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# Thiol oxidation coupled to DT-diaphorase-catalysed reduction of diaziquone

## Reductive and oxidative pathways of diaziquone semiquinone modulated by glutathione and superoxide dismutase

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DT-diaphorase [NAD(P)H:quinone oxidoreductase; EC 1.6.99.2] catalysed the two-electron reduction of the anti-tumour quinone 2,5-bis-(1-aziridinyl)-3,6-bis(ethoxycarbonylamino)-1,4-benzoquinone (AZQ) to the hydroquinone form ($AZQH_2$). Although DT-diaphorase catalysis of AZQ was not significantly affected by pH, the hydroquinone product was effectively stabilized by protonation at pH values below 7, whereas, above that pH, hyroquinone autoxidation, evaluated in terms of $H_2O_2$ production, increased exponentially. The autoxidation of $AZQH_2$ entailed the formation of diverse radicals, such as $O_2^{-\cdot}$, $HO^{\cdot}$, and the semiquinone form of AZQ ($AZQ^{-\cdot}$), which contributed to different extents to the e.p.r. spectrum. Superoxide dismutase enhanced the autoxidation of $AZQH_2$ and suppressed the e.p.r. signal ascribed to $AZQ^{-\cdot}$, in agreement with a displacement of the equilibrium of the semiquinone autoxidation reaction ($AZQ^{-\cdot} + O_2 \rightleftharpoons AZQ + O_2^{-\cdot}$) upon enzymic withdrawal of $O_2^{-\cdot}$. GSH increased the steady-state concentration of $AZQH_2$ formed during DT-diaphorase catalysis and inhibited temporarily its autoxidation. This effect was accompanied by oxidation of the thiol to the disulphide within a process involving glutathionyl radical ($GS^{\cdot}$) formation, the relative contribution of which to the e.p.r. spectrum was enhanced by increasing GSH concentrations. $GS^{\cdot}$ formation in this experimental model can be rationalized as originating from the reaction of GSH with $AZQ^{-\cdot}$, rather than with $O_2^{-\cdot}$ or $HO^{\cdot}$, for thiol oxidation was not affected significantly by superoxide dismutase, and $GS^{\cdot}$ formation was insensitive to catalase. In addition, GSH suppressed the e.p.r. signal attributed to $AZQ^{-\cdot}$. No glutathionyl–quinone conjugate was detected during the DT-diaphorase-catalysed reduction of AZQ; although the chemical requirements for alkylation were partly fulfilled (quinone ring aromatization and acid-assisted aziridinyl ring opening), the negligible dissociation of GSH ($GS^- + H^+ \rightleftharpoons GSH$) at low pH prevented any nucleophilic addition to occur. Therefore the redox transitions of AZQ during DT-diaphorase catalysis seemed to be centred on the semiquinone species, the fate of which was inversely affected by catalytic amounts of superoxide dismutase and large amounts of GSH: the former enhanced $AZQ^{-\cdot}$ autoxidation and the latter favoured $AZQ^{-\cdot}$ reduction. Accordingly, superoxide dismutase and GSH suppressed the semiquinone e.p.r. signal. These results are discussed in terms of three interdependent redox transitions (comprising one-electron transfer reactions involving the quinone, oxygen and the thiol) and the thermodynamic and kinetic properties of the reactions involved.

## INTRODUCTION

The overall biological activity of quinonoid compounds (expressed as toxicity and possibly carcinogenicity of many chemicals as well as chemotherapeutic activity) can be understood in terms of their physico-chemical properties, which are a function of their molecular structure and functional group chemistry (Brunmark & Cadenas, 1989; Gutierrez, 1989; Powis, 1989).

The chemical structure of diaziquone [2,5-bis-(1-aziridinyl)-3,6-bis(ethoxycarbonylamino)-1,4-benzoquinone; AZQ], an anti-tumour quinone, provides for dual functionalities on the following accounts: on the one hand, the *p*-benzoquinone moiety imparts the unique property of quinones, i.e., their ability to undergo reversible oxidation–reduction reactions with the formation of semiquinone intermediates. On the other hand, the basic aziridinyl ring substituents have the potential to undergo ring opening to form covalent adducts with nucleophiles. The latter reactions seem to require aromatization (i.e., upon one- or two-electron reduction) of the quinone ring and protonation of aziridinyl N atom (Driebergen *et al.*, 1986; Gutierrez, 1989). Thus the redox transitions centred in the quinone moiety and in the aziridinyl rings may be to some extent interdependent and they influence significantly the electron distribution in the overall molecule and the subsequent redox chemistry.

Despite the fact that a single molecular mechanism cannot explain the myriad of cytotoxic effects triggered by quinones, electron transfer seems to be a key reaction contributing to these effects. The bioreductive activation of quinones encompasses sole electron-transfer reactions and alkylation reactions, i.e., the reaction of electrophilic quinones with suitable nucleophiles. The former process is accomplished by one- and two-electron transfer enzymes. DT-diaphorase, a unique two-electron transfer flavoprotein (Lind *et al.*, 1990), catalyses the conversion of quinones into hydroquinones, the chemical reactivity of which is a function of the quinone substitution pattern (Buffinton *et al.*, 1989; Öllinger *et al.*, 1990), in addition, two-electron transfer reactions seem to be a requisite condition for the activation of quinones with leaving groups, i.e., bioreductive alkylating agents, leading to the formation of quinone methide intermediates (Moore, 1977; Abdella & Fisher, 1985; Sartorelli, 1986). GSH plays a key role among the latter, that is, alkylation reactions, because of its higher intracellular concentration and its self-evident significance in many drug metabolic pathways. Several aspects of thiol reactivity towards quinones with different substitution pattern,

Abbreviations used: AZQ or diaziquone, 2,5-bis-(1-aziridinyl)-3,6-bis(ethoxycarbonylamino)-1,4-benzoquinone; DMPO, 5,5′-dimethyl-1-pyrroline-*N*-oxide; $GS^{\cdot}$, glutathionyl radical; DTPA, diethylenetriaminepenta-acetic acid; $R_t$, retention time.

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including bioreductive alkylating agents, have been described (Wilson *et al.*, 1987; Brunmark & Cadenas, 1988; Goin *et al.*, 1991).

The reactivity of AZQ has been amply characterized at the chemical, biochemical and cellular level by a series of studies *in vitro* and *in vivo* (Driebergen *et al.*, 1986; Gutierrez, 1989). The chemical aspects have been extensively covered (Khan & Driscoll, 1978; Butler *et al.*, 1987; Gutierrez, 1989; Butler *et al.*, 1990) and broadly summarized above. AZQ is a substrate for the two-electron flavoprotein DT-diaphorase in MCF-7 (Fisher & Gutierrez, 1991) and HT-29 (Siegel *et al.*, 1990) cells and, in the latter instances, the two-electron activation of AZQ seemed to be a process leading to the formation of genotoxic and cytotoxic metabolites. Other cellular aspects of AZQ metabolism include its differential cytotoxicity under hypoxic and aerobic-exposure conditions (O'Brien *et al.*, 1990) and its production of DNA strand breakage and interstrand cross-linking in nuclei from human cells (Szmigiero & Kohn, 1984; Szmigiero *et al.*, 1984). Studies with various aziridinylbenzoquinone derivatives indicated that cytotoxicity could be a function of the reduction potential of the compounds and, subsequently, their capacity to form cross-links in DNA (Dzielendziak *et al.*, 1990). Overall, there seems to be a consensus that quinone aromatization, aziridinyl-N protonation and ring opening, and reaction with cellular nucleophiles, are sequentially involved in the expression of the cytotoxic effects triggered by diaziquone.

The present study was aimed at a kinetic and thermodynamic understanding of the molecular mechanisms inherent in the two-electron reduction of AZQ by DT-diaphorase, the subsequent redox transitions of the hydroquinone evaluated in terms of autoxidation as $H_2O_2$ and oxygen-centred radical ($HO^{\cdot}$, $O_2^{-\cdot}$) formation, as well as the effect on these transitions of pH, superoxide dismutase and GSH.

