Biochemical Pharmacology, Vol. 38, No. 5, pp. 803–809, 1989.
Printed in Great Britain.

0006-2952/89 $3.00 + 0.00

ENZYMATIC OXIDATIVE ACTIVATION OF 5-IMINODAUNORUBICIN

SPECTROPHOTOMETRIC AND ELECTRON PARAMAGNETIC RESONANCE STUDIES

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(*Received* 13 *January* 1988; *accepted* 22 *July* 1988)

Abstract—Horseradish peroxidase catalyzed oxidation of the antitumor agent 5-iminodaunorubicin by hydrogen peroxide was studied with both spectrophotometric and electron paramagnetic resonance methods. Kinetics of oxidation of the drug at pH 3, 6 and 8 were determined. Rapid formation of a nitrogen-centered free radical metabolite was demonstrated with electron paramagnetic resonance employing the ^{15}N-labeled drug and by deuterium exchange techniques. This enzymatic oxidative activation of 5-iminodaunorubicin suggests an alternative mode of metabolism and mechanism of action of this less cardiotoxic anticancer agent. By contrast, the parent compound, daunorubicin, did not undergo oxidation by the horseradish peroxidase–hydrogen peroxide system.

The anthracycline antibiotics, daunorubicin and doxorubicin, are widely employed in the treatment of various solid and hematologic malignancies [1–3]. However, their clinical use has been limited due to development of cumulative and dose-dependent cardiotoxicity. It has been found that the quinone-modified anthracycline, 5-iminodaunorubicin (5-IMDR†, I, Scheme 1), has little or no cardiac toxicity [4–7] and is less mutagenic than the parent daunorubicin (VIII), while still maintaining its antitumor activity [8]. The molecular basis of antitumor action of the anthracyclines, as well as their cardiac toxicity, are commonly explained, at least in part, by reductive activation of the drug with cytochrome P-450 reductase [9]. The mechanism involves one electron reduction of the quinone moiety of these drugs to a semiquinone radical. This semiquinone radical reacts with molecular oxygen initiating a cascade of reactions resulting in generation of a variety of reactive, oxygen-derived species, such as superoxide ion, hydrogen peroxide and hydroxyl radicals. These species have been implicated in the damage of cellular proteins, nucleic acids and components of cell membranes [9, 10].

5-IMDR has a markedly diminished capacity to form reactive oxy-radical species following enzymatic reduction [10–12]. Electrochemical measurements demonstrate that iminoquinones are more resistant to reduction than the corresponding quinones [13], and this is reflected in the more negative polarographic reduction potentials for 5-IMDR (−670 mV) compared with daunorubicin (−640 mV) [14, 15].

The pronounced differences in the ability to generate reactive oxygen-derived species, between 5-IMDR and daunorubicin, and the markedly lower mutagenicity of the former, imply not only quantitative but rather qualitative differences in their mechanisms of action. Moreover, 5-IMDR possesses structural features which suggest that this drug should readily undergo enzymatic oxidation. For example, oxidation of *p*-aminophenols leading to the formation of iminoquinone systems is well known and was studied for xenobiotics such as acetaminophen, *p*-aminophenol and ellipticines [16–20].

This potential for oxidative activation has not been recognized hitherto in the metabolism of 5-IMDR; however, such enzymatic activation, accompanied by the generation of free radicals, was reported recently for the structurally related anticancer agent mitoxantrone [21, 22]. For these reasons, we have investigated the capacity of 5-IMDR to undergo enzymatic oxidation. Horseradish peroxidase (HRP) and hydrogen peroxide (H_2O_2) were used as the oxidizing system, and EPR techniques were employed to detect free radical metabolites.

MATERIALS AND METHODS

Daunorubicin was obtained from the Laboratoire Rhône-Poulenc (France). Horseradish peroxidase (EC 1.11.1.7) was purchased from Boehringer Mannheim GmbH, West Germany. Hydrogen peroxide (30%) was obtained from Fisher Scientific. Gaseous [^{15}N]ammonia (99% isotope enrichment) was from Stohler Isotope Chemicals, Waltman, MA, U.S.A., and deuterium oxide ^2H_2O was purchased from the Aldrich Chemical Co. Inc., Milwaukee, WI, U.S.A. 5-IMDR was synthesized according to the method previously described [4] and, for the preparation of its ^{15}N-labeled analogue,

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† Abbreviations: 5-IMDR, 5-iminodaunorubicin; HRP, horseradish peroxidase; H_2O_2, hydrogen peroxide; EPR, electron paramagnetic resonance; and ^2H_2O, deuterium oxide.

