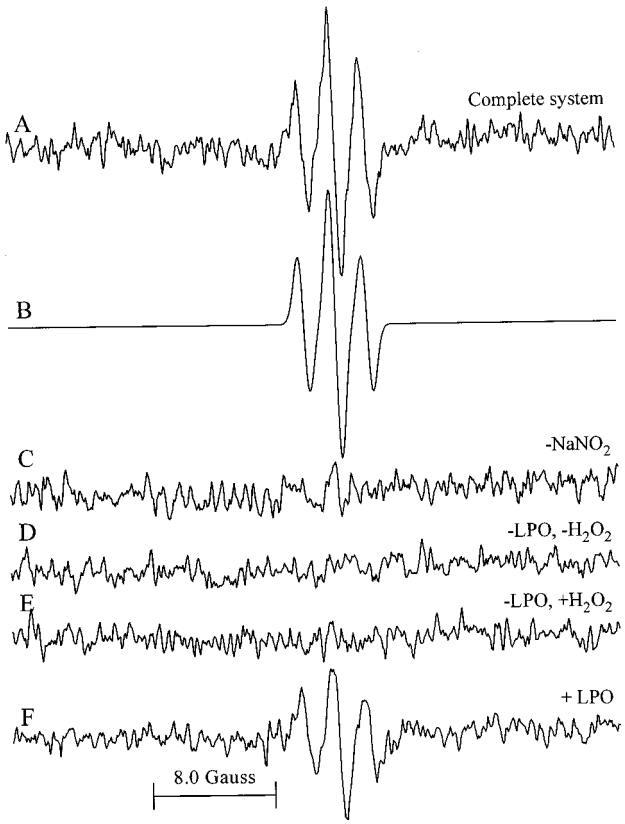


FIGURE 11: EPR spectra generated by oxidation of 2,5-DTBHQ (4.1 mM) by LPO (~0.25 μM), H_2O_2 (4.2 mM), and NO_2^- (4.2 mM) in pH 6.0/MeOH (2:1 v/v) mixture. (A) Complete system; (B) simulation of spectrum A using a hyperfine coupling constant of 2.08 (2H); (C) same as spectrum A but with nitrite omitted; (D) same as spectrum A but with LPO and H_2O_2 omitted; (E) after addition of H_2O_2 to sample D; (F) after addition of LPO to sample E. Instrumental settings: microwave power, 40 mW; modulation amplitude, 1 G; time constant, 8 ms; scan rate, 80 G/41.94 s.

DISCUSSION

The major finding of this study is the observation that the clinically important anthracyclines DXR and DNR undergo oxidation via a mechanism dependent on LPO, H_2O_2, and NO_2^-. This is a novel oxidative mechanism of metabolic transformation, an alternative to the more frequently studied reductive pathway of activation of these agents.

Anthracyclines appeared to be poor substrates for LPO/H_2O_2. This conclusion is based on the observation that the absorption spectra of DNR and DXR do not change in the presence of H_2O_2 and LPO (nanomolar concentrations), suggesting that oxidation of their hydroquinone moiety via reactions analogous to those given by eqs 2 and 3 must be very slow. The inability of the LPO/H_2O_2 system alone to oxidize the compounds is likely due to the restrictive nature of the heme center in LPO (*60*, *61*). In contrast to 1,4-DHB, an unsubstituted hydroquinone, which is readily oxidized by LPO and MPO (*23*−*25*), anthracyclines are relatively bulky compounds and may be unable to access the active sites in a way that would permit their oxidation. Although access to the heme is less restricted in HRP compared to LPO, HRP/H_2O_2 was equally inefficient in oxidation of DNR and DXR. However, both enzymes, when used at high concentrations (~1 μM), caused slow oxidation of the anthracyclines via a process which involves, primarily, LPO-I or HRP-I, since LPO-II and HRP-II could be readily detected during the

reaction. This suggests that interaction of DNR and DXR with the enzymes in the form of compound II is rate-limiting.

NO_2^- dramatically enhances the oxidizing capacity of LPO/H_2O_2. NO_2^- is a small molecule with high affinity to the heme center in LPO (*62*). NO_2^- binds to the enzyme with a dissociation constant of 2.43 mM at pH 5.5 (*62*) and 20 mM at pH 7.0 (*38*), indicating that the interaction between the ion and the enzyme is stronger at acid pH than at neutral pH. The oxidation of NO_2^- by LPO/H_2O_2 is usually described by a typical peroxidase cycle in which LPO-I is reduced by two NO_2^- molecules in two one-electron-transfer steps recovering native LPO and producing two $^{\bullet}NO_2$ radicals (eqs 4−6) (*38*):

