One- and two-electron reduction of 2-methyl-1,4-naphthoquinone bioreductive alkylating agents: kinetic studies, free-radical production, thiol oxidation and DNA-strand-break formation

Cecilia GIULIVI and Enrique CADENAS*
Department of Molecular Pharmacology and Toxicology, University of Southern California, Los Angeles, CA 90033, U.S.A.

The one- and two-electron enzymic reduction of the bioreductive alkylating agents 2-methylmethoxynaphthoquinone (quinone I) and 2-chloromethylnaphthoquinone (quinone II) was studied with purified NADPH–cytochrome P-450 reductase and DT-diaphorase respectively, and characterized in terms of kinetic constants, oxyradical production, thiol oxidation and DNA-strand-break formation. The catalytic-centre activity values indicated that DT-diaphorase catalysed the reduction of quinone I far more effciently than NADPH–cytochrome P-450 reductase, although the K_m values of the two enzymes for this quinone were similar (1.2–3.0 μM). The one-electron-transfer flavoenzyme also catalysed the reduction of quinone II, but the behaviour of DT-diaphorase towards this quinone did not permit calculation of kinetic constants. A salient feature of the redox transitions caused by the one- and two-electron catalysis of these quinones was the different contributions of disproportionation and autoxidation reactions respectively. In the former case, about 26 % of NADPH consumed was accounted for in terms of autoxidation (as H_2O_2 formation), whereas in the latter, the autoxidation component accounted for most (98 %) of the NADPH consumed.

This difference was abrogated by superoxide dismutase, which enhanced autoxidation during NADPH–cytochrome P-450 catalysis to a maximal value. E.s.r. analysis indicated the formation of superoxide radicals, the signal of which was suppressed by superoxide dismutase and unaffected by catalase. The one- and two-electron reduction of these quinones in the presence of GSH was accompanied by formation of thiyl radicals. Although superoxide dismutase suppressed the thiol radical e.s.r. signal in both instances, the enzyme enhanced GSSG accumulation during NADPH–cytochrome P-450 catalysis of quinone I, whereas it inhibited GSSG formation during reduction of the quinone by DT-diaphorase. One- and two-electron reduction of quinone I led to calf thymus DNA-strand-break formation, a process that (a) was substantially decreased in experiments performed with dialysed DNA and in the presence of desferal and (b) was partially sensitive to superoxide dismutase and/or catalase. These findings are rationalized in terms of the occurrence of metal ions ligated to DNA, protecting against the toxic effects of superoxide radicals generated during enzymic reduction of quinones.

INTRODUCTION

The biological activity of many quinones consists ultimately of a chemical modification, which may be a consequence of the bioreductive activation of the quinone and/or of its electrophilic character, the latter leading to covalent binding to essential biomolecules (Powis, 1989; Brunmark and Cadenas, 1989; O'Brien, 1991; Monks et al., 1992). The dominant features of quinone chemistry, namely their ability to undergo reversible oxidation–reduction reactions and their electrophilic character, have been addressed in studies that evaluated the relative contribution of redox cycling and macromolecule arylation to quinone toxicity (Thor et al., 1982; Gant et al., 1988). These features can be used to rationalize the diverse biological effects of quinones, such as the nephrotoxicant character of some quinone thioethers (Monks et al., 1988, 1990; van Ommen et al., 1991), the potential role of endogenous quinones derived from catecholamines in certain types of neuronal degeneration (Graham, 1984) and the carcinogenic and antitumour properties of quinones (Powis, 1989).

A number of antineoplastic agents possess the quinone nucleus and an appropriate substituent that permits them to function as bioreductive alkylating agents. These compounds are expected to exert cytotoxicity on a sequence of reactions, which involve their activation by two-electron transfers, elimination of the leaving group with formation of a quinone methide intermediate and macromolecule arylation by the quinone methide (Lin and Sartorelli, 1976). That a quinone methide is a chemical requisite inherent in the mechanism of action of these agents was suggested by the finding that compounds with the best leaving groups were the most efficacious as antineoplastic agents (Antonini et al., 1982). However, this property was evaluated as an increase in the survival time of mice bearing sarcoma 180 ascites cells, and the actual mechanism leading to this observation remained undefined. 2- and 6-methyl-1,4-naphthoquinone bioreductive alkylating agents belong to this category; the redox characteristics of these compounds (Lin and Sartorelli, 1976; Wilson et al., 1986), their reactivity towards sulphur nucleophiles (Wilson et al., 1987; Goin et al., 1991) and some aspects of their antitumour activity (Antonini et al., 1982) have been previously reported.

DT-diaphorase [NAD(P)H–quinone oxidoreductase; EC 1.6.99.2] is an obligate two-electron reductase (Ernster, 1987) and, as such, fulfils the chemical requirements for bioreductive alkylation and the ensuing formation of reactive electrophilic quinone methides. The two-electron reduction of quinones catalysed by DT-diaphorase cannot be viewed, however, as a

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detoxifying mechanism, because the redox stability of the hydro-quinones formed is an expression of their functional-group chemistry as shown for benzo- and naphtho-quinones with different substitution patterns (Brunmark and Cadenas, 1988; Buffinton et al., 1989).

The present study was aimed at evaluating the redox properties of 2-methylmethoxy-1,4-naphthoquinone (quinone I) and 2-chloromethyl-1,4-naphthoquinone (quinone II) in terms of their kinetic constants with purified one- and two-electron-transfer flavoproteins and subsequent autoxidation reactions, as well as their contribution to thiol oxidation/arylation and DNA impairment. The biological implications of these results are discussed in terms of the reductive pathways operative in normal and cancer cells under normoxic conditions.
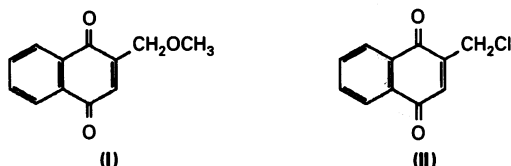
MATERIALS AND METHODS

Materials

Quinones I and II were kindly provided by Professor A. Sartorelli (Comprehensive Cancer Center, Yale University School of Medicine, New Haven, CT, U.S.A.). Chelating resin (imino-diacetic acid/chelex 100), calf thymus DNA and H_2O_2 were from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Bovine liver catalase was from Pharmacia (Uppsala, Sweden). NADPH, GSH, GSSG, horseradish peroxidase (grade I) and Cu–Zn superoxide dismutase were from Boehringer (Mannheim, Germany). Desferal mesylate was from Ciba Pharmaceutical Co. (Summit, NJ, U.S.A.). 5,5′-Dimethyl-1-pyrroline-N-oxide (DMPO) was from Aldrich Chemical Co. (Milwaukee, WI, U.S.A.). All DMPO solutions were filtered with activated charcoal (Darco G-60) repeatedly until the e.s.r. spectrum of DMPO alone was virtually signal-free. DT-diaphorase was a gift from Dr. S. Chen (Division of Immunology, Beckman Research Institute, City of Hope, Duarte, CA, U.S.A.) (Chen et al., 1992) and had a specific activity of 2790 nmol/min per μg of protein measured with 2-methyl-1,4-naphthoquinone as electron acceptor (Lind et al., 1990). The specific activity of NADPH–cytochrome P-450 reductase was 17.3 nmol/min per μg of protein, measured as rate of reduction of cytochrome c. All other reagents were of analytical grade. Potassium phosphate buffers were passed through Chelex-100 before use.