[^{15}N]ammonia was employed. Mass spectral analyses of 5-IMDR and its labeled analogue were performed on an MS-9 AEI high resolution instrument. The following molecular ions were detected: M+H, $m/z = 527$ for 5-IMDR; and M+H, $m/z = 528$ for the ^{15}N-labeled analogue, respectively. The fragmentation ions $m/z = 398$ (M − sugar); 381; 362 and 320 for 5-IMDR together with ions $m/z = 399$ (M − sugar); 382, 363 and 321 for the labeled analogue, respectively, confirm that labeling occurred at the C-5 position of daunorubicin.

Stock solutions of the drug (4 mg/ml) and H_2O_2 (70 mM and 7 mM) were prepared in distilled water. Enzyme stock solutions (2500 units/ml) were prepared in 0.1 M phosphate buffer, pH 6.0. Incubation mixtures containing 5-IMDR, H_2O_2 and HRP were prepared in 0.01 M and 0.1 M acetate (pH 3 and 4) or phosphate (pH 6 and 8) buffers for the EPR and spectrophotometric experiments respectively.

UV-VIS absorption spectra were recorded with an HP 8452A Diode Array Spectrophotometer using standard 1 cm quartz cuvettes. Kinetic spectrophotometric experiments were performed for drug concentrations in the range of 7–560 μM, [drug]/[H_2O_2] = 1 and containing 10–100 units of enzyme.

Oxidation was initiated by addition of a small aliquot (20 μl) of the HRP stock solution to an incubation sample. The final volume of samples for the spectrophotometric analysis was 1 ml. Heat denaturation of the enzyme was accomplished by heating the HRP in the pH 6 buffer at 100° for 10 min.

Samples for the EPR studies were introduced into a Suprasil quartz flat cell and transferred quickly to the microwave cavity of the Bruker ER-400 spectrometer operating at 9.5 GHz and 100 kHz field modulation. Time-dependent free radical concentration changes were determined measuring the amplitude of the first, low-field component of the EPR spectrum recorded at 15- to 30-sec intervals. All experiments were performed at room temperature.

RESULTS

The absorption spectrum of 5-IMDR in phosphate buffer pH 6 is shown in Fig. 1 (run a). Addition of HRP to the solution containing 5-IMDR and hydrogen peroxide caused a decrease of the drug absorbances at 550 and 588 nm, characteristic of the drug, and generation of a new absorption band in the region 400–420 nm. An isosbestic point at 450 nm was identified. No spectral changes of the drug were observed when either enzyme or hydrogen peroxide was omitted, or when thermally inactivated enzyme was used. Spectral changes similar to those shown in Fig. 1 were observed when buffer pH 8 was employed (not shown). It was found that the kinetics of the drug consumption depended on the [H_2O_2]/[drug] ratio. This dependence is presented in Fig. 2 for samples incubated at pH 6. It may be seen that, for the ratio <1, oxidation was incomplete and was followed by a partial increase of the absorbance at 550 nm. This may be interpreted as a partial recovery of the drug, after exhausting the H_2O_2, in comproportionation–disproportionation reactions, by processes similar to those observed for mitoxantrone [22].

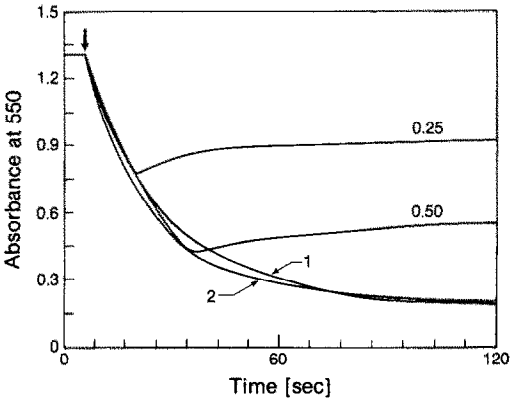


Fig. 2. Oxidation of 5-iminodaunorubicin by H_2O_2/HRP system in phosphate buffer, pH 6: Kinetics of the decrease of the absorbance at 550 nm. The incubation mixture contained 5-IMDR (140 μM) and various concentrations of H_2O_2. The numbers above the curves correspond to [H_2O_2]/[drug] ratios. The reaction was initiated by addition of HRP (100 units) as indicated with arrow.