## MATERIALS AND METHODS

### Chemicals and biochemicals

AZQ was supplied from the Drug Synthesis and Chemistry Branch, Division of Cancer Treatment, National Cancer Institute (Bethesda, MD). AZQ solutions (5 mM) were prepared daily in DMSO. NADPH, GSH, GSSG, superoxide dismutase, catalase and horseradish peroxidase were from Boehringer (Mannheim, Germany). 5,5′-Dimethyl-1-pyrroline-*N*-oxide (DMPO) was from Aldrich Chemical Co. (Milwaukee, WI, U.S.A.). Diethylenetriaminepenta-acetic acid (DTPA), chelating resin (iminodiacetic acid/chelex 100), and $H_2O_2$ were from Sigma Chemical Co. (St. Louis, MO). Activated carbon (Darco G-60) and h.p.l.c.-grade methanol were from Fisher Scientific (Fairlawn, NJ, U.S.A.). All buffers were passed through chelex-100 before use. DT-diaphorase was a gift from Dr. S. Chen (Division of Immunology, Beckman Research Institute, City of Hope, Duarte, CA, U.S.A.) and had a specific activity of 2790 nmol·min$^{-1}$·μg of protein$^{-1}$, measured with 2-methyl-1,4-naphthoquinone as electron acceptor (Lind *et al.*, 1990).

### Standard assay conditions

The standard assay mixture consisted of 100 μM-AZQ (final dimethyl sulphoxide concn. 2%, v/v) and 200 μM-NADPH in 0.25 M-sucrose/0.1 M-potassium phosphate buffer, pH 5–8. The reaction was initiated upon addition of DT-diaphorase (1.6 μg/ml; suspended in 0.25 M-sucrose/0.1% BSA/0.1 M-potassium phosphate buffer, pH 7.8). The assay temperature was 30 °C. Anaerobic conditions were achieved by purging the solutions with either high purity He or Ar for at least 5 min in rubber-septum-capped tubes or cuvettes. The reactions were then initiated by injecting the DT-diaphorase suspension through the septum.

### Spectrophotometric and fluorimetric assays

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 DT-diaphorase catalysis of AZQ was measured at the isosbestic point for AZQ reduction ($\epsilon_{313} = 2.84$ mM$^{-1}$·cm$^{-1}$), for the decrease of absorption at 340 nm reflected both NADPH oxidation and AZQ removal (Siegel *et al.*, 1990). Reduction of AZQ was monitored at 525 nm ($\epsilon$ 0.41 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 spectrophotofluorimeter (American Instrument Co., Silver Spring, MA, U.S.A.).

### Polarographic assays

$O_2$ consumption was monitored with a Clark-type oxygen electrode (Rank Brothers, Bottisham, Cambridge, U.K.) connected to a Gilson model 5/6H oxygraph (Gilson Medical Electronic, Middleton, WI, U.S.A.).

### H.p.l.c. with u.v. detection

GSH and GSSG were measured as dinitrophenol derivatives (Fariss & Reed, 1987). A 250 mm × 4.6 mm Spheri-5-amino 5 μm column (Applied Biosystems, Foster City, CA, U.S.A.) was connected to the 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 autoinjector (Shimadzu; model SIL-9A; injection vol. 100 μl) and the eluate was monitored at 365 nm using a u.v.–visible spectrophotometer 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.

AZQ and its metabolites were analysed by using a 15 cm × 3.9 mm Nova-pak 5 μm C-18 reverse-phase column (Millipore/Waters Chromatography Division, Milford, MA, U.S.A.) with a C-18 Guard pre-column (Altech Associates, Deerfield, IL, U.S.A.). A binary gradient elution was employed (Ross *et al.*, 1990) using 50 mM-ammonium acetate, pH 6.2 (solution A) and methanol (solution B). The linear gradient was 20–80% solution B over 20 min at a flow rate of 1 ml/min. The eluate was measured at 260 nm.

### H.p.l.c. with electrochemical detection

AZQ and its metabolites were analysed by electrochemical detection utilizing dual glassy carbon electrodes, a Ag/AgCl reference electrode with a dual LC-4B/17AT amperometric detector (BAS, West Lafayette, IN, U.S.A.) as previously described (Cadenas & Ernster, 1990). The mobile phase utilized was 50 mM-sodium phosphate buffer, pH 6.5, in methanol/water (7:13, v/v), and was purged continuously with high-purity He. Chromatograms were recorded with a strip chart recorder (model SE 120; ABB Goerz AB, Vienna, Austria). The flow rate and the injection volume were 0.5 ml/min and 20 μl respectively.

### E.p.r. spectroscopy

E.p.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 into heat-sealed capillary ends of Pasteur pipettes. Assay conditions were as described above, except that 80 mM-DMPO was present in the reaction mixture. All DMPO solutions were filtered with activated charcoal (Darco G-60) repeatedly until the e.p.r. spectrum of DMPO alone was virtually signal-free. E.p.r. simulation spectra were obtained using the e.p.r. Simulation Program, version 42.1 (Oehler & Dubose; provided by the Oklahoma Medical Research Foundation, Oklahoma City, OK, U.S.A.).

## RESULTS AND DISCUSSION

### DT-diaphorase-catalysed two-electron reduction of AZQ

The time courses of NADPH oxidation and hydroquinone formation during the DT-diaphorase catalysis of AZQ under aerobic and anaerobic conditions are shown in Figs. 1(*a*) and 1(*b*) under the former conditions. NADPH oxidation proceeded at an initial rate of $27.5 \pm 4$ $\mu$M·min$^{-1}$, and the pyridine nucleotide was completely oxidized within 8 min. Hydroquinone formation reached a steady-state concentration of about $30 \pm 3$ $\mu$M (Fig. 1*b*), encompassing the equilibrium of the oxidized and reduced forms ($AZQ \rightleftharpoons AZQH_2$; $[AZQH_2]/[AZQ] = 0.4$). At the time when NADPH was exhausted, the reduced form of AZQ decayed by autoxidation.

Under anaerobic conditions, the absence of $O_2$ prevented quinone redox cycling and, hence, NADPH oxidation, though proceeding at an initial rate similar to that under aerobiosis, levelled off at about 8–9 min without reaching a complete oxidation of the pyridine nucleotide. Simultaneously, AZQ was completely reduced. The $[NADPH]_{oxidized}/[AZQ]_{reduced}$ ratio was slightly above unity (1.15), indicating that a fraction of NADPH might be oxidized by reactions other than hydroquinone autoxidation, e.g., disproportionation–comproportionation reactions.

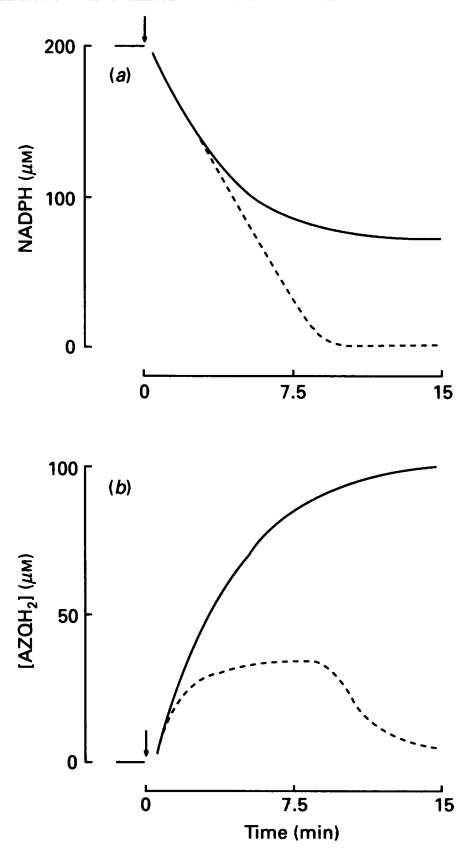
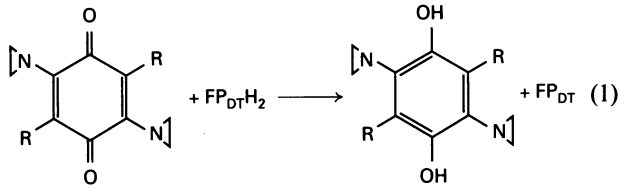



**Fig. 1. NADPH oxidation and AZQ reduction during DT-diaphorase catalysis under aerobic and anaerobic conditions**

Assay conditions: 100 $\mu$M-AZQ and 200 $\mu$M-NADPH in 0.25 M-sucrose/0.1 M-potassium phosphate buffer, pH 7.8, were supplemented with DT-diaphorase (1.6 $\mu$g/ml) to initiate the reaction. (*a*) NADPH oxidation under aerobic (----) and anaerobic (——) conditions. (*b*) $AZQH_2$ formation in aerobiosis (----) and anaerobiosis (——). Other experimental conditions as described in the Materials and methods section.