$$\text{LPO} + H_2O_2 \rightarrow \text{LPO-I} \quad (4)$$

$$\text{LPO-I} + NO_2^- \rightarrow \text{LPO-II} + {}^{\bullet}NO_2 \quad (5)$$

$$\text{LPO-II} + NO_2^- \rightarrow \text{LPO} + {}^{\bullet}NO_2 \quad (6)$$

A similar mechanism has been proposed for the oxidation of NO_2^- by HRP/H_2O_2 (*36*, *37*) and MPO/H_2O_2 (*39*) systems. Oxidation of NO_2^- by LPO/H_2O_2 is a fast process. At pH 7, the rate constants for the reaction of NO_2^- with LPO-I and LPO-II (eqs 5 and 6) have been determined to be $\sim 10^5$ and 3.5×10^4 M^{-1} s^{-1}, respectively (*38*). Recently a different mechanism has been proposed for NO_2^- oxidation by LPO/H_2O_2. Based on the observation that in the presence of NO_2^- LPO-I was converted into native LPO, without the apparent formation of any transient absorption from LPO-II, it was deduced that NO_2^- undergoes direct oxidation to nitrate, NO_3^-, in one step, without formation of the $^{\bullet}NO_2$ radical (eq 7) (*55*). The rate constant for this reaction has been determined to be 2.3×10^7 M^{-1} s^{-1} at pH 7.2.

$$\text{LPO-I} + NO_2^- \rightarrow \text{LPO} + NO_3^- \quad (7)$$

The same study confirmed the ability of LPO-II to oxidize NO_2^- to $^{\bullet}NO_2$ (reaction 6) (*55*). Thus, the catalytic role of NO_2^- could result from its rapid metabolism to the highly reactive $^{\bullet}NO_2$ radical or, alternatively, from the fast reduction of LPO-II to native LPO, which re-enters the peroxidative cycle, forming the more reactive LPO-I. A simultaneous operation of these two mechanisms is also possible.

In contrast to LPO/H_2O_2, oxidation of NO_2^- by HRP/H_2O_2 is much slower. Previous studies have reported rate constants for reaction of NO_2^- with HRP-I (analogous to reaction 5) to be 6.7×10^2 M^{-1} s^{-1} (pH 6.93) (*37*) and 4.5×10^2 M^{-1} s^{-1} (pH 7.0) (*38*), and those for reaction of NO_2^- with HRP-II (analogous to reaction 6) to be 25 M^{-1} s^{-1} (pH 6.83) (*37*) and 13.3 M^{-1} s^{-1} (pH 7.0) (*38*). Thus, oxidation of NO_2^- by LPO/H_2O_2 is approximately 3 orders of magnitude faster than by HRP/H_2O_2. We found that HRP/H_2O_2/NO_2^- was unable to oxidize DNR (and DXR) at low (nanomolar) HRP concentrations, although at comparable LPO concentrations, oxidation by LPO/H_2O_2/NO_2^- was very efficient (Figure 9A). Given the above, the probable reason LPO/H_2O_2/NO_2^- is considerably more effective than HRP/H_2O_2/NO_2^- in oxidation of the anthracyclines is the markedly faster metabolism of NO_2^- by LPO/H_2O_2.

We have also shown that $^{\bullet}NO_2$ can react directly with DNR, causing its oxidation (Figure 6). The reaction can be described by eq 8, in which NO_2^- is recovered and the hydroquinone is converted in a semiquinone radical.

$$^{\bullet}NO_2 + QH_2 \rightarrow NO_2^- + Q^{\bullet -} + 2H^+ \quad (8)$$

The generation of semiquinone radicals is expected because $^{\bullet}NO_2$ is a one-electron oxidant known to react via electron abstraction (*54*, *63*). Thus, if LPO/H_2O_2/NO_2^-/hydroquinone generates $^{\bullet}NO_2$, it can contribute to DNR (DXR) oxidation.