Assay conditions and kinetic studies

The standard assay mixture, once the kinetic constants were calculated, consisted of 10 μM quinone, 100 μM NADPH and 1.32 μg/ml NADPH–cytochrome P-450 reductase in 0.2 M potassium phosphate buffer, pH 7.4. For studies on purified DT-diaphorase, the reaction mixture consisted of 10 μM quinone, 200 μM NADPH and 30 ng/ml enzyme in 0.25 M sucrose/0.1 M potassium phosphate buffer, pH 7.4. Kinetic constants for purified NADPH–cytochrome P-450 reductase and DT-diaphorase were calculated from double-reciprocal plots of v against [S] at different fixed concentrations of NADPH. $1/V_{max.}$ was plotted against 1/[NADPH] to obtain $V_{max.}$ values at [NADPH] $= \infty$.

Absorption and fluorescence spectroscopy

Spectrophotometric measurements were carried out on a double-beam u.v.–visible spectrophotometer (model U-3110; Hitachi Instruments, Danbury, CT, U.S.A.). NADPH oxidation during NADPH–cytochrome P-450 reductase and DT-diaphorase catalysis of 2-methyl-1,4-naphthoquinone bioreductive alkylating agents was measured at 340 nm ($\epsilon = 6.22$ mM^{-1}·cm^{-1}). The concentration of the hydroquinone from quinone II was calculated after absorption in the u.v. region ($\epsilon_{255} = 5.8$ mM^{-1}·cm^{-1} and $\epsilon_{293} = 2.2$ mM^{-1}·cm^{-1}). H_2O_2 was measured fluorimetrically using the horseradish peroxidase/H_2O_2 assay coupled to p-hydroxyphenylacetic acid dimerization ($\lambda_{excitation}$ 315 nm; $\lambda_{emission}$ 410 nm) in an Aminco–Bowman spectrofluorimeter (American Instrument Co., Silver Springs, MD, U.S.A.).

E.s.r.

E.s.r. spectra were recorded on a Bruker ECS 106 equipped with a TM 8810 microwave cavity. Measurements were carried out at room temperature at a microwave frequency of 9.81 GHz and 100 KHz field modulation. Samples to be measured were transferred to heat-sealed capillary ends of Pasteur pipettes. E.s.r. simulation spectra were obtained using the e.s.r. simulation program, version 42.1 (Oehler and Dubose; provided by the Oklahoma Medical Research Foundation, Oklahoma City, OK, U.S.A.).

H.p.l.c. with u.v. and electrochemical detection

GSH and GSSG were measured as dinitrophenol derivatives by h.p.l.c. with u.v. detection (Fariss and Reed, 1987). A Spheri-5-amino 5 μm column (250 mm × 4.6 mm) (Applied Biosystems, Forster City, CA, U.S.A.) was connected to a high-pressure mixing chamber of a dual liquid-chromatography pump system (model LC-600; Shimadzu Scientific Instruments, Columbia, MA, U.S.A.). The mobile phases and gradient program used were as previously described (Goin et al., 1991). The flow rate was 1.5 ml/min. Samples were placed and accessed in an auto-injector (Shimadzu; model SIL-9A; injection volume 100 μl) and the eluate was monitored at 365 nm using a u.v.–visible spectrophometer detector (Shimadzu; model SPD-6AV). Concentrations of GSH and GSSG were calculated from standard curves, and γ-glutamylglutamate was used as the internal standard in all measurements. Quinones and quinone conjugates were analysed by electrochemical detection as previously reported (Cadenas and Ernster, 1990).

Measurement of DNA-strand breaks

Calf thymus DNA (1 mg/ml) was incubated under the conditions described in Table 2 for 3 h at room temperature. After precipitation with ethanol at −70 °C, the supernatants were saved and dried in a Speed-Vac. The residues in 0.2 ml of buffer A (10 mM sodium phosphate buffer, 10 μM CaCl$_2$, 0.02 % NaN$_3$, pH 6.8) and analysed by h.p.l.c. The samples were injected into a hydroxyapatite column (Bio-Gel HPHT; 100 mm × 7.8 mm) (injection volume 50 μl) and eluted with a linear gradient 0–20 min in 100 % solvent B (0.35 M sodium phosphate buffer, 10 μM CaCl$_2$, 0.02 % NaN$_3$, pH 6.8). The samples were recorded at 260 nm with a u.v.–visible detector attached to the h.p.l.c. Strand breaks were quantified against a calibration curve using pure DNA as a standard and expressed as a percentage. Another set of experiments was performed with dialysed DNA: calf thymus

Table 1 Kinetic constants of purified NADPH–cytochrome P-450 reductase and DT-diaphorase for NADPH and quinones and autoxidation of the semiquinone or hydroquinone products

Assay conditions were as described in the Materials and methods section. $K_m^{quinone}$ values were calculated from double-reciprocal plots of v against [S] at different fixed concentrations of NADPH. Secondary plots derived by plotting $1/V_{max.}$ against $1/$[NADPH] were used to caculate $V_{max.}$ at [NADPH] $= \infty$.

	Quinone I		Quinone II
	P-450 reductase	DT-diaphorase	(P-450 reductase)
Kinetic parameters			
K_m^{NADPH} (μM)	33.5	186.8	75.9
$K_m^{quinone}$ (μM)	3.0	1.2	1.1
$V_{max.}$ (nmol/min per μg of enzyme)	120.0	3267.0	37.2
$k_{cat.}$ (s^{-1})	156.6	2995.0	48.5
$k_{cat.}/K_m^{quinone}$ (M$^{-1}\cdot$s^{-1})	5.2×10^7	2.5×10^9	4.4×10^7
Autoxidation			
$-$d[NADPH]/dt(nmol/min per μg of enzyme)			
Control	12.1	2071.0	7.7
$+$Superoxide dismutase (0.5 μM)	12.5	3167.0	12.9
$+$d[H$_2$O$_2$]/dt (nmol/min per μg of enzyme)			
Control	3.2	2027.0	3.1
$+$Superoxide dismutase (0.5 μM)	11.7	2904.0	7.3
$+$d[H$_2$O$_2$]/dt/$-$d[NADPH]/dt			
Control	0.26	0.98	0.40
$+$Superoxide dismutase (0.5 μM)	0.94	0.92	0.57
Oxygen-independent transitions			
d[NADPH]/dt (nmol/min per μg of enzyme)			
Anaerobiosis	9.1	314.0	4.8

DNA (1 mg/ml) in PBS buffer was dialysed against 0.1 M sodium phosphate buffer/0.1 M disodium EDTA, pH 7.4, for 3 h at 0–4 °C using a Spectrapor membrane with an M_r cut-off of 12000–14000. The dialysis bag was removed and dialysed overnight at 0–4 °C using a Spectrapor membrane with an M_r cut-off of 12000–14000. The dialysis bag was removed and dialysed overnight at 0–4 °C against PBS containing 1 mM desferal.