These results are consistent with the initial reduction of AZQ by DT-diaphorase (reaction 1):




(1)

(R = —NHCOOCH$_2$CH$_5$; FP$_{DT}$H$_2$ and FP$_{DT}$ are the reduced and oxidized forms of DT-diaphorase flavin respectively), the specific activity of which towards this quinone was 14.8 nmol·min$^{-1}$·$\mu$g of enzyme$^{-1}$, a value similar to that obtained with the enzyme purified from rat liver (Siegel *et al.*, 1990). This $V_{max.}$ value is about 150-fold lower than that displayed by the enzyme towards 2-methyl-1,4-naphthoquinone and comparable with that observed with naphthoquinone derivatives bearing an –OH substituent in the quinonoid ring (e.g., 2-hydroxy-1,4-naphthoquinone) (Buffinton *et al.*, 1989). In addition, and unlike the cases of NADPH:cytochrome *P*-450 reductase (Powis & Appel, 1980) and xanthine oxidase (Butler *et al.*, 1987), this and our previous studies (Buffinton *et al.*, 1989) show that there is no correlation between the reduction potential of a quinone substrate and the rate of two-electron transfer to the quinone catalysed by DT-diaphorase. For example, kinetic studies performed on purified DT-diaphorase showed $V_{max.}$ values of 1301 and 15 nmol·min$^{-1}$·$\mu$g of protein$^{-1}$ for 5,8-dihydroxy-1,4-naphthoquinone (Öllinger *et al.*, 1989) and AZQ respectively, and the $E(Q/Q^{2-})$ (the two-electron reduction potential of the quinone) values for these quinones are −62 (Land *et al.*, 1983) and −50 mV (obtained from the corresponding hydrodynamic voltamogram).

### $AZQH_2$ autoxidation: effects of pH and superoxide dismutase

The initial rates of NADPH oxidation during DT-diaphorase catalysis of AZQ did not change significantly over the pH range 5–8, in agreement with what was reported concerning the DT-diaphorase catalysis of various benzo- and naphtho-quinones (Ernster *et al.*, 1962). However, after this initial rate, the subsequent rate of NADPH oxidation increased with increasing pH (Fig. 2*a*). This behaviour could be rationalized in terms of the chemical requirements for hydroquinone autoxidation (Steenken, 1979), i.e., as the pH increases, protonation of the two-electron-reduced quinone decreases, and, hence, it can participate in electron-transfer reactions, mainly autoxidation.

This view is supported by the dependence of $H_2O_2$ formation (originated during hydroquinone autoxidation) on pH shown in Fig. 2(*b*): at low pH values, e.g., 5, no $H_2O_2$ was detected over a 20 min incubation period, whereas at higher pH values the onset of $H_2O_2$ formation was preceded by a lag phase, the duration of which was shortened as the pH increased. The rates of $H_2O_2$ formation (Fig. 2*c*) (as well as those of $O_2$ consumption; results not shown) accompanying DT-diaphorase catalysis of AZQ remained consistently low at pH values below 7, whereas they increased exponentially above that pH (Fig. 2*c*). Therefore protonation at pH < 7 effectively stabilized the hydroquinone (i.e., $[AZQH_2] \gg [AZQ^{2-}]$), expectedly decreasing the rate of

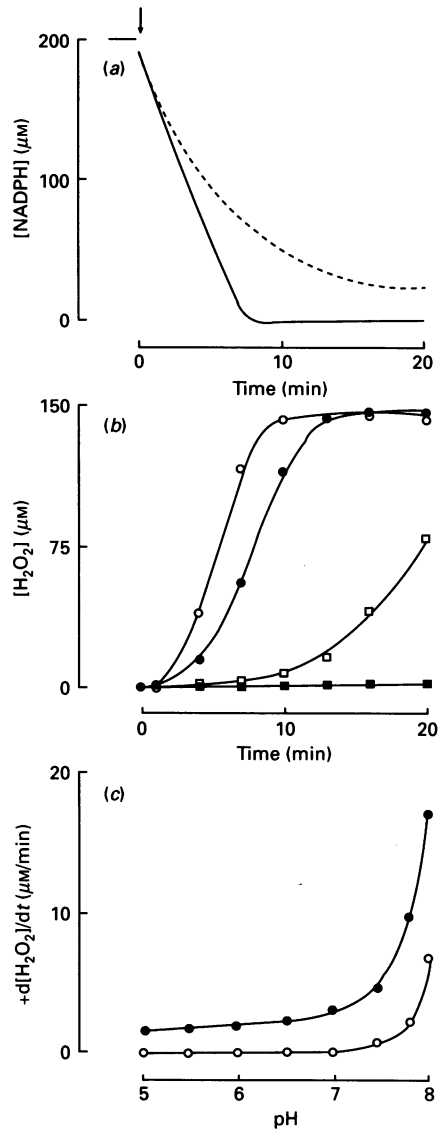



**Fig. 2. Effect of pH on the autoxidation of $AZQH_2$**

Assay conditions were as in Fig. 1, but the pH of the phosphate buffer ranged from 5 to 8. (*a*) Time course of NADPH oxidation at pH 5 (----) and pH 7.8 (——). (*b*) Time course of $H_2O_2$ formation at pH 7.0 (■), 7.5 (□), 7.8 (●), and 8.0 (○). (*c*) Dependence of the rate of $H_2O_2$ formation during $AZQH_2$ oxidation on pH and effect of superoxide dismutase on hydroquinone autoxidation: ○, control; ●, plus 5 μM-superoxide dismutase.

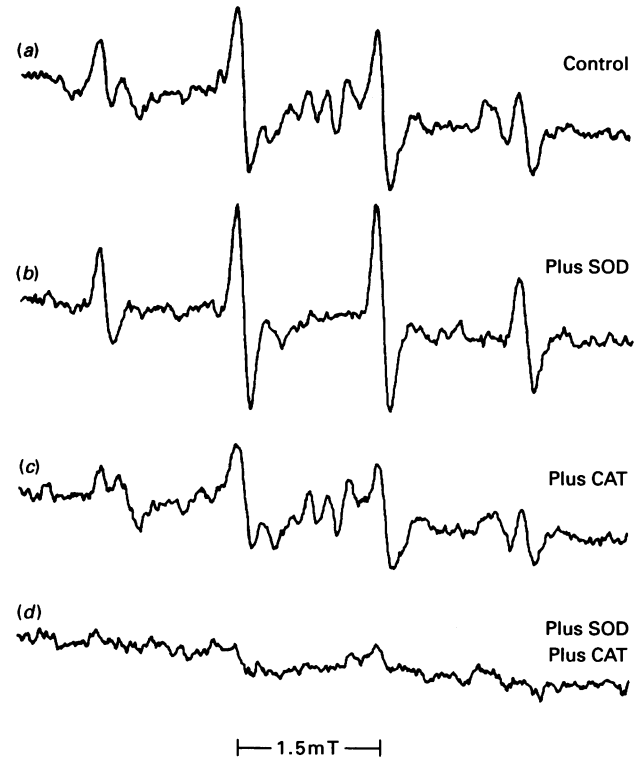



**Fig. 3. $O_2^{-\cdot}$ and $HO^{\cdot}$ generation during the redox transitions of $AZQH_2$ subsequent to DT-diaphorase catalysis**

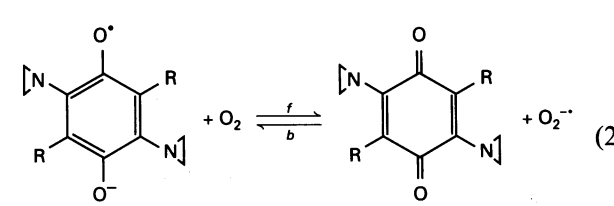
Experimental conditions: 100 μM-AZQ (water solution) 200 μM-NADPH and 80 mM-DMPO in 0.25 M-sucrose/0.1 M-potassium phosphate buffer, pH 7.8, were supplemented with DT-diaphorase (1.6 μg/ml) to initiate the reaction. E.p.r. spectra were taken 5 min after the addition of DT-diaphorase. (*a*) As under the experimental conditions above, without further additions. (*b*) As in (*a*) plus superoxide dismutase (SOD) (5 μM). (*c*) As in (*a*) plus catalase (CAT) (500 units/ml). (*d*) plus superoxide dismutase (5 μM) and catalase (500 units/ml). The signal centred at 0.38436 T (2.0040 *g*) in (*a*) and (*c*) is attributed to the semiquinone form of AZQ. Instrument settings: receiver gain, $2 \times 10^6$; microwave power, 20 mW; modulation amplitude, 0.1922 mT; time constant = 1.3 s; scan time = 5.6 min.

electron-transfer processes and, consequently, the reduction of $O_2$ to $H_2O_2$ during autoxidation.

Superoxide dismutase did not affect significantly the initial rates of NADPH oxidation during DT-diaphorase catalysis of AZQ, but it increased consistently with rates of $H_2O_2$ formation (Fig. 2*c*). This effect was particularly noticeable at pH < 7: no $H_2O_2$ formation could be detected in the absence of superoxide dismutase, whereas the addition of the enzyme to the assay mixture allowed detection of the peroxide (Fig. 2*c*).