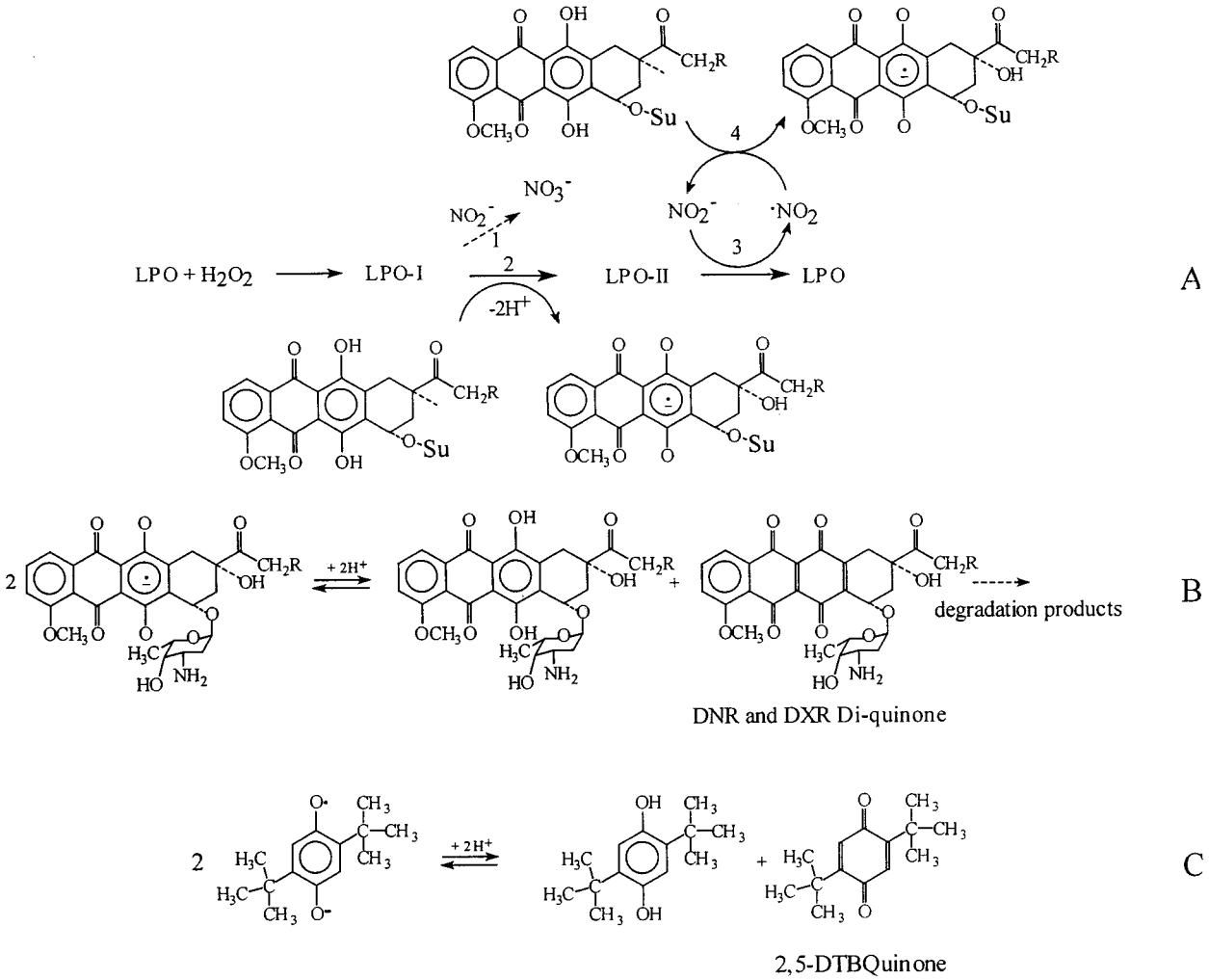
To explain the observed pH dependence of the oxidation of DNR, DXR, and 2,5-DTBHQ by LPO/H_2O_2/NO_2^-, it is necessary to consider the following three factors: (1) the effect of pH on ionization and redox properties of anthracyclines and 2,5-DTBHQ; (2) the effect of pH on the LPO metabolism of NO_2^-; and (3) the effect of pH on the redox potential of the $^{\bullet}NO_2$/NO_2^- couple.

With respect to the first factor, the observed dependence of the oxidation by LPO/H_2O_2/NO_2^- of DNR and DXR on pH suggests that ionization of a group with p$K_a \sim 6$ strongly affects the oxidation. The group is most likely the distal histidine residue in LPO (*22*). In the pH range 5.0−7.0 (Figure 5B), the relationship is similar to that found for other hydroquinones and phenolic compounds and shows similar pK_a (*20*, *22*, *25*). The pK_a for the first ionization of the hydroquinone moiety in DNR and DXR has been reported to be in the range 8−9.6 (*12*, *48*, *64*, *65*), with values ~9.6 being more frequently quoted. The hydroquinone group in 1,4-dihydroxyanthraquinones, close structural analogues of DNR and DXR, ionizes with pK_a of 9.1 (*58*). The p$K_a \sim$ 9.6 would indicate that in the pH range 5−7.4 the hydroquinone moiety in anthracyclines exists in nonionized form. Interestingly, deprotonation of naphthazarin (1,4-dihydroxynaphthoquinone, a compound comprised of rings B and C of anthracyclines, Figure 1) occurs with a markedly lower pK_a of 7.8 (*66*). This, and the conflicting data regarding the pK_a for anthracyclines, does not allow us to exclude the possibility that at near neutral pH, DNR and DXR could be partially ionized and therefore more susceptible to oxidation. An interaction between the −OH group at C6 in DNR (DXR) and the ammonium group in daunosamine might contribute to this ionization. The possibility of such an interaction has been postulated earlier based on subtle changes in the absorption spectra of DXR observed at various pH values (*64*). Ionization of the hydroquinone group in DNR (DXR) might be especially important for oxidation by $^{\bullet}NO_2$, as it has been shown that the species reacts more readily with ionized than with neutral phenolics (*54*, *63*). The lower rates of DNR oxidation observed at pH 7.4 and 8.0 (Figure 5) could be due to markedly slower metabolism of NO_2^- at these pHs. The combined effect of these factors, each of which influences the reaction in opposite directions, is that oxidation of anthracyclines by LPO/H_2O_2/NO_2^- is efficient only in a narrow pH range, around pH 7.