RESULTS AND DISCUSSION

Kinetic studies of purified NADPH–cytochrome P-450 reductase and DT-diaphorase

Table 1 lists the kinetic constants of the purified one- and two-electron-transfer flavoproteins, NADPH–cytochrome P-450 reductase and DT-diaphorase respectively for quinones I and II. Comparison of the catalytic-centre activity ($k_{cat.}$) and the $k_{cat.}/K_m$ quinone values indicated that (a) DT-diaphorase catalysed the reduction of quinone I far more efficiently than the one-electron-transfer flavoprotein, although the K_m values of the two enzymes for this quinone were similar and (b) NADPH–cytochrome P-450 reductase catalysed the reduction of the methylmethoxy-substituted naphthoquinone (I) slightly more efficiently than that of the chloromethyl-substituted quinone (II). The affinity of DT-diaphorase for quinose I was slightly higher than that reported for menadione (2.3 μM) (Hall et al., 1972).

The kinetic constants of purified DT-diaphorase could not be obtained for quinone II because of (a) a lack of linear correlation between enzyme activity and enzyme concentration and (b) the occurrence of v versus [S] plots revealing intermediary plateau and trough regions when NADPH and quinone were the varying substrates. In the former instance, increasing rates of NADPH oxidation were observed up to 25 ng of DT-diaphorase/ml, but above this concentration strong inhibition was observed, amount-

ing to about 94 % with 50 ng of enzyme/ml. The latter behaviour has been extensively studied with dichlorophenol-indophenol as substrate (Hollander et al., 1975). The non-hyperbolic v versus [S] curves seem to occur with chlorine-substituted quinones, such as quinone II studied here and dichlorophenol-indophenol (Hollander and Ernster, 1975; Hollander et al., 1975); similar kinetic behaviour was also observed with chloride-substituted diaziri-dinylbenzoquinones (J. Goin and E. Cadenas, unpublished work).

Autoxidation of 2-methyl-naphthosemiquinone and -naphthohydroquinone bioalkylating agents

Semi- and hydro-quinone autoxidation were evaluated in terms of H_2O_2 production from univalent reduction of O_2 to O_2^- and its subsequent disproportionation to H_2O_2. Formation of O_2^- during semiquinone autoxidation is understood in terms of the conventional reaction (1) described below. O_2^-, and hence H_2O_2, formation during hydroquinone autoxidation requires consideration of comproportionation reactions to yield the semiquinone species. The contribution of autoxidation to overall NADPH consumption during these redox transitions was determined by calculating the actual amounts of H_2O_2 formed and NADPH consumed as well as the ratio of the rates of NADPH oxidation to H_2O_2 production.

Semiquinone autoxidation

After NADPH–cytochrome P-450 catalysis, autoxidation of these semiquinones proceeded at considerable rates: rates of H_2O_2 were similar for the two quinones and accounted for 26–40 % of the rate of NADPH oxidation (Table 1 and Figure 1a). A large fraction (62–75 %) of the latter may be accounted for by reactions other than autoxidation, e.g. disproportionation, as suggested by results obtained under anaerobic conditions (Table 1). This was expected because the equilibrium rate constant (K_1)
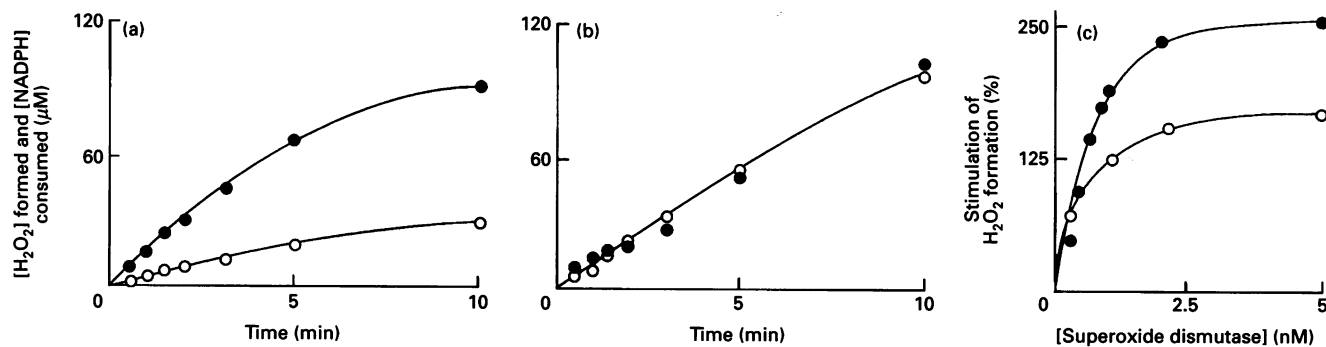
Figure 1 Effect of superoxide dismutase on autoxidation of 2-methylmethoxyseminaphthoquinone during NADPH–cytochrome P-450 catalysis

(a) NADPH consumed (●) and H_2O_2 formed (○) during NADPH–cytochrome P-450 catalysis of quinone I. Assay conditions: 0.2 mM NADPH and 10 μM quinone I in 0.2 M potassium phosphate buffer, pH 7.4, were supplemented with NADPH–cytochrome P-450 reductase (1.32 μg/ml) to initiate the reaction. (b) As in (a) in the presence of superoxide dismutase (50 nM). (c) Dependence of stimulation of H_2O_2 formation during semiquinone autoxidation on superoxide dismutase concentration: ●, quinone I; ○, quinone II.

values for autoxidation of the semiquinone forms [reaction (1)] of quinones I and II are less than 1 (Wilson et al., 1986), i.e.

$$E_{Q/Q^{-\bullet}} > E_{O_2/O_2^{-\bullet}}$$

$$Q^{-\bullet} + O_2 \rightleftharpoons Q + O_2^{-\bullet} \tag{1}$$

The relative contributions of autoxidation and disproportionation to semiquinone decay and hence the overall rate of NADPH oxidation was affected by superoxide dismutase: in the presence of the enzyme 94 % of NADPH oxidation could be ascribed to 2-methylmethoxynaphthosemiquinone autoxidation (Table 1; Figure 1b), whereas 57 % of NADPH oxidation could be ascribed to autoxidation of the halogen-substituted semiquinone (Table 1). The concentration of superoxide dismutase required to elicit half-maximal stimulation of H_2O_2 formation was 0.53 and 0.4 nM for compounds I and II respectively (Figure 1c), values that represent catalytic amounts of the enzyme. This effect of superoxide dismutase is understood in terms of perturbation of the equilibrium of semiquinone autoxidation [reaction (1)] towards the right, on removal of $O_2^{-\bullet}$ (Winterbourn, 1981; Wardman and Wilson, 1987; Winterbourn et al., 1989).