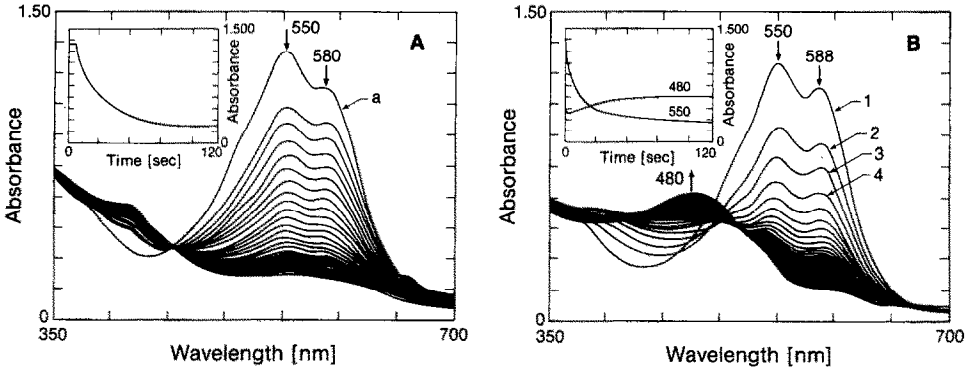


Fig. 1. (A) Oxidation of 5-iminodaunorubicin by H_2O_2/HRP system: Optical spectra. The incubation mixture contained 5-IMDR (140 μM), H_2O_2 (140 μM) and HRP (100 units) in phosphate buffer, pH 6. HRP was added after the first scan (a) and then spectra were recorded every 3 sec. The insert shows changes of the absorbance at 550 nm vs time. (B) As in panel A but reaction was performed in acetate buffer, pH 3. The insert shows changes of the absorbance at 550 and 480 nm. Arrows indicate direction of changes (numbers show consecutive scans).

When oxidation was performed at pH 3, quite a different pattern of spectral changes was recorded (Fig. 1B). The decrease of absorbance at 550 and 588 nm was followed by generation of another absorption band in the region of 460–500 nm and the formation of an isosbestic point around 510 nm. This newly formed product was isolated and, by chromatographic and mass spectroscopy analyses, was found to be identical with daunorubicin.

No change in the UV-VIS spectra of 5-IMDR was observed when the drug was incubated at pH 3 without HRP. Therefore, it can be concluded that, at pH 3, oxidative enzymatic activation of the drug is required for the transformation which can be described by the following equation:

$$\text{5-IMDR} \xrightarrow[\text{pH 3}]{\text{HRP}/H_2O_2} \longrightarrow \text{an intermediate} \xrightarrow[\text{pH 3}]{} \ldots \text{Daunorubicin.}$$

Introduction of ascorbic acid (280 μM) to the incubation mixture, prior to HRP addition, totally prevented oxidation of 5-IMDR (not shown). Ascorbic acid was also able to prevent the oxidation of 5-IMDR by HRP/H_2O_2, when added soon after initiation of the reaction by HRP. For samples incubated at pH 3, addition of ascorbic acid, 12 sec after the reaction had been initiated with HRP, prevented the oxidation and caused partial recovery (*ca.* 60%) of the absorbance at 550 nm (Fig. 3). Apparently some oxidation products can be reduced back to 5-IMDR by ascorbic acid under these conditions. No recovery of absorbance was observed for samples incubated at pH 8 (Fig. 3).

Addition of ascorbic acid to fully oxidized 5-IMDR (no absorbance at 550 nm and 588 nm) did not regenerate the substrate. The decrease in absorbance at 550 nm and 588 nm versus time was used to determine the rate of oxidation of 5-IMDR with the HRP/H_2O_2 system. K_m and V_{max} values for the reaction at pH 3 and 8 were estimated from the Lineweaver–Burke plot and are shown in Table 1. The kinetic parameter V_{max} at pH 6 was found not to differ significantly from that reported for pH 8. Analogous experiments employing daunorubicin showed no change in the absorption spectrum, implying resistance of this drug to oxidation with the HRP/H_2O_2 system.