The pK_a for ionization of 2,5-DTBHQ is likely to be ~10, similar to that of 1,4-DHB, and therefore in the pH range studied, the compound exists in nonionized form. The reduction potentials of 2,5-DTBHQ are expected to be close to those of 1,4-DHB (*53*).

Regarding the second factor, the effect of pH on NO_2^- metabolism, it is known that oxidation of NO_2^- by peroxidases/H_2O_2 is pH-dependent. The rate constants for the interaction of HRP-I and HRP-II with NO_2^- at pH ~6 were

Scheme 1: Proposed Mechanism for Oxidation of Anthracyclines and 2,5-DTBHQ by LPO/H_2O_2/NO_2^- [a]



[a] "Su" denotes the sugar moiety in DNR and DXR. The broken arrow in panel A (path 1) is to emphasize that the exact mechanism of the conversion of NO_2^- to NO_3^- (direct one step, through NO_2^+, or peroxynitrite) is not known.

reported to be 6.5×10^3 and 1.8×10^2 M^{-1} s^{-1}, respectively (*37*), or roughly 10 times higher than at pH 7. Similar dependence of rate constants on pH was observed for oxidation of NO_2^- by MPO compounds I and II (*39*). It is likely that the rate of interaction of LPO-I and LPO-II with NO_2^- at acid pH is higher compared to neutral pH. As to the third factor, it is expected that due to the low pK_a for ionization of HNO_2, 3.35, in the investigated pH range the redox potential of the $^{\bullet}NO_2/NO_2^-$ couple remains virtually constant, close to 1040 mV (*67*).

All these data taken together suggest that the observed dependence of oxidation of DNR and DXR on pH may be due to the unique redox properties of anthracyclines, rather than to these other factors. Although no data on redox potentials of hydroquinone moieties in DNR, DXR, or related anthracycline analogues (i.e., naphthazarin, or 1,4-dihydroxyanthraquinones) are available, we speculate that they should be markedly higher than those for simple hydroquinones (i.e., 1,4-DHB or 2,5-DTBHQ). This could be because in DNR and DXR the hydroquinone moiety is directly coupled to the highly electrophilic quinone group (Figure 1, rings B and C, respectively). This should increase the reduction potential of the hydroquinone group above that of 1,4-DHB or 2,5-DTBHQ. Thus, in anthracyclines, the quinone moiety may stabilize the hydroquinone group, rendering it more resistant to oxidation. This hypothesis is supported by the observation that 5-iminodaunorubicin, a DNR analogue in which the oxygen at C5 (Figure 1) has been replaced by an electron-donating imino group (=NH), readily undergoes oxidation by HRP/H_2O_2, even in the absence of NO_2^- (*29*).

The proposed mechanism for the oxidative metabolism of DNR, DXR, and 2,5-DTBHQ is depicted in Scheme 1A. It shows that NO_2^- and anthracyclines compete for LPO-I, and their oxidation leads to NO_3^- (path 1) and a semiquinone radical from the agents along with LPO-II (path 2). In the next step, NO_2^- reduces LPO-II to native LPO, forming concomitantly the $^{\bullet}NO_2$ radical (path 3). Reaction of $^{\bullet}NO_2$ with DNR (DXR) yields NO_2^- and an oxidized drug (path 4).

The formation of the anthracycline- and 2,5-DTBHQ-derived radicals was confirmed by EPR measurements (Figures 10 and 11). The semiquinone radicals decay most likely by disproportionation to the parent compound and the respective quinone, as shown in Scheme 1 for radicals from DNR (DXR) (panel B) and from 2,5-DTBHQ (panel C). Formation of 2,5-DTBquinone has been confirmed by its characteristic absorption spectrum with a maximum at 260 nm (Figure 7). Recent studies have shown that this pattern of decay is characteristic of semiquinones formed by pulse radiolytic

oxidation of 1,4-dihydroxyanthraquinones and naphthazarin, which disproportionate rapidly (rate constants $>10^9$ M^{-1} s^{-1}) to the respective parent compounds and di-quinone-type products (*58*, *59*). Importantly, di-quinones are strong electrophiles and therefore are potentially cytotoxic, if produced in vicinity of important biological targets. Our observation that reducing agents were unable to recover the original absorption of the drugs suggests that the di-quinone products do not accumulate, but undergo further reactions that lead to degradation of their chromophores. The nature of these products requires further study. Results of our preliminary mass spectrometry experiments did not reveal formation of any single major metabolite.[3] Recent reports that 3-methoxysalicylic acid has been identified among products of photochemically oxidized DXR confirm that oxidation of anthracyclines may lead to extensive degradation of their anthraquinone skeleton (*68*, *69*).