This effect was different from that observed with 1,4-naphthohydroquinone derivatives bearing $-CH_3$, $-OCH_3$ or -SG substituents, the autoxidation of which was inhibited effectively by superoxide dismutase (Öllinger *et al.*, 1990). Conversely, the stimulation of hydroquinone autoxidation by superoxide dismutase was encountered with aromatic-ring hydroxy-substituted hydroquinones, such as 5-hydroxy-1,4-naphthoquinone and 5,8-dihydroxy-1,4-naphthoquinone (Öllinger *et al.*, 1989, 1990). The stimulation of the autoxidation of the hydroquinone forms of the latter compounds as well as that of AZQ by superoxide dismutase can be rationalized on the following accounts.

(*a*) The reduction potentials [$E(Q/Q^{-\cdot})$] of these quinones is generally lower than that of the $O_2/O_2^{-\cdot}$ couple. For the particular case of AZQ, pulse-radiolysis studies (Butler *et al.*, 1987) indicated that the quinone can be readily reduced by $O_2^{-\cdot}$ ($k_{2f} = 1.1 \times 10^7$ and $k_{2b} = 2.7 \times 10^8$ $M^{-1} \cdot s^{-1}$), as expected from the individual reduction potentials of the redox couples involved [$E(AZQ/AZQ^{-\cdot}) = -61 \pm 15$ mV (Butler *et al.*, 1990); $E(O_2/O_2^{-\cdot}) = -155$ mV (Ilan *et al.*, 1974)]. Thus enhancement of hydroquinone autoxidation by superoxide dismutase could be partly attributed to a displacement of the equilibrium of semiquinone oxidation (reaction 2):




(2)

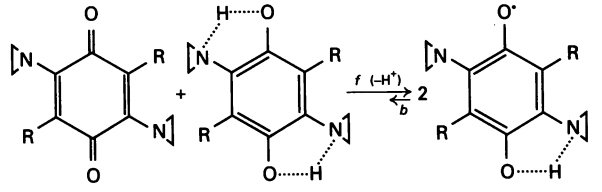
towards the right upon withdrawal of $O_2^{-\cdot}$ by the enzyme (reaction 3) (Winterbourn, 1981).

$$O_2^{-\cdot} + O_2^{-\cdot} + 2H^+ \longrightarrow H_2O_2 + O_2 \quad (3)$$

(*b*) The above effect is an expected feature of semiquinone autoxidation (Winterbourn, 1981; Öllinger *et al.*, 1990), but it cannot explain entirely on its own the stimulation of hydroquinone autoxidation by superoxide dismutase. For that, the redox transitions during hydroquinone autoxidation ought to be centred on the semiquinone species. The aromatic-ring hydroxy-substituted quinones referred to above tend to form strong intramolecular H-bonding that leads to stabilization of the semiquinone transient species involving displacement over to the right of the comproportionation reaction (reaction 4) (Dodd & Mukherjee, 1984):

$$Q + Q^{2-} \rightleftarrows 2Q_2^{-\cdot} \quad (4)$$

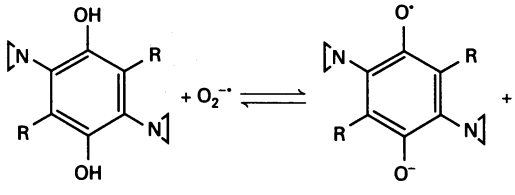
A similar behaviour could be expected during the redox transitions involving AZQ, where H-bonding between the –OH groups in the quinonoid ring and the –N in the aziridinyl substituents (Gutierrez *et al.*, 1986) would lead to semiquinone stabilization (reaction 5):



(5)

Expectedly, the decay of $AZQ^{-\cdot}$ via disproportionation proceeds at a slow rate ($k_{5b} = 8.9 \times 10^5$ M$^{-1}\cdot$s$^{-1}$) (Butler *et al.*, 1987).

In summary, both features of $AZQ^{-\cdot}$ described above should be summoned in order to rationalize the enhancement of hydroquinone autoxidation by superoxide dismutase. $AZQ^{-\cdot}$ is a key species in the redox transitions of the hydroquinone, and its steady-state concentration is enhanced on reduction of the quinone by $O_2^{-\cdot}$ and stabilized by intramolecular H-bonding. This would favour the appearance of the semiquinone under aerobic conditions. Also, and because of this effect by superoxide dismutase, it could be assumed that $O_2^{-\cdot}$ would not behave as a free-radical-propagating species in $AZQH_2$ autoxidation and that the contribution of reaction (6):



(6)

to the overall redox transitions is less significant than for other hydroquinones previously studied (Öllinger *et al.*, 1990).

The DT-diaphorase-catalysed reduction of AZQ and subsequent autoxidation of the hydroquinone encompasses the formation of diverse free radicals which can be detected by e.p.r. either directly or in conjunction with the spin trap DMPO (Fig. 3).

A composite of the quartet signals of the DMPO/OH (hyperfine splitting constants: $a^N = 1.49$ mT; $a^H = 1.49$ mT; $g = 2.0050$) and DMPO/OOH (further split to give a total of eight peaks; hyperfine splitting constants: $a^H = 1.43$ mT, $a^H{}_\beta = 1.17$ mT, $a^H{}_\gamma = 0.125$ mT and $a^N = 1.49$ mT, $a^H = 1.49$ mT; $g = 2.0050$) adducts was observed under control conditions (Fig. 3*a*). In addition, the five-line e.p.r. signal of the semiquinone form of AZQ ($g = 2.0046$) at the centre of the DMPO adducts quartet was detected (Fig. 3*a*). A simulated e.p.r. spectrum equivalent to that in Fig. 3(*a*) was obtained with a 1:2 mixture of the DMPO/OOH and DMPO/OH adducts (results not shown). As previously reported, the semiquinone species can be observed under aerobic and anaerobic conditions (Gutierrez *et al.*, 1982), despite the fact that the $[AZQH_2]/[AZQ]$ ratio (= 0.4) described above in connection with Fig. 1(*b*) is not optimal for the detection of the semiquinone (Nguyen *et al.*, 1988). The stability of $AZQ^{-\cdot}$ in aerobic solutions could be partly accounted for in terms of the facile reduction of the quinone by $O_2^{-\cdot}$ ($k_{2b} = 2.7 \times 10^8$ M$^{-1}\cdot$s$^{-1}$) (Butler *et al.*, 1987).

In the presence of superoxide dismutase, the e.p.r. spectrum became more distinctly that of DMPO/OH adduct, as indicated by the characteristic quartet spectrum in Fig. 3(*b*). The fact that the DMPO/OH signal was not suppressed by superoxide dismutase also suggests that this adduct was not originating solely from the spontaneous decay of the DMPO/OOH adduct. In addition, superoxide dismutase suppressed the five-line signal at the centre of the DMPO/OH quartet attributed to the AZQ semiquinone, in agreement with the displacement of the equilibrium of reaction (2) towards the right by the enzyme (reaction 3).

Conversely, the e.p.r. spectrum exhibited the more characteristic profile of the DMPO/OOH adduct when $H_2O_2$ was removed by catalase (Fig. 3*c*; a similar spectrum was simulated with a 17:20 mixture of the DMPO/OOH and DMPO/OH adducts). With time, the signal in Fig. 3(*c*) changed into the characteristic DMPO/OH spectrum, owing to spontaneous internal conversion of DMPO/OOH into DMPO/OH (Finkelstein *et al.*, 1982). When both superoxide dismutase and catalase were present in the reaction mixture, the system became e.p.r.-silent. The effect of catalase on the e.p.r. spectrum arising from the AZQ redox transitions during DT-diaphorase catalysis shown here differed from that reported during the redox transitions of the quinone observed upon incubation with a MCF-7 S9 fraction (Fisher & Gutierrez, 1991). In the former case, the semiquinone and the DMPO/OOH adduct persisted in the presence of catalase, whereas in the latter case (Fisher & Gutierrez, 1991) the enzyme rendered the system e.p.r.-silent.

In summary, $O_2^{-\cdot}$, $HO^\cdot$ and the semiquinone are generated during the redox transitions of $AZQH_2$ subsequent to reduction of the oxidized counterpart by DT-diaphorase. $HO^\cdot$ seems to originate partly from the internal conversion of the DMPO/OOH adduct into DMPO/OH adduct and partly from the decomposition of $H_2O_2$. It is worth mentioning that, in the latter instances, the DMPO/OH e.p.r. signal was insensitive to metal chelators such as EDTA and DTPA.