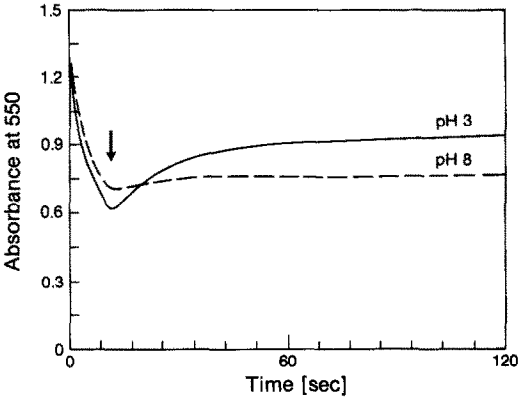


Fig. 3. Oxidation of 5-iminodaunorubicin by H_2O_2/HRP system: Effect of ascorbic acid. The incubation mixture contained 5-IMDR (140 μM) and H_2O_2 (140 μM) in acetate buffer, pH 3, and phosphate buffer, pH 8, respectively. The reaction was initiated by addition of HRP (100 units), and absorbance at 550 nm was recorded every 3 sec. After 12 sec ascorbic acid (280 μM) was added, as indicated with the arrow.

EPR study. A strong, well defined, EPR signal was recorded when 5-IMDR was oxidized by H_2O_2 in the presence of HRP (Fig. 4). The signal reached maximum intensity within 60 sec from initiation of reaction with HRP and then decayed (Fig. 5). Owing to very fast generation of the drug-derived radicals at various [drug]/[H_2O_2] ratios and at the enzyme concentrations employed, we were not able to follow the kinetics of generation of the free radicals; however, the kinetics of their decay could be

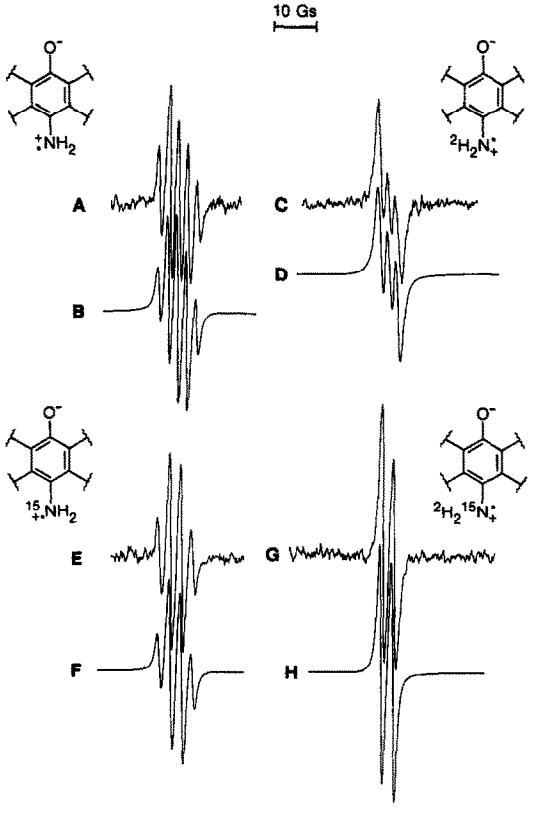


Fig. 4. (A) EPR spectra recorded during oxidation of 5-iminodaunorubicin with H_2O_2/HRP in phosphate buffer, pH 6; [5-IMDR] = 600 μM, [H_2O_2] = 600 μM, and HRP (10 units). Spectrometer settings: scan range, 150 G; scan time, 50 sec; time constant, 0.5 sec; microwave power, 20 mW; modulation amplitude, 0.67 G. The g-value for the spectrum was found to be equal to 2.0048. (B) Simulated spectrum. (C) Oxidation of 5-iminodaunorubicin with H_2O_2/HRP in ^2H_2O buffer. Conditions as in A except that ^2H_2O was used as a solvent. (D) Simulated spectrum. (E) Oxidation of [^{15}N]-5-iminodaunorubicin by H_2O_2/HRP. Conditions as in A except that [^{15}N]-5-IMDR was used. (F) Simulated spectrum. (G) Oxidation of [^{15}N]-5-iminodaunorubicin in ^2H_2O. Conditions as in A except that [^{15}N]-5-IMDR and ^2H_2O were used. (H) Simulated spectra. Parameters for simulation of the EPR spectra are given in Table 2.