Experiments using $^{\bullet}NO_2$ gas have shown that $^{\bullet}NO_2$ can oxidize DNR. Oxidation of hydroquinones by $^{\bullet}NO_2$ should lead to recovery of NO_2^- (eq 8 and path 4 in Scheme 1, panel A). However, the observation that NO_2^- can be depleted prior to complete oxidation of the agents by LPO/H_2O_2/NO_2^- suggests that $^{\bullet}NO_2$ may play only a minor role in the drug's oxidation. Based on data in Figure 3A and Figure 4B, we estimate that at pH 7, 1 molecule of DNR was oxidized per approximately 20 NO_2^- molecules metabolized and 17−20 molecules of H_2O_2 consumed. The reaction of NO_2^- via path 1 may be the most effective drain through which NO_2^- is consumed. This pathway could be especially important at acid pH, at which the ion's affinity toward LPO is increased and, consequently, the percentage of DNR(DXR) molecules interacting with LPO-I could dramatically decrease. This could explain why at pH 5.0 all NO_2^- was consumed, causing only a minimal oxidation of DNR (DXR) (Figure 5). Interestingly, we observed that oxidation of 2,5-DTBHQ was fast even at pH 5.0 suggesting that this compound was able to compete effectively with NO_2^- for LPO-I, even at this low pH. In the suggested mechanism of the oxidative metabolism of anthracyclines (Scheme 1), a step that is required for nitrite catalysis is the formation of LPO-II. This can occur, presumably only, through reduction of LPO-I by DNR (DXR) or 2,5-DTBHQ. It may be that the reason DNR and DXR were oxidized less efficiently at acid pH is that in acidic solutions they are unable to reduce LPO-I to LPO-II. In contrast, 2,5-DTBHQ can perform the reduction even at pH 5.0.

The question remains whether the reaction yields NO_3^- directly in one step, or through reactive intermediates such as free or enzyme-bound nitronium (NO_2^+) or peroxynitrite (*44*, *46*), given the known ability of LPO to catalyze two-electron oxidation (*20*, *23*, *72*) and oxygen-transfer processes (*73*), and the fact that both nitronium and peroxynitrite can give rise to nitrate. Thus, the exact nature of the species involved in NO_2^- depletion and in the metabolism of anthracyclines by LPO/H_2O_2/NO_2^- is not, as yet, unambiguously established.

Other mechanisms via which NO_2^- could be depleted are possible. One mechanism involves nitration of anthracyclines, but it would account for only a small decrease in NO_2^- concentration, given that in some samples NO_2^- was present in large excess over DNR (e.g., 250 and 500 μM NO_2^- versus 43 μM DNR, traces *b* and *c* in Figure 3A, respectively). Another possible reaction is dimerization of $^{\bullet}NO_2$, a process known to proceed with a high rate constant (4.5×10^8 M^{-1} s^{-1}, *54*, *63*). The resulting dimer (N_2O_4) hydrolyzes to nitrate (NO_3^-) and NO_2^-. Although the recovered NO_2^- could re-enter the reaction cycle, all nitrite will eventually be depleted, providing that H_2O_2 is still present. This reaction of $^{\bullet}NO_2$ may be especially relevant in the presence of poor $^{\bullet}NO_2$ substrates, such as DNR and DXR at acid pH.

The oxidative metabolism of anthracyclines is an alternative to the widely accepted reductive mode of activation and may be pertinent to the biological activity of these agents in vivo. LPO has been identified in breast tissues (*32*, *33*), in airway mucosa (*34*), and in some body fluids (*35*). Spectroscopic investigations have shown that in LPO the heme and its environment are similar to those in other heme-based peroxidases (*74*, *75*). It is therefore likely that reactions catalyzed by LPO may also be supported by related enzymes such as EPO and MPO. MPO is secreted by activated phagocytes at sites of inflammation and bacterial infections (*76*, *77*), and a recent study identified EPO in human breast cancer tissues (*78*). This, together with the recent finding that NO_2^- accumulates in breast cancer tissues (*79*, *80*), suggests that the occurrence of reactions described in this study in vivo is feasible. Nitrite itself is cytotoxic, especially at acid pH (*45*, *81*, *82*), but its antibiotic activity is markedly enhanced in the presence of LPO, MPO, or EPO enzymes (*45*). In addition, several studies point at the role DXR may play in $^{\bullet}NO$ production and possible participation of $^{\bullet}NO$ (or $^{\bullet}NO$-derived products) in DXR biological activities (*83*−*85*). Oxidatively modified anthracyclines may also play a role in cardiotoxicity since it has been found that DXR treated with HRP/H_2O_2 inactivates creatine kinase (*31*), an enzyme involved in ATP generation and contractile function in the heart.

In summary, this study demonstrates a novel oxidative mechanism of metabolic transformation of anthracycline anticancer antibiotics, which is dependent on LPO, H_2O_2, and NO_2^-. Because anthracyclines may co-localize in tissues in various pathological situations, including inflammation and cancer in which elevated levels of NO_2^-, peroxidases, and peroxides have been reported, the likelihood of reactions described in this study occurring in vivo is high. Thus, our observations may be pertinent to the biological activities (anticancer and/or cardiotoxic) of the anthracycline antibiotics in vivo. However, it is not known whether oxidation of anthracyclines leads to enhanced biological activity or whether it is merely a mechanism of their detoxification. In any case, it can be hypothesized that NO_2^- could be exploited to modulate biological activities of anthracycline agents.