### Effect of GSH on the redox transitions of AZQ

Thiol oxidation has been shown to accompany the redox transitions of quinones with different substitution patterns during DT-diaphorase catalysis (Llopis *et al.*, 1990) and GSH nucleophilic addition (Öllinger *et al.*, 1989; Goin *et al.*, 1991), as well as during the reduction of naphthoxyl (d'Arcy Doherty *et al.*, 1986) and alloxan (Winterbourn & Munday, 1990) radicals and during the course of the peroxidase-catalysed oxidation of several drugs (Ross & Moldéus, 1985).

The effects of GSH on the redox transitions of AZQ listed below were observed at pH 7.8. These effects were absent at pH 5, probably because the dissociation of GSH ($GSH \rightleftharpoons GS^- + H^+$; $pK \approx 9$), a requisite condition for nucleophilic addition and electron transfer, was negligible: 0.1 $\mu$M-$GS^-$ was present in a 1 mM-GSH solution at pH 5 ($[GS^-]/[GSH] = (1 + 10^{pK-pH})^{-1} = 10^{-4}$).

***(a)* GSH-mediated increase of the steady-state concentration of $AZQH_2$.** GSH exerted a dual effect on the time course of $AZQH_2$ formation: on the one hand, it induced an increase in the total amount of $AZQH_2$ formed and, on the other, it temporarily prevented the oxidation of $AZQH_2$ (Fig. 4). The magnitude of

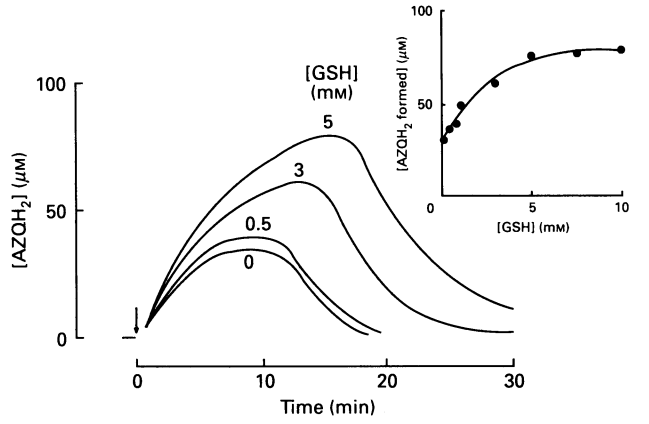



**Fig. 4. Effect of GSH on the steady-state concentration of $AZQH_2$ formed during DT-diaphorase-catalysed reduction of AZQ**

Assay conditions: 100 $\mu$M-AZQ and GSH (different concentrations) in 0.25 M-sucrose/0.1 M-potassium phosphate buffer, pH 7.8, were supplemented with DT-diaphorase (1.6 $\mu$g/ml) to initiate the reaction. The main Figure shows time-course traces of $AZQH_2$ formation in the absence or presence of different concentrations of GSH. Inset: Dependence of total $AZQH_2$ formed on GSH concentration. Assay conditions were as in the main Figure. Other assay conditions were as described in the Materials and methods section.

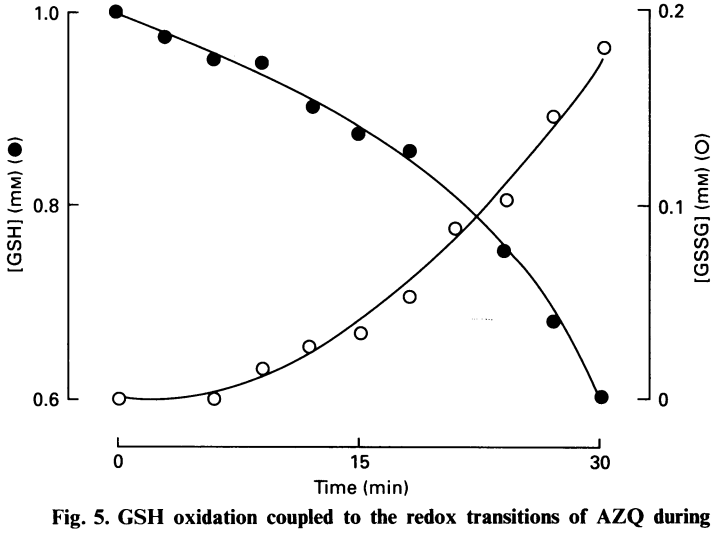



**Fig. 5. GSH oxidation coupled to the redox transitions of AZQ during DT-diaphorase catalysis**

Assay conditions: 100 $\mu$M-AZQ, 200 $\mu$M-NADPH and 1 mM-GSH in 0.25 M-sucrose/0.1 M-potassium phosphate buffer, pH 7.8, were supplemented with DT-diaphroase (1.6 $\mu$g/ml) to initiate the reaction. Samples for GSH and GSSG analysis by h.p.l.c. with u.v. detection were treated as described in the Materials and methods section.

both effects was related to increasing concentrations of the thiol up to about 10 mM (Fig. 4, inset); beyond this concentration no further increases were observed. This effect of GSH was accompanied by a decrease in the rates of NADPH oxidation and $O_2$ uptake (results not shown), thus indicating that hydroquinone autoxidation was temporarily inhibited by GSH.

***(b)* Thiol oxidation coupled to the redox transitions of AZQ.** The increase in total $AZQH_2$ formed along with the temporary inhibition of its autoxidation described above were coupled to GSH oxidation to its disulphide (Fig. 5*a*). After 30 min incubation, GSH consumption ($\sim$ 425 $\mu$M) could be accounted for almost entirely in terms of its oxidation to GSSG ($\sim$ 200 $\mu$M)

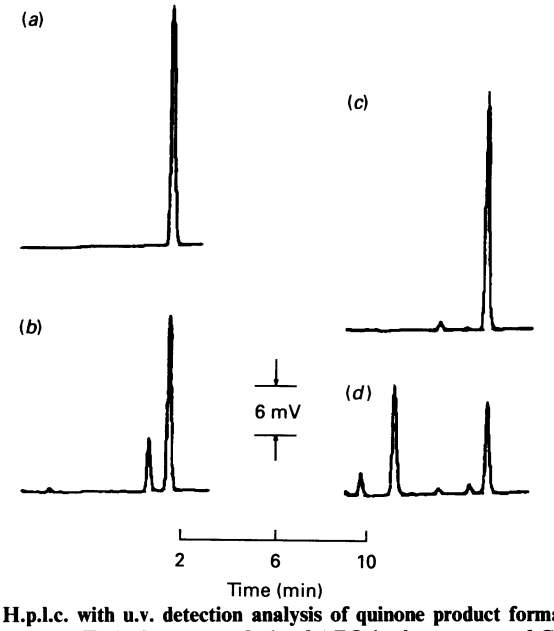



**Fig. 6. H.p.l.c. with u.v. detection analysis of quinone product formation during DT-diaphorase catalysis of AZQ in the presence of GSH**

Assay conditions: (*a*) 100 $\mu$M-AZQ in 0.1 M-potassium phosphate buffer, pH 7.8. (*b*) 100 $\mu$M-AZQ, 200 $\mu$M-NADPH and 1 mM-GSH in 0.1 M-potassium phosphate buffer, pH 7.8, were supplemented with DT-diaphorase (1.6 $\mu$g/ml). The reaction mixture was analysed by h.p.l.c. after 30 min incubation. (*c*) The same as in (*b*) after 60 min incubation. (*d*) Chromatogram resulting from the incubation of 100 $\mu$M-AZQ in 0.1 M-Tris/HCl buffer, pH 7.4, with 2 mM-GSH for 24 h.

(Fig. 5*a*). Superoxide dismutase did not exert significant changes on either the profile of the GSH $\rightarrow$ GSSG conversion or the individual rates.

Nucleophilic addition to the aziridinyl groups of AZQ did not take place during the DT-diaphorase-catalysis of the quinone as evidenced in Fig. 6. The chromatogram resulting from the addition of GSH to the reaction mixture revealed two peaks ascribed to the reduced [retention time ($R_t$) = 7.6 min] and oxidized ($R_t$ = 8.5 min) forms of AZQ (Fig. 6*b*), the identity of which was confirmed by h.p.l.c. with electrochemical detection; the intensity of the latter peak increased with time at expense of the former peak (Fig. 6*c*). This is in agreement with the results shown in Fig. 4, indicating that the increase in the steady-state concentration of $AZQH_2$ mediated by GSH is a temporary event that is eventually followed by oxidation of the hydroquinone.