Table 1. Kinetic constants for oxidation of 5-IMDR by HRP/H_2O_2 at different pH values

pH	V_{max} [μmol/sec unit]	K_m [μM]
3.0	1.92	93
6.0	1.06	42
8.0	1.10	84

followed. Figure 5 shows such kinetics for samples incubated at pH 6 for several different [drug]/[H_2O_2] ratios. Similar profiles were found for the radical decay at pH 4 and 8 (not shown).

Because the highest stability of the radicals at pH 6 was observed for [drug]/[H_2O_2] = 1 (Fig. 5), the complete EPR spectrum was recorded under these conditions and is shown in Fig. 4A. A simulated spectrum (Fig. 4B) was obtained assuming interaction of the unpaired electron with the amine (position 5) nitrogen ^{14}N and two, non-equivalent protons (see Table 2 for the hyperfine coupling constants). Confirmation of the interaction of the unpaired spin with two exchangeable protons comes from the experiments performed in ^2H_2O which produced a three-line spectrum, as shown in Fig. 4C. When ^{15}N-labeled 5-IMDR was incubated in H_2O buffer and under the conditions described above, the EPR spectrum shown in Fig. 4E was observed. The four-line spectrum was assigned to the interaction of the unpaired electron with the ^{15}N nucleus and two equivalent protons. A simulated spectrum (Fig. 4F) was obtained using hyperfine coupling constants from Table 2. Figure 4G shows the EPR spectrum from the incubation mixture containing ^{15}N-labeled drug in ^2H_2O buffer (pD = 6). The spectrum was assigned to that originating from hyperfine splittings on the ^{15}N nucleus in position 5 of the chromophore. Reduction in the number of hyperfine components in the EPR spectra from ^2H_2O incubations, in comparison with incubations in H_2O, was consistent with the effect of substitution of the exchangeable protons by deuterium [17]. No EPR spectra were detected from incubations containing daunorubicin under similar conditions.

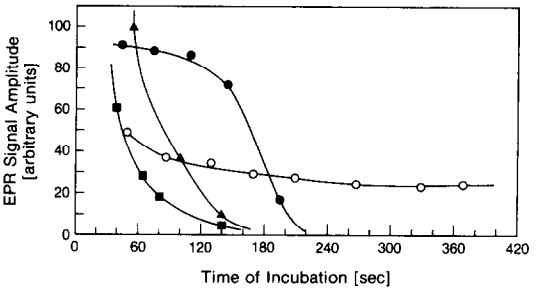


Fig. 5. Dependence of the amplitude of EPR signal on [drug]/[H_2O_2] ratios. Incubations consisted of 5-iminodaunorubicin (600 μM), HRP (10 units) and H_2O_2 (33–600 μM) in buffer, pH 6. Employed [drug]/[H_2O_2] ratios were: (■) 18, (▲) 9, (●) 4.5 and (○) 1.

DISCUSSION

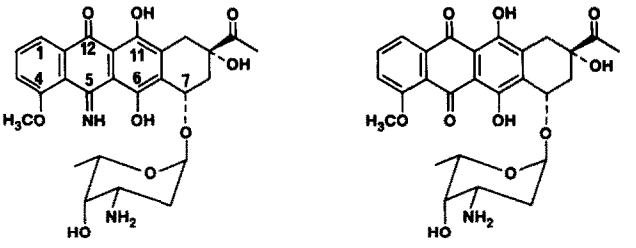
5-IMDR is considered to be a less cardiotoxic anticancer agent than the parent drug daunorubicin [5–7]. This property may possibly be attributed to the more negative reduction potential of 5-IMDR, when compared to that of daunorubicin, on the basis of the commonly accepted explanation for the low augmentation of microsomal oxygen consumption and diminished production of reactive, oxygen derived species via redox cycling [14, 15]. It is plausible that this difference in redox potentials is responsible for the inefficient reductive metabolism of 5-IMDR, compared with that of the parent drug daunorubicin. The physiological expression of this property may then be the markedly lower cardiotoxicity of this semisynthetic drug. We recently conducted studies on the oxidative metabolism of the structurally related drug, mitoxantrone, which is also known to be resistant to reductive enzymatic activation [21, 22]. These studies revealed that mitoxantrone undergoes extensive oxidative metabolism, involving generation of free radicals and structural transformation to a novel metabolite.