[3] We have measured mass spectra (electrospray ionization and MALDI) of DNR and DNR oxidized by LPO/H_2O_2/NO_2^- in the *m*/*z* range of 100−800. The spectra showed the presence of molecular ions $(M+H)^+$ of *m*/*z* 528 (parent molecule), 383 (aglycon), 363 (aglycon with aromatized ring A), 337, 321, which are characteristic of fragmentation of an intact DNR (*70*, *71*). In the sample containing oxidized DNR, relative peak intensities for ions at *m*/*z* 321 and 363 were markedly decreased, and the ion at 528 was absent. An ion of *m*/*z* 148 (daunosamine) was observed in a sample containing oxidized DNR. Molecular ions from corresponding Na^+ adducts were also detected since samples were prepared in sodium phosphate buffer. No peaks indicating formation of nitrated DNR were detected. Molecular ions at *m*/*z* 427 and 471 were detected only in the sample containing oxidized DNR, but no unambiguous assignment could be made at present.

Although the present study has focused on the role of nitrite in the oxidative metabolism of anthracyclines, we foresee that other physiologically relevant molecules might also be capable of catalyzing these transformations. Work is currently in progress to address these various possibilities.

ACKNOWLEDGMENT

We are thankful to Mr. Sean Martin (EPR Core Facility, University of Iowa) for chemiluminescence measurements of NO_2^-/NO_3^-.