However, and as reported previously (Ross *et al.*, 1990), GSH conjugation to AZQ indeed does occur after prolonged incubations of the quinone with the thiol ($R_t$ = 4.3 min; Fig. 6*d*). The latter chromatogram also shows two small peaks with $R_t$ of about 3.4 and 6.0 min flanking that of the conjugate ($R_t$ = 4.3 min) and attributed to the double- and single ring-opening of the aziridinyl substituent in AZQ (Poochikian & Cradock, 1981; Poochikian & Kelley, 1981). Although these derivatives could be observed during DT-diaphorase-catalysed reduction of AZQ, preferentially at low pH, they were not a consequence of the quinone-ring aromatization facilitated by enzyme, for aqueous solutions of the quinone underwent similar changes with time. Moreover, experiments carried out with [$^3$H]GSH indicated that these peaks were not due to glutathionylquinone derivatives (results not shown). It can be concluded that, although sulphur nucleophilic addition to the aziridinyl groups takes place during prolonged incubation of the quinone with GSH (Ross *et al.*, 1990; Fig. 6*d*), it does not occur

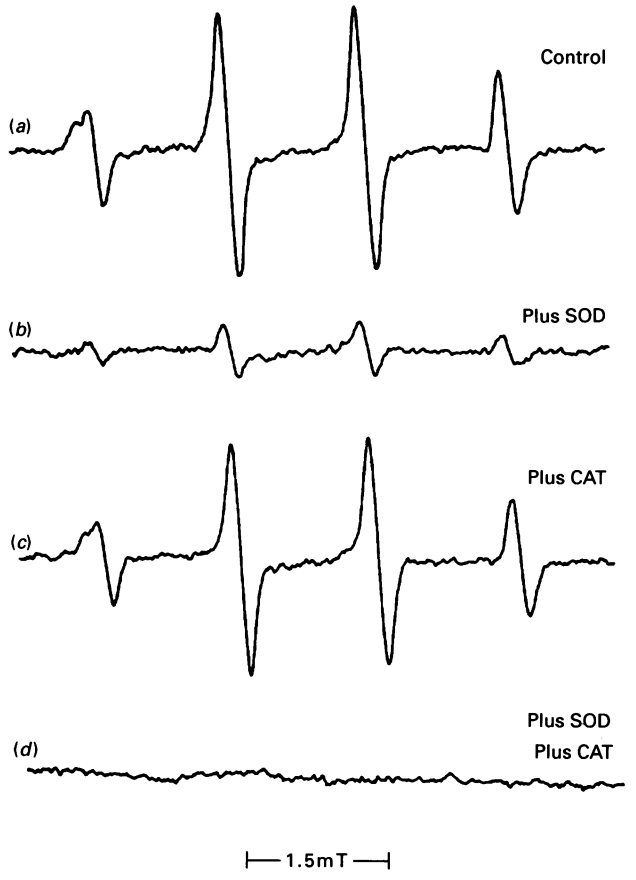



**Fig. 7. Formation of HO$^{\cdot}$, $O_2^{-\cdot}$, and GS$^{\cdot}$ during the redox transitions of AZQ in the presence of GSH**

Assay conditions: as in Fig. 3, but in the presence of 12.5 mM-GSH. (*a*) No further additions. Note that the signal centred at 0.34863 T ($g = 2.0046$) is suppressed by GSH. (*b*) As in (*a*) plus superoxide dismutase (SOD) (5 $\mu$M). (*c*) As in (*a*) plus catalase (CAT) (500 units/ml). (*d*) Plus superoxide dismutase (5 $\mu$M) and catalase (500 units/ml). Instrument settings: receiver gain, $2 \times 10^6$; microwave power, 20 mW; modulation amplitude, 0.1922 mT; time constant, 1.3 s; scan time, 5.6 min.

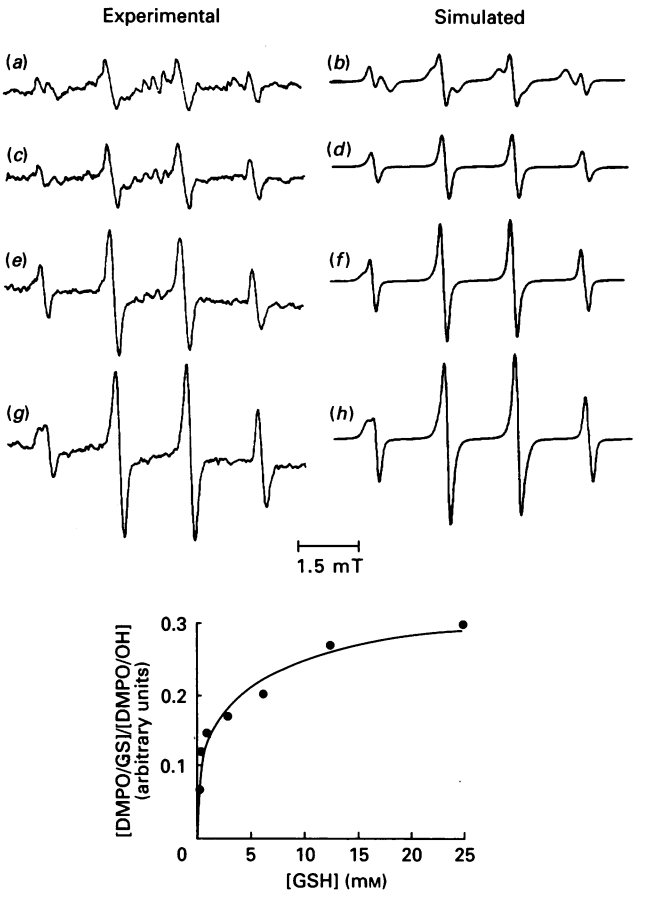
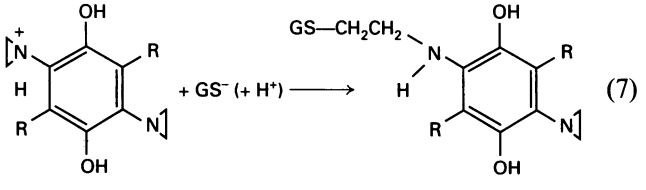



**Fig. 8. Effect of GSH concentration on the relative contribution of DMPO/OH and DMPO/GS adducts to the overall e.p.r. spectrum**

Assay conditions were as in Fig. 7, but with various concentrations of GSH. (*a*) No GSH added; (*c*) plus 0.5 mM-GSH; (*e*) Plus 3 mM-GSH; (*g*) Plus 12.5 mM-GSH. Simulated spectra corresponding to the experimental spectra are indicated on the right side (spectra *b*, *d*, *f*, and *h*). Inset: dependence of the relative contribution of DMPO/GS and DMPO/OH (expressed as the [DMPO/GS]/[DMPO/OH] ratio) on GSH concentration. Data were obtained from spectra simulating the experimental data and calculated after mathematical addition of the individual simulated spectra of each species.

during DT-diaphorase catalysis of AZQ. This observation is important for the one- or two-electron reduction of AZQ (reaction 1), along with acid-assisted aziridinyl ring-opening seem to be requisite conditions of the thiol arylation of the aziridinyl substituents (indicated in reaction 7 as monoconjugate formation) and for binding of DNA to AZQ (Gutierrez, 1989).




(7)

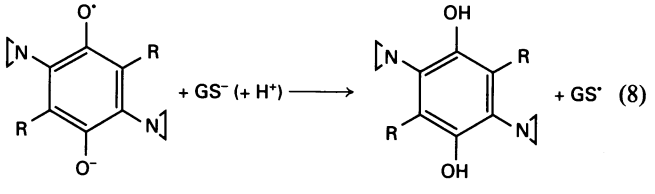
Moreover, protonation of the latter, facilitated by lowering the pH to 5 and without affecting the rate of electron transfer catalysed by DT-diaphorase, did not lead to conjugate formation either, probably because at this pH only a minute amount of GSH existed as $GS^-$, a requirement for nucleophilic addition. Of note, a study on the interactions of 2,5-bis-(1-aziridinyl)-1,4-benzoquinone derivatives and GSH showed that no GSH adduct formation occurred with AZQ and that nucleophilic Michael addition required H or halogen substitution of the quinonoid ring (Lusthof *et al.*, 1990).