NADPH–cytochrome P-450 catalysis of compounds I and II in the presence of the spin trap DMPO led to generation of e.s.r. signals consistent with the formation of superoxide radical adducts (Figure 2). Albeit small differences in signal intensity, the one-electron reduction of both quinones was accompanied by the formation of a composite spectrum ascribed to a 9:1 mixture of $O_2^{-\bullet}/HO^\bullet$ (Figure 2g). The signal was suppressed by superoxide dismutase (Figure 2c), indicating that the DMPO–HO$^\bullet$ adduct originated from the spontaneous internal conversion of DMPO–HOO$^\bullet$ into DMPO–HO$^\bullet$ (Finkelstein et al., 1980) and therefore that a single species is formed during these redox transitions, $O_2^{-\bullet}$. Moreover, the e.s.r. spectrum exhibited a more characteristic profile of the DMPO–HO$^\bullet$ adduct in the presence of catalase (Figure 2b), in agreement with the reported effect of H_2O_2 of facilitating the spontaneous internal conversion of DMPO–HOO$^\bullet$ into DMPO–HO$^\bullet$ (Finkelstein et al., 1980).

In summary, the results obtained with these 2-methyl-substituted naphthoquinone bioreductive alkylating agents for their one-electron reduction by NADPH–cytochrome P-450 reductase seem to involve only electron-transfer reactions such as autoxidation and disproportionation. A previous pulse-radiolysis study found no evidence for the unimolecular elimination of a leaving group at the semiquinone stage of reduction (Wilson et al., 1986), a process that appears to occur only during two-electron reduction of these compounds (Lin and Sartorelli, 1974).

Hydroquinone autoxidation

H_2O_2 formation during redox transitions of 2-methylmethoxy-1,4-naphthohydroquinone subsequent to DT-diaphorase catalysis proceeded at a rate of 2027 nmol/min per μg of enzyme under standard conditions (Table 1). Hydroquinone autoxidation, in terms of H_2O_2 generation, was enhanced 1.4-fold (2904 nmol/min per μg of enzyme) by superoxide dismutase. However, this enzyme did not change the $+d[H_2O_2]/dt/-d[NADPH]/dt$ ratio significantly, suggesting that hydroquinone decay was primarily by autoxidation. Stimulation of hydroquinone autoxidation by superoxide dismutase has been observed previously during autoxidation of aromatic-ring hydroxy-substituted naphthohydroquinones (Öllinger et al., 1990) and diaziridinylbenzohydroquinone (Ordoñez and Cadenas, 1992). This effect suggests a relative significance of the semiquinone species in the redox transitions of 2-methylmethoxy-1,4-naphthohydroquinone.

The e.s.r. spectrum accompanying the two-electron reduction of compound I was similar, albeit of lower intensity, to that observed during NADPH–cytochrome P-450 catalysis, i.e. a composite spectrum of superoxide and hydroxyl adducts insensitive to catalase and suppressed by superoxide dismutase (Figures 2d–2f).

Reactivity of 2-methyl-1,4-naphthoquinone bioreductive alkylating agents toward glutathione

Two processes accompany the redox transitions of quinones I and II during their one- and two-electron reductive activation in the presence of GSH: thiol oxidation and sulphur nucleophilic substitution/addition.

Thiol oxidation during NADPH–cytochrome P-450 catalysis

The time course of GSH oxidation during the redox transitions is illustrated in Figure 3 for one-electron enzyme catalysis of quinone I: at 20 min incubation, 38 % of the GSH had been oxidized to GSSG (Figure 3a). Disulphide formation was accompanied by H_2O_2 accumulation (Figure 3c) and proceeded via the intermediate formation of thiol radicals as exemplified in Figure 4(a). The DMPO–$^\bullet$SG adduct [a^N = 15.4 G; a^H_β = 16.2 G (Mason and Rao, 1990)] was not affected by catalase (Figure 4b). The simulated spectrum in Figure 4(d) indicates that those shown in Figures 4(a) and 4(b) corresponded to a mixture of $HO^\bullet/O_2^{-\bullet}/GS^\bullet$ adducts in the ratio 1:0.5:1. Again, the sensitivity
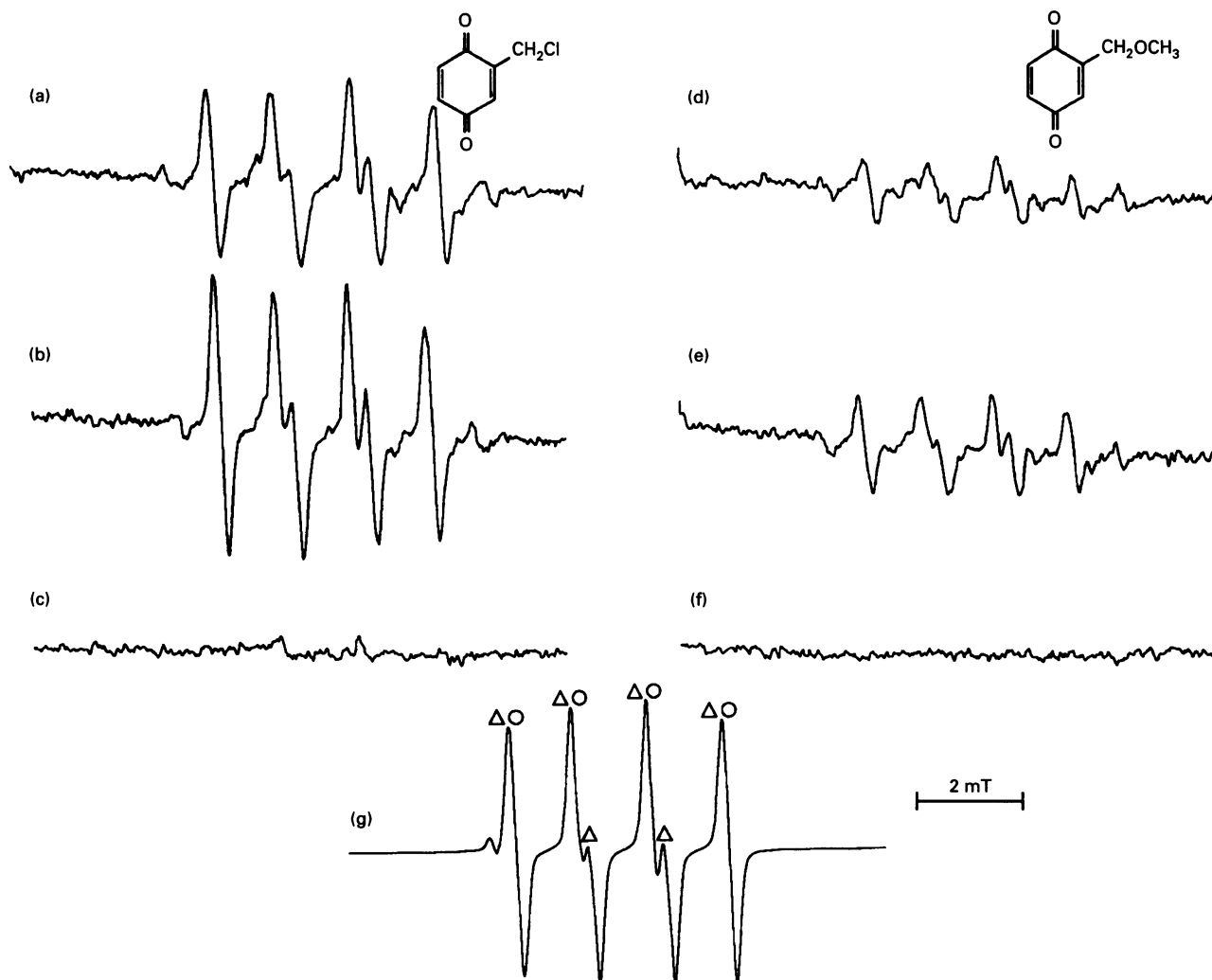
Figure 2 $O_2^{-\cdot}$ generation during the redox transitions of the semi- and hydro-quinone forms of 2-methylnaphthoquinone alkylating agents