REFERENCES

1. Young, R. C., Ozols, R. F., and Myers, C. E. (1981) *N. Engl. J. Med. 305*, 139−153.
2. Keizer, H. G., Pinedo, H. M., Schuurhuis, G. J., and Joenje, H. (1990) *Pharmacol. Ther. 47*, 219−231.
3. Olson, R. D., and Mushlin, P. S. (1990) *FASEB J. 4*, 3076−3086.
4. Bachur, N. R., Gordon, S. L., Gee, M. V., and Kon, H. (1979) *Proc. Natl. Acad. Sci. U.S.A. 76*, 954−957.
5. Kalyanaraman, B., Perez-Reyes, E., and Mason, R. P. (1980) *Biochim. Biophys. Acta 630*, 119−130.
6. Schreiber, J., Mottley, C., Sinha, B. K., Kalyanaraman, B., and Mason, R. P. (1987) *J. Am. Chem Soc. 109*, 348−351.
7. Gille, L., and Nohl, H. (1997) *Free Radical Biol. Med. 23*, 775−782.
8. Handa, K., and Sato, S. (1976) *Gann 67*, 523−528.
9. Doroshow, J. H. (1986) *Proc. Natl. Acad. Sci. U.S.A. 83*, 4514−4518.
10. Garner, A. P., Paine, M. J. I., Rodriguez-Crespo, I., Chinje, E. C., De Montellano, P. O., Stratford, I. J., Tew, D. G., and Wolf, C. R. (1999) *Cancer Res. 59*, 1929−1934.
11. Vasquez-Vivar, J., Martasek, P., Hogg, N., Masters, B. S. S., Pritchard, K. A., Jr., and Kalyanaraman, B. (1997) *Biochemistry 36*, 11293−11297.
12. Pietronigro, D. D., McGinness, J. E., Koren, M. J., Crippa, R., Seligman, M. L., and Demopoulos, H. B. (1979) *Physiol. Chem. Phys. 11*, 405−414.
13. Chinami, M., Kato, T., Ogura, R., and Shingu, M. (1984) *Biochem. Int. 8*, 299−304.
14. Zweier, J. L. (1984) *J. Biol. Chem. 259*, 6056−6058.
15. Zweier, J. L. (1985) *Biochim. Biophys. Acta 839*, 209−213.
16. Zweier, J. L., Gianni, L., Muindi, J., and Myers, C. E. (1986) *Biochim. Biophys. Acta 884*, 326−336.
17. Gianni, L., Zweier, J. L., Levy, A., and Myers, C. E. (1985) *J. Biol. Chem. 260*, 6820−6826.
18. Beraldo, H., Garnier-Suillerot, A., Tosi, L., and Lavelle, F. (1985) *Biochemistry 24*, 284−289.
19. Malisza, K. L., and Hasinoff, B. B. (1995) *Arch. Biochem. Biophys. 321*, 51−60.
20. Dunford, H. B. (1999) *Heme peroxidases*, Wiley-VCH, New York.
21. McCormick, M. L., Gaut, J. P., Lin, T.-S., Britigan, B. E., Buettner, G. R., and Heinecke, J. W. (1998) *J. Biol. Chem. 273*, 32030−32037.
22. Monzani, E., Gatti, A. L., Profumo, A., Casella, L., and Gullotti, M. (1997) *Biochemistry 36*, 1918−1926.
23. Nakamura, M., Yamazaki, I., Kotani, T., and Ohtaki, S. (1985) *J. Biol. Chem. 260*, 13546−13552.
24. Zhang, H., and Dunford, H. B. (1993) *Can. J. Chem. 71*, 1990−1994.
25. Kettle, A. J., and Winterbourn, C. C. (1992) *J. Biol. Chem. 267*, 8319−8324.
26. Reszka, K., Kolodziejczyk, P., and Lown, J. W. (1986) *Free Radical Biol. Med. 2*, 25−32.
27. Kolodziejczyk, P., Reszka, K., and Lown, J. W. (1988) *Free Radical Biol. Med. 5*, 13−25.
28. Kolodziejczyk, P., Reszka, K., and Lown, J. W. (1988) in *Oxy-Radicals in Molecular Biology and Pathology* (Cerutti, P. A., Fridovich, I., and McCord, J. M., Eds.) pp 525−539, Alan, R. Liss, Inc., New York.
29. Kolodziejczyk, P., Reszka, K., and Lown, J. W. (1989) *Biochem. Pharmacol. 38*, 803−809.
30. Davies, A., Malone, M. E., Martin, E. A., Jones, R. M., Jukes, R., Lim, C.-K., Smith, L. L., and White, I. N. (1997) *Free Radical Biol. Med. 22*, 423−431.
31. Miura, T., Muraoka, S., and Fujimoto, Y. (2000) *Biochem. Pharmacol. 60*, 95−99.
32. Josephy, P. D. (1996) *Mutagenesis 11*, 3−7.
33. Shin, K., Tomita, M., and Lonnerdal, B. (2000) *J. Nutr. Biochem. 11*, 94−102.
34. Gerson, C., Sabater, J., Scuri, M., Torbati, A., Coffey, R., Abraham, J. W., Lauredo, I., Forteza, R., Wanner, A., Salathe, M., Abraham, W. M., and Conner, G. E. (2000) *Am. J. Respir. Cell Mol. Biol. 22*, 665−671.
35. Tenovuo, J. O. (1985) in *The Lactoperoxidase System* (Pruitt, K. M., and Tenovuo, J. O., Eds.) Chapter 8, pp 101−122, Marcel Dekker, Inc., New York.
36. Chance, B. (1952) *Arch. Biochem. Biophys. 41*, 416−424.
37. Roman, R., and Dunford, H. B. (1973) *Can. J. Chem. 51*, 588−596.
38. Gebicka, L. (1999) *Acta Biochim. Pol. 46*, 919−927.
39. Burner, U., Furtmuller, P. G., Kettle, A. J., Koppenol, W. H., and Obinger, C. (2000) *J. Biol. Chem. 275*, 20597−20601.
40. Reszka, K. J., Matuszak, Z., and Chignell, C. F. (1997) *Chem. Res. Toxicol. 10*, 1325−1330.
41. Reszka, K. J., Matuszak, Z., McCormick, M. L., Britigan, B. E., and Chignell, C. F. (1999) *Free Radical Biol. Med. 27 (Suppl. 1)*, S148.
42. Reszka, K. J., Matuszak, Z., Chignell, C. F., and Dillon, J. (1999) *Free Radical Biol. Med. 26*, 669−678.
43. McCormick, M. L., Reszka, K. J., Hazen, S. L., Buettner, G. R., and Britigan, B. R. (1999) *Free Radical Biol. Med. 27 (Suppl. 1)*, S81.
44. van der Vliet, A., Eiserich, J. P., Halliwell, B., and Cross, C. E. (1997) *J. Biol. Chem. 272*, 7617−7625.
45. Klebanoff, S. (1993) *Free Radical Biol. Med. 14*, 351−360.
46. Sampson, J. B., Ye, Y. Z., Rosen, H., and Beckman, J. S. (1998) *Arch. Biochem. Biophys. 356*, 207−213.
47. Chaires, J. B., Dattagupta, N., and Crothers, D. M. (1982) *Biochem*istry *21*, 3927−3932.
48. Razzano, G., Rizzo, V., and Vigevani, A. (1990) *Farmaco 45* (2), 215−222.
49. Bardsley, W. G. (1985) in *The Lactoperoxidase System* (Pruitt, K. M., and Tenovuo, J. O., Eds.) Chapter 4, pp 55−87, Marcel Dekker, Inc., New York.
50. Venkataraman, S., Martin, S. M., Schafer, F. Q., and Buettner, G. R. (2000) *Free Radical Biol. Med. 29*, 580−585.
51. Taatjes, D. J., Gaudiano, G., Resing, K., and Koch, T. H. (1997) *J. Med. Chem. 40*, 1276−1286.
52. Tong, G., Lee, W. W., Black, D. R., and Henry, D. W. (1976) *J. Med. Chem. 19*, 395−398.
53. Wardman, P. (1989) *J. Phys. Chem. Ref. Data 18*, 1637−1755.
54. Forni, L. G., Mora-Arellano, V. O., Packer, J. E., and Willson, R. L. (1986) *J. Chem. Soc., Perkin Trans. 2*, 1−6.
55. Bruck, T. B., Fielding, R. J., Symons, M. C. R., and Harvey, P. J. (2001) *Eur. J. Biochem. 268*, 3214−3222.
56. Reszka, K. J., Matuszak, Z., and Chignell, C. F. (1998) *Free Radical Biol. Med. 25*, 208−216.
57. Floris, R., Piersma, S. R., Yang, G., Jones, P., and Wever, R. (1993) *Eur. J. Biochem. 215*, 767−775.
58. Pal, H., Palit, D. K., Mukherjee, T., and Mittal, J. P. (1992) *J. Chem. Soc., Faraday Trans. 88*, 681−687.
59. Mukherjee, T., Land, E. J., Swallow, A. J., Bruce, J. M., Beaumont, P. C., and Parsons, B. J. (1988) *J. Chem. Soc., Faraday Trans. 1 84*, 3423−3434.
60. Hu, S., Treat, R. W., and Kincaid, J. R. (1993) *Biochemistry 32*, 10125−10130.
61. Sievers, G. (1980) *Biochim. Biophys. Acta 624*, 249−259.
62. Ferrari, R. P., Ghibaudi, E. M., Traversa, S., Laurenti, E., De Gioia, L., and Salmona, M. (1997) *J. Inorg. Biochem. 68*, 17−26.
63. Huie, R. E. (1994) *Toxicology 89*, 193−216.
64. Beraldo, H., Garnier-Suillerot, A., and Tosi, L. (1983) *Inorg. Chem. 22*, 4117−4124.