The chromophore of daunorubicin was found to be resistant to oxidative enzymatic activation by the H_2O_2/HRP system frequently used as a model for bio-oxidation of xenobiotics [23]. However, 5-IMDR

Table 2. Hyperfine coupling constants from the EPR spectra of the quinoneimine radical from 5-IMDR

Compound and solvent	Coupling constants* (G) a_N	a_H	Modulation amplitude*	Linewidth*
[^{14}N]-5-IMDR H_2O	2.3	2.8, 2.5	0.4	1.0
[^{14}N]-5-IMDR ^2H_2O	2.1	—	0.8	1.4
[^{15}N]-5-IMDR H_2O	3.0	2.8 (2H)	0.6	1.0
[^{15}N]-5-IMDR ^2H_2O	3.0	—	0.4	1.0

* Parameters used for simulation of the EPR spectra.



I VIII

Scheme 1. Structures of 5-iminodaunorubicin (I) and daunorubicin (VIII).

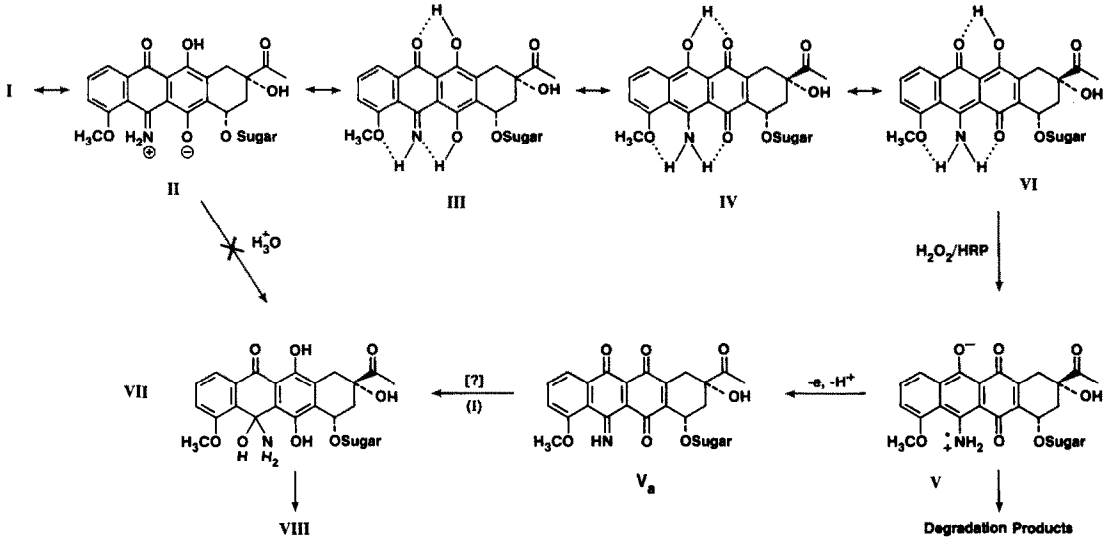
(a derivative in which the C-ring quinone is regiospecifically transformed into an iminoquinone) readily undergoes oxidation when incubated with H_2O_2 and HRP.

In aqueous solutions 5-IMDR (structure I, Scheme 1) can exist in equilibrium with several tautomeric forms: II, III, IV, and VI as shown in Scheme 2 [4, 24].

The existence of form VI with hydrogen bonds, as indicated in Scheme 2, has been suggested on the basis of an NMR study [4]. Analogous bonds could be present in IV. The form in which the drug exists in aqueous solution seems to be critical for the oxidation process. Structure I, containing a quinoneimine moiety, is the most frequently represented form of this agent [4, 14, 15]. It is known, however, that quinoneimines are extremely reactive species and readily undergo hydrolysis in aqueous solutions, most readily in the pH 2–3 range [25]. The apparent stability of the absorption spectra of solutions containing 5-IMDR at the lower pH values does not favor structure I. However, it may be argued that steric factors from groups flanking the quinoneimine moiety within structure I, and the possible role of aggregation of the drug molecules in aqueous solution, may influence stability and thus affect the rate of hydrolysis. The results seem to be more in accord with structure IV and/or VI (presented in Scheme 2 in the non-ionized form). The EPR measurements indicated formation of a nitrogen-centered radical which may be ascribed to structure V. Radical V may then undergo further oxidation with formation of the quinoneimine (V_a).