(a), (b) and (c) Autoxidation of 2-chloromethyl-1,4-naphthosemiquinone. Experimental conditions: (a) 10 μM quinone, 200 μM NADPH and 130 mM DMPO in 0.2 M potassium phosphate buffer, pH 7.4, were supplemented with NADPH–cytochrome P-450 reductase (1.32 μg/ml) to initiate the reaction. (b) As above in the presence of catalase (500 units/ml). (c) As in (a) in the presence of superoxide dismutase (50 nM). (d), (e) and (f) Autoxidation of 2-methylmethoxy-1,4-naphthohydroquinone. Experimental conditions: (d) 10 μM quinone, 200 μM NADPH and 130 mM DMPO in 0.1 mM potassium phosphate buffer, pH 7.4, were supplemented with DT-diaphorase (30 ng/ml) to initiate the reaction. (e) As in (d) in the presence of catalase (500 units/ml). (f) As in (d) in the presence of superoxide dismutase (50 nM). Instrument settings: receiver gain, 2×10^6; microwave power, 20 mW; modulation amplitude, 0.1922 mT; time constant, 1.3 s; scan time, 5.6 min. (g) Stimulated e.s.r. spectrum corresponding to a composite of DMPO–HOO· and DMPO–HO· adducts (9:1). Linewidth for $O_2^{-\cdot}$ and HO· was 1.7 G and 1.5 G respectively. HO· has a G shift (-2). $\bigcirc$ and $\triangle$, DMPO–HO· and DMPO–HOO· respectively.

of the signal to superoxide dismutase indicated that the contribution of the DMPO–HO· adduct to the spectrum was due to spontaneous internal conversion of DMPO–HOO· into DMPO–HO· (Finkelstein et al., 1980).

The effects of superoxide dismutase on the above parameters were as follows: (1) it enhanced thiol oxidation (Figure 3b) and H_2O_2 formation substantially (Figure 3c); (2) it suppressed the e.s.r. signals corresponding to $O_2^{-\cdot}$ and GS· (Figure 4c). These two apparently divergent effects can be explained by reactions (2)–(5), in which thiyl-radical is formed on oxidation of GSH by the semiquinone [reaction (1)]; superoxide dismutase suppresses the thiyl-radical signal by favouring the rapid oxidation of a disulphide anion intermediate [reactions (3)–(4)] on removal of $O_2^{-\cdot}$, followed by its catalysed disproportionation to H_2O_2 [reaction (5)].

$$Q^{-\cdot} + GS^-(H^+) \rightarrow QH_2 + GS^\cdot \tag{2}$$

$$GS^\cdot + GS^- \rightarrow GSSG^{-\cdot} \tag{3}$$

$$GSSG^{-\cdot} + O_2 \rightarrow GSSG + O_2^{-\cdot} \tag{4}$$

$$O_2^{-\cdot} + O_2^{-\cdot} + 2H^+ \rightarrow H_2O_2 + O_2 \tag{5}$$

It is difficult to explain the suppression of the thiyl-radical signal by superoxide dismutase in terms of reaction (6), because such a process would be associated with a decrease in GSSG accumulation. Also, the inhibitory effect of superoxide dismutase indirectly rules out the contribution of HO· to thiyl-radical formation.
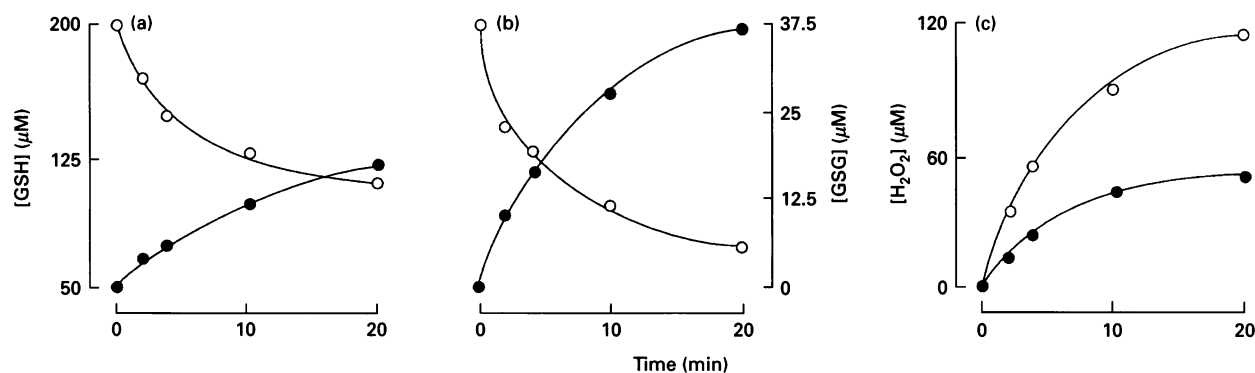
$$O_2^{-\cdot} + GS^-(H^+) \rightarrow H_2O_2 + GS^\cdot \tag{6}$$

Figure 3 Thiol oxidation during NADPH–cytochrome P-450 catalysis of quinone I

(a) Assay conditions: 0.2 mM NADPH, 0.2 mM GSH and 50 μM quinone I in 0.2 M potassium phosphate buffer, pH 7.4, were supplemented with NADPH–cytochrome P-450 reductase (1.32 μg/ml) to initiate the reaction. (b) As in (a) in the presence of 50 nM superoxide dismutase. $\bigcirc$, [GSH]; $\bullet$, [GSSG]. (c) H_2O_2 formation during enzymic catalysis of quinone I in the absence ($\bullet$) and presence ($\bigcirc$) of superoxide dismutase [assay conditions as in (a) and (b) respectively].
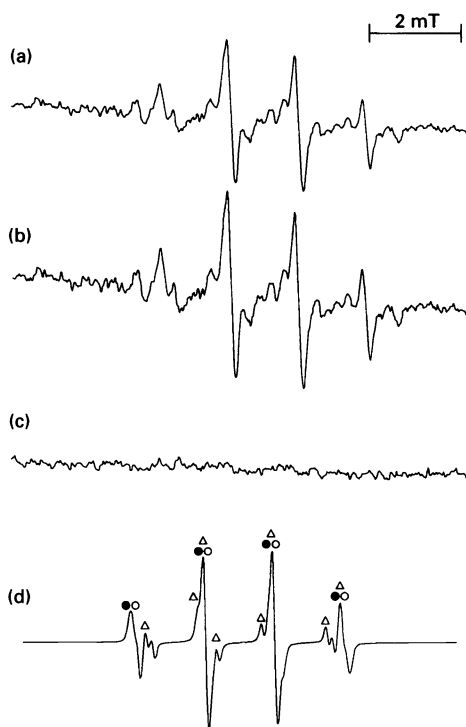