65. Kano, K., Konse, T., and Kubota, T. (1985) *Bull. Chem. Soc. Jpn. 58*, 424−428.
66. Land, E. J., Mukherjee, T., Swallow, A. J., and Bruce, J. M. (1983) *J. Chem. Soc., Faraday Trans. 1*, 391−404.
67. Stanbury, D. M. (1989) *Adv. Inorg. Chem. 33*, 69−138.
68. Ramu, A., Mehta, M. M., Liu, J., Turyan, I., and Aleksic, A. (2000) *Cancer Chemother. Pharmacol. 46*, 449−458.
69. Ramu, A., Mehta, M. M., Leaseburg, Th., and Aleksic, A. (2001) *Cancer Chemother. Pharmacol. 47*, 338−346.
70. Monneret, C., and Sellier, N. (1986) *Biomed. Environ. Mass Spectrom. 13*, 319−326.
71. Bloom, J., Lehman, P., Israel, M., Rosario, O., and Korfmacher, W. A. (1992) *J. Anal. Toxicol. 16*, 223−227.
72. Kohler, H., and Jenzer, H. (1989) *Free Radical Biol. Med. 6*, 323−339.
73. Sun, W., and Dunford, H. B. (1993) *Biochem*istry *32*, 1324−1331.
74. Andersson, L. A., Bylkas, S. A., and Wilson, A. E. (1996) *J. Biol. Chem. 271*, 3406−3412.
75. Rae, T. D., and Goff, H. M. (1998) *J. Biol. Chem. 273*, 27968−27977.
76. Miller, R. A., and Britigan, B. E. (1997) *Clin. Microbiol. Rev. 10*, 1−18.
77. Hampton, M., Kettle, A. J., and Winterbourn, C. C. (1998) *Blood 92*, 3007−3017.
78. Samoszuk, M. K., Nguyen, V., Gluzman, I., and Pham, J. H. (1996) *Am. J. Pathol. 148*, 701−706.
79. Thomsen, L. L., Miles, D. W., Happerfield, L., Bobrow, L. G., Knowles, R. G., and Moncada, S. (1995) *Br. J. Cancer 72*, 41−44.
80. Jenkins, D. C., Charles, I. G., Thomsen, L. L., Moss, D. W., Holmes, L. S., Baylis, S. A., Rhodes, P., Westmore, K., Emson, P. C., and Moncada, S. (1995) *Proc. Natl. Acad. Sci. U.S.A. 92*, 4392−4396.
81. Stuehr, D. J., and Nathan, C. F. (1989) *J. Exp. Med. 169*, 1543−1555.
82. Kono, Y., Shibata, H., Adachi, K., and Tanaka, K. (1994) *Arch. Biochem. Biophys. 311*, 153−159.
83. Keller, R., Geiges, M., and Keist, R. (1990) *Cancer Res. 50*, 1421−1425.
84. Lind, D. S., Kontaridis, M. I., Edwards, P. D., Josephs, M. D., Moldawer, L. L., and Copeland, E. D., III (1997) *J. Surg. Res. 69*, 283−287.
85. Douzono, M., Suzu, S., Yamada, M., Yanai, N., Kawashima, T., Hatake, K., and Motoyoshi, K. (1995) *Jpn. J. Cancer Res. 86*, 315−321.

BI011869C