(*c*) **Thiyl- and hydroxyl-radical formation during the redox transitions of AZQ.** GSSG formation has been shown to proceed in some instances through the generation of intermediate thiyl radicals (see Mason & Ramakrishna Rao, 1990) identified by e.p.r. in conjunction with the spin trap DMPO (hyperfine splitting constants: $a^N = 1.53$ mT, $a^H = 1.62$ mT; $g = 2.0065$–$2.0067$). The oxidation of GSH to GSSG during the AZQ redox transitions in the presence of the spin trap DMPO led to the generation of e.p.r. signals consistent with a composite of the quartet spectra of the hydroxyl- and thiyl-radical adducts (Fig. 7*a*), with the former being predominant (a simulated spectrum equivalent to this one was obtained with a 13:7 mixture of DMPO and HO and DMPO/GS adducts; results not shown). Under the experimental conditions of Fig. 7, superoxide dismutase decreased significantly the DMPO/GS signal along with a less pronounced decrease on the DMPO/HO signal (Fig. 7*b*; a simulated spectrum equivalent to this one was obtained with a 9:1 mixture of DMPO/HO and DMPO/GS adducts). Catalase had virtually no effect on the e.p.r. signal (Fig. 7*c*), thus suggesting that neither the presence or removal of $H_2O_2$ or its breakdown to HO$^{\cdot}$ was of consequence for thiyl-radical formation. Furthermore, this also

suggests that the signal shown in Fig. 7(*a*), partly attributed to the DMPO/HO adduct, is mainly due to trapping of $O_2^{-\cdot}$ and not of $HO^{\cdot}$; this view is supported by (*a*) the short half-life of the DMPO/OOH adduct (~ 2 min), which spontaneously decays to DMPO/HO (Finkelstein *et al.*, 1982) and (*b*) the fact that thiols can carry out the direct reduction of DMPO/OOH to DMPO/HO by a hydroxyl-radical-independent mechanism (Finkelstein *et al.*, 1980). The addition of both superoxide dismutase and catalase rendered the system e.p.r.-silent (Fig. 7*d*), an effect which was obviously consequential to that observed during DT-diaphorase-catalysed reduction of AZQ in the absence of GSH (Fig. 3*d*).

The relative contributions of the hydroxyl- and thiyl radical adducts of DMPO to the e.p.r. signal during the redox transitions of AZQ depended on the thiol concentration in the reaction mixture (Fig. 8). At low GSH concentration (e.g., 0.5 mM; Fig. 8*c*), the DMPO/GS appeared as a small shoulder slightly downfield of the characteristic DMPO/HO four-line spectrum. A simulated spectrum equivalent to this one was obtained at a [DMPO/GS]/[DMPO/HO] ratio ≈ 0.11 (Fig. 8*d*). At a GSH concentration equivalent to 3 mM, the above shoulder was more pronounced, and a decrease of the five-line signal at the centre of the DMPO adduct quartet (ascribed to the semiquinone form of AZQ) was observed (Fig. 8*e*). The simulated spectrum (Fig. 8*f*) indicated a [DMPO/GS]/[DMPO/HO] ratio of ≈ 0.21. At high GSH concentration (> 5 mM-GSH), the profile of the overall e.p.r. spectrum clearly indicated a composite of the DMPO adducts of thiyl and hydroxyl radicals (Fig. 8*g*), which could be simulated with a [DMPO/GS]/[DMPO/HO] ratio ≈ 0.3 (Fig. 8*h*). These findings are summarized in the inset of Fig. 7, where the dependence of the [DMPO/GS]/[DMPO/HO] ratios was plotted against thiol concentration. A half-maximal value (≈ 0.15) for the [DMPO/GS]/[DMPO/HO] ratio was achieved with 1.55 mM-GSH. Of note, the effect of GSH concentration on the [DMPO/GS]/[DMPO/HO] ratio resembled that on total reduced AZQ formed (Fig. 4).

In addition, GSH suppressed the signal located at a magnetic field of 0.34863T (attributed to the semiquinone form of AZQ) as shown in Figs. 7(*a*) and 8(*g*), and further exemplified in the e.p.r. spectrum obtained in the absence of the spin trap (Fig. 9). This effect of GSH may be consistent with reduction of the semiquinone by the thiol according to reaction 8:




(8)

An experiment similar to that in Fig. 9(*a*) carried out under anaerobic conditions yielded a two-fold more intense semiquinone e.p.r. signal, which was also suppressed by GSH. Under these conditions and in the presence of DMPO, however, the thiyl radical adduct ensuing from reaction (8) was not observed. This was expected, for reduction of the DMPO/GS adduct to the e.p.r.-silent hydroxylamine (Janzen *et al.*, 1985) can be achieved by the hydroquinone or the large amounts of GSH present.

### Mechanistic aspects

Despite the complexity of this experimental model, three types of interdependent redox transitions could be envisaged, which – except for the initial two-electron transfer to AZQ catalysed by DT-diaphorase (reaction 1) – imply one-electron transfer reactions involving the quinone:

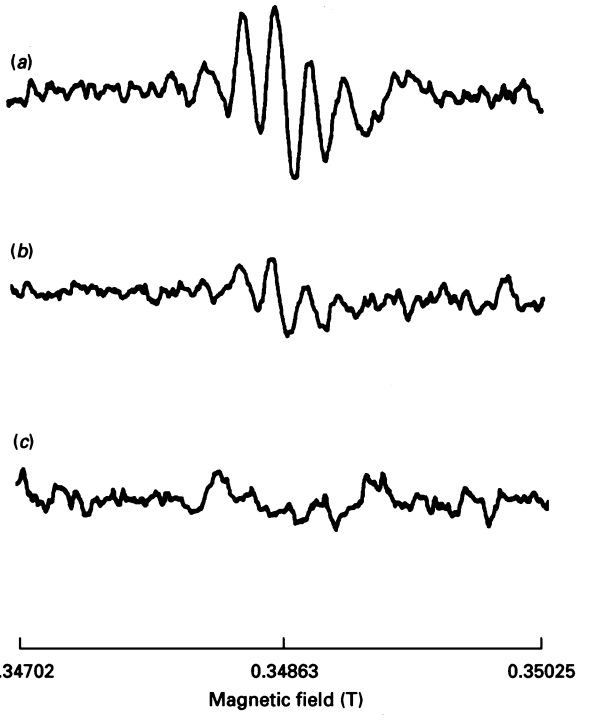



**Fig. 9. GSH-mediated suppression of the e.p.r. signal corresponding to $AZQ^{-\cdot}$**

Assay conditions: 100 μM-AZQ and 200 μM-NADPH in helium-saturated 0.25 M-sucrose/0.1 M-potassium phosphate buffer, pH 7.8, were supplemented with DT-diaphorase (1.6 μg/ml) to initiate the reaction. (*a*) No further additions. (*b*) As in (*a*) plus 10 mM-GSH. (*c*) As in (*a*) plus 20 mM-GSH. Instrument settings: receiver gain, $2 \times 10^6$; microwave power, 20 mW; modulation amplitude, 0.1922 mT; time constant, 1.3 s; scan time, 5.6 min.

$$AZQ \rightleftharpoons AZQ^{-\cdot} \rightleftharpoons AZQ^{2-}$$

oxygen:

$$O_2 \rightleftharpoons O_2^{-\cdot} \rightarrow H_2O_2 \rightarrow HO^{\cdot}$$

and the thiol:

$$GS^- \rightleftharpoons GS^{\cdot}$$

Of necessity, the above redox transitions are coupled with each other, and the individual rate of each step will be a function of the physico-chemical properties of the quinone (including its dissociation constants), the steady-state concentration and reduction potential of the redox couples involved, and the pH of the environment.

**Redox transitions involving quinone and oxygen.** Analysis of the interdependence of the two first redox transitions above requires the following considerations.

First, like all hydroquinones, the dissociation of the $AZQH_2$ ($AZQH_2 \rightleftharpoons AZQ^{2-} + 2H^+$) is a requisite condition for observing electron transfer (Steenken, 1979) and, hence, autoxidation, as indicated by the pH-dependence of $H_2O_2$ formation (Fig. 2*b*).

Secondly, the semiquinone form of AZQ is a central species in the redox transitions of AZQ, despite the initial two-electron transfer to the quinone catalysed by DT-diaphorase. This can be rationalized on the basis of two key features of this redox chemistry: (*a*) $AZQ^{-\cdot}$ is readily formed upon reduction of the quinone by $O_2^{-\cdot}$ (reaction 2*b*) (Butler *et al.*, 1987); (*b*) $AZQ^{-\cdot}$ decays slowly by disproportionation (reaction 5*b*); this may be understood in terms of displacement of the equilibrium of the disproportionation–comproportionation reaction and stabilization of the semiquinone by intramolecular H-bonding between the –O in the quinone moiety and the aziridinyl N atom. Previous

reports (Gutierrez *et al.*, 1982; Gutierrez, 1989) and the present one indicate that $AZQ^{-\cdot}$ is detectable by e.p.r. under aerobic conditions (Fig. 3*a*), a fact that can be explained by (*a*) and (*b*) above. Aromatic-ring hydroxy-substituted semiquinones are also stabilized by intramolecular H-bonding and detected easily by e.p.r. (Dodd & Mukherjee, 1984).