Figure 4 Thiyl-radical formation during NADPH–cytochrome P-450 catalysis of quinone II

Experimental conditions: (a) 10 μM quinone, 200 μM NADPH and 130 mM DMPO in 0.2 M potassium phosphate buffer, pH 7.4, were supplemented with NADPH–cytochrome P-450 reductase (1.32 μg/ml^{-1}) to initiate the reaction. (b) As in (a) in the presence of catalase (500 units/ml). (c) As in (a) in the presence of superoxide dismutase (50 nM). (d) Simulated e.s.r. spectrum corresponding to a composite of HO$^{\cdot}$, $O_2^{-\cdot}$ and GS$^{\cdot}$ adducts in the ratio 1:0.5:1. $\bigcirc$, $\triangle$, and $\bullet$, DMPO–HO$^{\cdot}$, DMPO–HOO$^{\cdot}$ and DMPO–$^{\cdot}$SG spin adducts respectively.

Thiol oxidation during DT-diaphorase catalysis

The time course of thiol oxidation during DT-diaphorase catalysis of quinone I is shown in Figure 5(a); this process was accompanied by accumulation of H_2O_2 (Figure 5c) and intermediate formation of thiyl radicals (not shown). Similarly to

that described for the one-electron catalysis of quinone I, superoxide dismutase suppressed the thiyl-radical signal and enhanced H_2O_2 formation (Figure 5c). However, the enzyme decreased the accumulation of the disulphide substantially (Figure 5b), suggesting that, in these instances, thiyl-radical formation originated partly from the oxidation of GSH by $O_2^{-\cdot}$ [reaction (6)]. Although the second-order rate constant for reaction (6) is low (Bielski et al., 1985), thiyl radicals are detected in $O_2^{-\cdot}$-generating systems in the presence of GSH (Ross and Moldéus, 1986).

Table 1 indicates that autoxidation (implying H_2O_2 formation) is a far larger component of the redox transitions during DT-diaphorase catalysis than during NADPH–cytochrome P-450 catalysis. Accordingly, the contribution of reaction (6) to thiol oxidation ought to be larger during the former than during the latter.

Sulphur nucleophilic addition

Regardless of the molecular mechanism underlying thiol oxidation, GSSG formation did not account for the total GSH consumed during the redox transitions of these quinones. Previous research has shown that 2- and 6-methyl-1,4-naphthoquinone-alkylating agents reacted with GSH with rate constants of about 0.2×10^2–20×10^2 M$^{-1} \cdot$s^{-1} (Wilson et al., 1987) and that the thioether derivative thereby formed participated in redox transitions, yielding oxygen and thiol radicals (Goin et al., 1991). H.p.l.c. with electrochemical detection analysis of quinone/GSH mixtures showed a loss of the quinone peak (R_t = 52.1 min; Figure 6a) and concomitant appearance of a peak with a shorter retention time (7.6 min; Figure 6b), which could be attributed to a quinone thioether derivative in a manner analogous to 3-glutathionyl-2-methyl-1,4-naphthoquinone formation. The u.v.-absorption spectral properties of the compound originating from the reaction of GSH with compound I revealed an increase and shift towards shorter wavelengths (330 nm) of the 340 nm band along with the development of a broad absorption at 420–430 nm with isosbestic points at 300 and 500 nm (Figure 6c); the absorption at 420–430 nm is indicative of formation of a thioether linkage (Nishibayashi et al., 1967; Nakai and Hase, 1968). Glutathionyl substituents do not exert a large effect on the $E_{1/2}$ values of the quinone [−240 mV against a standard calomel electrode (Lin and Sartorelli, 1976)], as also shown for the case
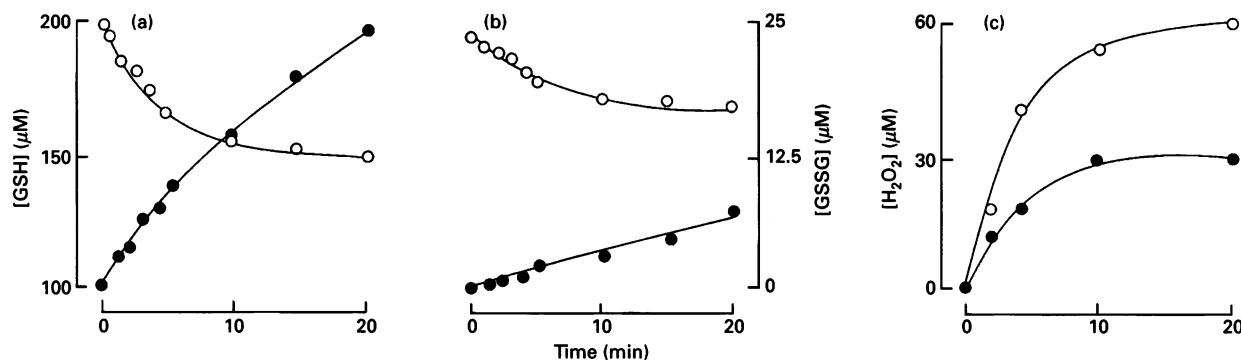
Figure 5   Thiol oxidation during DT-diaphorase catalysis of quinone I

(a) GSH consumption (○) and GSSG formation (●) during DT-diaphorase catalysis of quinone I. Assay conditions: 0.2 mM NADPH, 0.2 mM GSH and 50 μM quinone I in 0.1 M potassium phosphate buffer, pH 7.4, were supplemented with DT-diaphorase (20 ng/ml) to initiate the reaction. (b) As in (a) in the presence of 50 nM superoxide dismutase. (c) H_2O_2 formation in the absence (●) and presence (○) of superoxide dismutase [assay conditions as in (a) and (b) respectively].
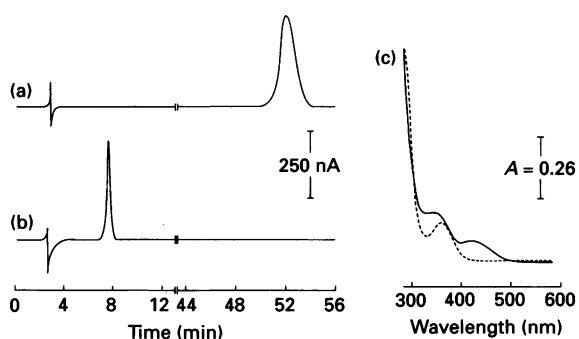

Figure 6   Thiol arylation by quinone I

H.p.l.c. with electrochemical detection chromatograms of (a) 20 nmol of quinone I and (b) 20 nmol of quinone incubated with 40 nmol of GSH in 0.1 M potassium phosphate buffer, pH 7.4, for 10 min. (c) Absorption spectral properties of the product formed during the reaction of quinone I with GSH. ——, Quinone I [assay conditions as in (a)]; ————, quinone I plus GSH [assay conditions as in (b)].

of menadione and its thioether derivative (Buffinton et al., 1989). Likewise, thioether formation does not alter significantly the one-electron reduction potential of quinones (E_{Q/Q^-}) (Wilson et al., 1987), and quinone–glutathione conjugates do redox cycle with oxygen in a similar way to the parent quinones (Öllinger et al., 1990).