The oxidation of IV (or VI), as shown in Scheme 2, is analogous to enzymatic oxidation of *p*-aminophenol [17, 18] and is in accord with the EPR data. It appears plausible that the partial recovery of 5-IMDR, observed at pH 3, when ascorbic acid was added soon after the reaction was initiated with HRP (Fig. 3), could be explained by reduction of the radical V to the form IV (or VI).

At pH 3 daunorubicin was formed among the



Scheme 2. Schematic depiction of H_2O_2/HRP oxidation of 5-iminodaunorubicin (I) in one of its tautomeric forms (III, IV or VI) towards radical cation V and further oxidation to the quinoneimine V_a form. No hydrolysis of protonated form II to daunorubicin (VIII) was observed without previous enzymatic activation. The formation of daunorubicin (VIII) upon enzymatic activation may involve hydrolysis of the iminoquinone form (V_a) and/or disproportionation-comproportionation of the radical cation V and regeneration of II–IV forms.

products of the oxidation. One possible pathway in which daunorubicin could be formed may involve hydrolysis and reduction of form V_a (possibly by unreacted IV) to VII which after elimination of ammonia yields VIII (daunorubicin) (Scheme 2). Accumulation of an intermediate preceding formation of VIII is evident from the spectra in Fig. 1B. The appearance of an absorption band at 480 nm is concomitant with formation of an isosbestic point but delayed until *ca.* 50% of the substrate is oxidized (this corresponds to the fourth scan in Fig. 1B). The fact that hydrolysis of 5-IMDR to daunorubicin at pH 3 must be preceded by enzymatic activation with the H_2O_2/HRP system, may suggest different behavior from that of "typical" quinoneimines [25].

The saturation analysis carried out with respect to substrate 5-iminodaunorubicin indicates that the apparent K_m (and hence the substrate–enzyme affinity) does not change appreciably with the pH. This indicates that the same species intervene in the oxidation reaction, within the pH range investigated.

In contrast, the 2-fold increase in V_{max} found at pH 3 may be attributed to a partial hydrolysis of the substrate, an irreversible process that does not appear to occur at higher pH.

Many aromatic amines are oxidized efficiently by peroxidases, such as HRP. For example, quinoneimines are the final products of the HRP-catalyzed oxidation of *p*-aminophenol [17] and acetaminophen [18, 19], and the reaction involves free radical metabolites, as shown by EPR. In addition, the formation of adducts from ellipticine with macromolecules has been shown to involve a quinoneimine form of the drug generated by H_2O_2/HRP oxidation [16].

Mammalian tissues contain a microsomal enzyme and prostaglandin synthase systems which possess hydroperoxidase activity [18, 26]. The metabolites from xenobiotics such as aminophenols, include reactive species capable of binding to macromolecules, resulting in the formation of polymers, but in most cases their chemical structure is unknown [18]. However, it has been demonstrated that quinoneimines form adducts with glutathione *in vivo*. When large doses of such drugs are administered, hepatic glutathione levels are depleted and covalent binding to hepatic proteins occurs [18–20]. There is also evidence for oxidative activation of mitoxantrone by prostaglandin synthase [27].

Therefore, the involvement of 5-IMDR in an alternative redox process, quite different from that already established for anthracyclines, may be important for understanding of the biological properties, particularly the diminished cardiotoxicity, of this agent. The fact that the more cardiotoxic anthracyclines do not undergo such oxidative activation as found for mitoxantrone, ametantrone [22], and 5-IMDR (all of which are less cardiotoxic agents), should be taken into account.

On the other hand, the mechanism of action of 5-IMDR may be related to the scavenging of primary or induced organic hydroperoxides by the peroxidative pathway, or to inactivation of particular enzymatic systems by "suicidal inhibition" of free radicals generated in the oxidative activation process as suggested earlier for mitoxantrone [22].

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