Thirdly, it can be inferred that the hydroquinone of AZQ does not autoxidize as described for various compounds of the *p*-benzo- and 1,4-naphtho-quinone series (Öllinger *et al.*, 1990), in that $O_2^{-\cdot}$ is not a free-radical-propagating species formed during semiquinone autoxidation ($Q^{-\cdot} + O_2 \rightarrow Q + O_2^{-\cdot}$) and consumed during hydroquinone oxidation ($Q^{2-} + 2H^+ + O_2^{-\cdot} \rightarrow Q^{-\cdot} + H_2O_2$; reaction 6). If this mechanism were operative, inhibition of hydroquinone autoxidation on withdrawal of the free-radical-propagating species ($O_2^{-\cdot}$) by the enzyme would be expected. Hence, reaction (6) cannot contribute significantly to the redox transitions of AZQ after its reduction by DT-diaphorase. Conversely, the enhancement of $AZQH_2$ autoxidation by superoxide dismutase, similar to the reported enhancement of hydroxypyrimidine autoxidation by the enzyme (Winterbourn *et al.*, 1989), strengthens the importance of the contribution of reaction (2) to the overall process. This view is further substantiated by the suppression of the e.p.r. signal attributed to $AZQ^{-\cdot}$ by superoxide dismutase (Fig. 3*b*).

**Redox transitions involving GSH.** GSH oxidation coupled to the redox transitions of AZQ (Fig. 5) seems to proceed via $GS^{\cdot}$ formation (Figs. 7 and 8), which could originate upon reaction of the thiol with $O_2^{-\cdot}$, $AZQ^{-\cdot}$ or $HO^{\cdot}$. The latter species seems not to contribute to $GS^{\cdot}$ formation, for the DMPO/GS adduct could be observed in the presence of catalase. Thiol reactivity towards $O_2^{-\cdot}$ (reaction 9):

$$GS^-(H^+) + O_2^{-\cdot} \rightleftharpoons GS^{\cdot} + H_2O_2 \qquad (9)$$

is low ($k_9 = 6.7 \times 10^5\ M^{-1} \cdot s^{-1}$) (Bielski *et al.*, 1985), and that towards $AZQ^{-\cdot}$ (reaction 8) is thermodynamically unlikely [$E(AZQ^{-\cdot}/AZQ^{2-}) = -39$ mV; $E(GS^{\cdot}, H^+/GSH) = +840$ mV (Surdhar & Armstrong, 1986)]. [The $E(AZQ^{-\cdot}/AZQ^{2-})$ value was calculated from the relationship:

$$E(Q^{-\cdot}/Q^{2-}) = 2\,E(Q/Q^{2-}) - E(Q/Q^{-\cdot})$$ (Swallow, 1982)

where $E(Q/Q^{2-})$ and $E(Q/Q^{-\cdot})$ were $-50$ mV (from hydrodynamic voltamograms) and $-61$ mV (Butler *et al.*, 1987) respectively]. This thermodynamic restriction, however, can be overcome kinetically (Wardman, 1990) when the equilibrium of reaction (8) is displaced towards the right upon efficient removal of the thiyl-radical product by dimerization or conjugation with $GS^-$ (Wardman, 1988). Although both pathways for $GS^{\cdot}$ decay lead to GSSG accumulation, the conjugation of $GS^{\cdot}$ with $GS^-$ proceeds with intermediate formation of $GSSG^{-\cdot}$ (reaction 10; $k_{10} = 6.6 \times 10^8\ M^{-1} \cdot s^{-1}$):

$$GS^{\cdot} + GS^- \rightarrow GSSG^{-\cdot} \qquad (10)$$

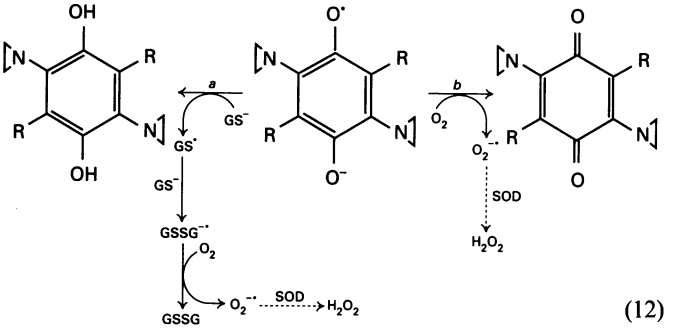
which decays rapidly by electron transfer to $O_2$ (reaction 11; $k_{11} = 1.6 \times 10^8\ M^{-1} \cdot s^{-1}$):

$$GSSG^{-\cdot} + O_2 \rightleftharpoons GSSG + O_2^{-\cdot} \qquad (11)$$

(Quintiliani *et al.*, 1977). The equilibrium of the latter reaction could also be displaced upon removal of $O_2^{-\cdot}$ by superoxide dismutase (reaction 3).

The effect of superoxide dismutase solely does not permit to distinguish between a direct interaction of $GS^-$ with $O_2^{-\cdot}$ (reaction 9) or competition between $O_2$ (reaction 2) and GSH (reaction 8) for $AZQ^{-\cdot}$, especially since the equilibrium of the last two reactions could be equally affected by dismutase (involving the sequence of reactions $2 \rightarrow 3$, $8 \rightarrow 10$ and $11 \rightarrow 3$ respectively). Accordingly, superoxide dismutase suppressed the DMPO/GS signal (Fig. 7*b*), and yet it did not significantly prevent or modify GSSG formation during DT-diaphorase catalysis of AZQ (Fig. 5). The suppression of the $AZQ^{-\cdot}$ signal by GSH under anaerobic conditions (similar to Fig. 9) suggested the direct interaction of the thiol with the semiquinone.

In summary, it seems likely that catalytic amounts of superoxide dismutase and large amounts of GSH are able to determine the fate of $AZQ^{-\cdot}$ in terms of an oxidative and a reductive pathway (reaction 12): the former by enhancing the autoxidation of $AZQ^{-\cdot}$ and the latter by enhancing its reduction. In compliance with this, superoxide dismutase (Fig. 3*b*) and GSH (Figs. 7*g* and 9*b* and 9*c*) abolished the semiquinone e.p.r. signal. The reductive pathway (reaction 12*a*):




(12)

is ultimately sensitive to superoxide dismutase, owing to its oxidative component represented by reaction (11).

## Conclusions

There are two previous reports in the literature describing DT-diaphorase-catalysed two-electron reduction of AZQ. The enzyme, purified from rat liver or human HT-29 cells (Siegel *et al.*, 1990), as well as the dicoumarol-sensitive enzymic activity of the MCF7 S9 fraction (Fisher & Gutierrez, 1991), represent an efficient electron-transfer process leading to AZQ bioactivation, which, in the former case (Siegel *et al.*, 1990) is further accompanied by DNA inter-strand cross-linking. That work (Siegel *et al.*, 1990) seems to summarize the chemical requirements for AZQ cytotoxicity entailing the sequence of two-electron reduction and, hence quinone-ring aromatization, aziridinyl ring-opening and reaction with a cellular nucleophile.

The present work, on the other hand, shows that the redox transitions of AZQ during DT-diaphorase catalysis lead to oxidation of the most abundant cellular sulphur nucleophile, GSH, but not to alkylation of the thiol. We have attempted to dissociate autoxidation from alkylation reactions involving the hydroquinone form of AZQ. The former reactions are a function of the redox transitions of the quinone moiety, whereas the latter are an expression of the electrophilic character of the aziridinyl N after acid-assisted ring-opening. At first sight this was not difficult, for low pH does not affect significantly the catalysis of AZQ by DT-diaphorase and it leads to both stabilization of the hydroquinone and protonation of the aziridinyl N. The first effect entails inhibition of electron transfer to $O_2$, whereas the second one fulfills the chemical requirements for nucleophilic addition. However, whereas these conditions potentiate the electrophilic character of the aziridinyl groups, they have an opposite effect on the thiol nucleophile: at low pH, the concentration of $GS^-$, required for nucleophilic addition, is negligible, and no conjugate of AZQ is formed.

The biological significance of these findings requires evaluation of two-electron transfer processes in quinone bioactivation, which entail not solely those implied in DT-diaphorase catalysis but also those in the 1,4 Michael addition with sulphur nucleophiles. DT-diaphorase activity is enhanced in several cancer cell

lines and preneoplastic nodules (Schor, 1987), whereas the concentration of GSH in typical tumours in mice is within the range found in normal tissues (Minchinton *et al.*, 1984). In addition, Cu,Zn-superoxide dismutase activity is generally, but always, lowered in the tumour cell (Oberley, 1982). These cellular relationships, along with the findings described in here and centred on reaction (12), would make GSH oxidation a salient feature accompanying the redox transitions of AZQ.

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