These observations are consistent with a mechanism involving both oxidation of the thiol by an O_2^-- and/or semiquinone-dependent mechanism and sulphur nucleophilic attack on the quinone leading to quinone thioether formation. The latter process is not expected to compete with the overall redox cycling process, because thioether derivatives are also substrates for one- and two-electron-transfer flavoenzymes (Buffinton et al., 1989; Öllinger et al., 1990).

Calf thymus DNA-strand-break formation during 2-methyl-1,4-naphthoquinone reduction

Table 2 shows that the one- and two-electron reduction of quinone I under the aerobic conditions was associated with DNA-strand break formation (background level 0.5%). The percentage of strand breaks was 4.3-fold higher during DT-

diaphorase-catalysed reduction of the quinone than during its one-electron reduction (Table 2, column A). Under the former conditions, the presence of superoxide dismutase, catalase or a combination of the two decreased the formation of strand break by 85–90%. This effect was less pronounced during the one-electron reduction of quinone I amounting to about 55–64% inhibition of strand-break formation (Table 2, column A).

The e.s.r. data provided in Figure 2 showed that autoxidation of these quinones entailed mainly formation of O_2^- and that the DMPO–HO• adduct detected (Figure 2) originated from the internal conversion of a DMPO–HOO• adduct. Given their individual chemical reactivities, it is unlikely that either O_2^- or H_2O_2 per se are involved in the induction of DNA-strand breaks.

Table 2   DNA strand-break formation during reduction of quinone I

Assay conditions in column A for one-electron reduction: 1 mg/ml DNA, 50 μM quinone I and 0.1 mM NADPH in PBS, pH 7.4, were supplemented with 1.32 μg/ml NADPH–cytochrome P-450 reductase to initiate the reaction; for two-electron reduction: 1 mg/ml DNA, 50 μM quinone I and 0.2 mM NADPH in PBS, pH 7.4, were supplemented with 30 ng/ml DT-diaphorase to initiate the reaction. Column B: experimental conditions as in A, but with dialysed calf thymus DNA and 1 mM desferal. Assay conditions were as decribed in the Materials and methods section.

	Strand breaks (%)	
	A	B
One-electron reduction		
Quinone I + NADPH + P-450 reductase		
Control	3.3	2.2
+ Superoxide dismutase	1.5	0.6
+ Catalase	1.2	0.8
+ Superoxide dismutase/catalase	1.2	0.6
Anaerobiosis	1.6	0.5
Two-electron reduction		
Quinone I + NADPH + DT-diaphorase		
Control	14.3	4.5
+ Superoxide dismutase	2.1	1.5
+ Catalase	1.5	1.6
+ Superoxide dismutase/catalase	1.5	1.5
Anaerobiosis	1.0	1.1

The participation of HO· in the observed strand-break formation along with the requirement of transition metals to catalyse H_2O_2 reduction are addressed in column B in Table 2, where the results listed were obtained with dialysed DNA and in the presence of desferal. During the one-electron reduction of the quinone, DNA-strand-break formation was decreased by 33% with respect to experiments carried out with non-dialysed DNA and in the absence of metal chelators. Superoxide dismutase and/or catalase further inhibited DNA-strand-break formation by 63–73%. During the two-electron reduction of the quinone, the percentage of DNA-strand breaks decreased by about 3.2-fold when dialysed DNA was used in the presence of desferal, and superoxide dismutase, catalase or a combination of the two further decreased (by 64–67%) but did not completely suppress DNA-strand-break formation.

Overall the results shown in Table 2 suggest the participation of adventitious metals and therefore HO· in DNA-stand-break formation. Considering that the latter radical species was not detected during the one- or two-electron reduction of quinones (see Figure 2), it can be surmised that it originated on metal catalysis of H_2O_2 formed during semi- or hydro-quinone autoxidation. The chelating agent desferal decreased DNA-strand-break formation substantially, suggesting that HO· radicals were partly generated in the bulk solution and elicited a random attack on DNA. However, the residual formation of DNA-strand breaks (2.2 and 4.5% during one- and two-electron reduction of the quinone respectively) indicated that metals ligated to the DNA surface were not completely removed by extensive dialysis (see the Materials and methods section). In these instances, it could be assumed that the DNA-strand breaks occurred as an HO·-mediated site-specific damage mechanism (Goldstein and Czapski, 1986), which is supported by the occurrence of metals ligated to the surface of DNA which acted as sensitizers of the toxic effects of $O_2^{-·}$ or H_2O_2. Moreover, the residual DNA-stand breaks were only partially sensitive to superoxide dismutase and/or catalase, and 0.6–1.5% strand breakage might be attributed to mechanisms that do not involve oxygen radical production, such as electrophilic quinone attack on DNA via a reactive quinone methide intermediate formed on oxidative elimination of the leaving group.

Concluding remarks

A salient feature of the redox transitions of quinone I on its activation by one- or two-electron-transfer flavoenzymes is the distinct contribution of autoxidation to the overall redox cycling process. In the former case, only 26% of NADPH oxidized can be accounted for in terms of H_2O_2 formation, whereas in the latter, i.e. during DT-diaphorase catalysis, almost all NADPH oxidized (98%) is accounted for in terms of autoxidation. These differences were abrogated by the presence of superoxide dismutase, a situation in which almost all NADPH oxidized during the one-electron reduction of the quinones could be accounted for as H_2O_2 production.

These observations permit the study of quinone reactivity, and its implications for cytotoxicity, in terms of bioreductive activation systems and inherent redox reactions. Thiol oxidation constitutes an example: the GS⁻ →GS· transition is mainly coupled to semiquinone reduction during NADPH–cytochrome P-450 catalysis and superoxide radical reduction during DT-diaphorase catalysis. The numerous reports supporting thiyl-radical formation via reaction of thiols with superoxide radical or semiquinones indicates that these processes are kinetically driven (Ross and Moldéus, 1986; Wardman, 1988). The prevalence of either reaction is probably a function of the steady-state

concentration of oxidants, as inferred from the data in Table 1. The effect of superoxide dismutase on these redox transitions merits further comment. In general, semiquinone autoxidation is enhanced by superoxide dismutase on displacement of the equilibrium of reaction (1) towards the right (Winterbourn, 1981; Wardman and Wilson, 1987). In the presence of GSH, however, the semiquinone appears to decay by a reductive pathway and the focus of superoxide dismutase activity would be centred on another reaction further down the free-radical chain, i.e displacing the equilibrium of reaction (4) via reaction (5). The physiological implications of such mechanisms (Winterbourn, 1993) along with their thermodynamic appraisal (Koppenol, 1993) have been recently addressed. Thus the radical character is transferred according to the sequence:

$$Q^{-·} \rightarrow GS· \rightarrow GSSG^{-·} \rightarrow O_2^{-·}$$
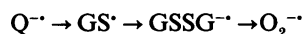
and, finally, via a disproportionation reaction to a non-radical product, H_2O_2. Two radical species with positive $E^{0'}$ values are generated during this sequence, $Q^{-·}$ and GS· {the $E^{0'}$ value of the former $[E_{Q^{-·}/QH_2}]$ could be estimated to be about $+0.12$–0.14 V and that of the latter is $+0.85$ V (Surdhar and Armstrong, 1986)} and two radicals with negative $E^{0'}$ values, GSSG⁻· and $O_2^{-·}$ [with $E^{0'} = -1.6$ V and -0.155 V respectively (Surdhar and Armstrong, 1986; Ilan et al., 1974)]. The significance of the conjugation of thiyl radicals with thiolate [reaction (3)] is self-evident (Wardman, 1988) inasmuch as it shifts the oxidizing radicals to reducing radicals. Conversely, during the course of two-electron reduction of quinone I, a situation ensues in which $[O_2^{-·}]_{ss} > [Q^{-·}]_{ss}$ (where SS is steady state) and thiyl-radical formation proceeds mainly via reaction (6). It was not possible to establish an analogous comparison between one- and two-electron-reduction mechanisms for quinone II because kinetic constants for purified DT-diaphorase could not be obtained.

The mechanisms for DNA-strand breakage produced by oxygen free-radical attack is complex, key reactions appearing to be hydrogen abstraction from one of the carbons of the deoxyribose backbone, followed by base release and β-elimination (von Sonntag et al., 1981; Schulte-Frohlinde, 1983; Goldberg, 1987). The requirement of a potent electrophilic radical to initiate DNA-strand breakage is well established, and the data listed in Table 2 suggest that metal ions bound to DNA protect against the toxic effects of $O_2^{-·}$ and H_2O_2 produced during the one- and two-electron reduction of the bioreductive alkylating agent, quinone I. It has been previously shown that iron binds to DNA (Floyd, 1981; Shires, 1982; Imlay and Linn, 1988) and suggested that this metal might already be present on the DNA in vivo (Halliwell, 1987) and participate in production of HO·, a species that reacts with DNA with second-order rate constants of $\sim 8 \times 10^8$ $M^{-1} \cdot s^{-1}$ (Scholes et al., 1969).

In line with these concepts, the higher yield of oxygen radicals during the DT-diaphorase catalysis of this quinone (Table 1) was associated with a larger percentage of DNA-strand breakage (4.3-fold higher than that observed during NADPH–cytochrome P-450-mediated quinone reduction) (Table 2), a value comparable with that observed during the metabolism of benzopyrene quinone in human fibroblasts (Morrison et al., 1985). The results in this study and elsewhere (Imlay and Linn, 1988; Kyle et al., 1988; Coleman et al., 1989) suggest that reduction of DNA-bound metal ions involves $O_2^{-·}$, and alternative reductants, such as a semiquinone species (Winterbourn et al., 1985), may partly account for strand breakage during NADPH–cytochrome P-450 catalysis of quinone I. However, evaluation of this possibility requires knowledge of the steady-state concentration of reactants and absolute rate constants for the reactions of the semiquinone with itself and with ligated iron. It is expected that the con-

tribution of this reaction would be more significant at lower oxygen concentrations.

The superoxide dismutase and/or catalase-insensitive strand breakage (slightly above background levels) suggests a mechanism that probably involves quinone electrophilic attack on DNA resulting in a highly labile modified guanine (Singer and Kusmierek, 1982). In this regard, the binding of adriamycin and daunorubicin has been shown to assist DNA cleavage (Lown et al., 1977). However, the data in Table 2 indicate that DNA-strand breakage denotes mainly oxygen free-radical attack which is not an expression of alkylation, although these quinones are expected to alkylate via a reactive methide intermediate in hypoxic or anaerobic conditions.

The reduction of the bioreductive alkylating agent quinone I by DT-diaphorase is more efficient than that of the chemotherapeutic agent diaziquone (Ordoñez and Cadenas, 1992) and probably that of mitomycin C at low pH (Siegel et al., 1992). Although characterization of the quinones studied here in normoxic conditions is a necessary step towards understanding their biological activity, an assessment of their efficacy as anticancer quinones is complicated. First, the processes described in this study cannot be viewed as a 'deactivation' of the bioalkylating agents because they entail oxygen free-radical formation rather than rearrangement of the quinones to potent electrophilic methides. Although the latter is expected to cause cytotoxicity partly by DNA alkylation, the former is associated with DNA-strand breakage. Secondly, the suggested role of DT-diaphorase, an enzyme that is overexpressed in some cancer cells (Cresteil and Jaiswal, 1991), in enzyme-directed bioreductive drug development (Riley and Workman, 1992) is obscured by controversial issues, such as quinone bioreductive activation in hypoxic and normoxic conditions and the participation of additional bioreductive systems. The one- and two-electron reduction of quinones with leaving groups described here along with the diverse effect of these processes on cellular nucleophiles, such as GSH and DNA, and their different modulation by superoxide dismutase, suggest a complex network of reactions with distinct implications for cytotoxicity.

Finally, the molecular basis of quinone cytotoxicity has been attributed to one-electron-transfer reactions; two-electron transfers, such as occur during DT-diaphorase catalysis, have been regarded as processes that lead to quinone detoxification or protection against quinone toxicity. This and previous studies (Buffinton et al., 1989; Öllinger et al., 1989; Ordoñez and Cadenas, 1992) have re-examined the function of DT-diaphorase in quinone metabolism. The concept that two-electron transfers, as catalysed by this enzyme, represent a protective mechanism against quinone toxicity is not entirely correct; for example, the hydroquinone forms of menadione and 1,4-naphthoquinone are redox stable and their formation during DT-diaphorase catalysis suggests an antioxidant or protective function of the enzyme. Conversely, the hydroquinone forms of aromatic-ring hydroxyl-substituted naphthoquinones (Öllinger et al., 1989), certain diaziridinylbenzoquinones (Ordoñez and Cadenas, 1992) and the quinones studied here autoxidize readily after their formation by DT-diaphorase. In conclusion, whether DT-diaphorase-mediated reduction of quinones results in protection or toxicity depends on the functional-group chemistry and physicochemical properties of the hydroquinone generated.

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