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DT-diaphorase-catalysed reduction of 1,4-naphthoquinone derivatives and glutathionyl-quinone conjugates

Effect of substituents on autoxidation rates

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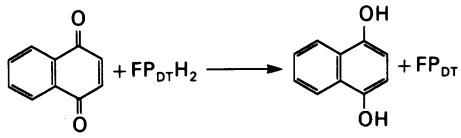
DT-diaphorase catalysed the reduction of 1,4-naphthoquinones with hydroxy, methyl, methoxy and glutathionyl substituents at the expense of reducing equivalents from NADPH. The initial rates of quinone reduction did not correlate with either the half-wave reduction potential ($E_{\frac{1}{2}}$) value (determined by h.p.l.c. with electrochemical detection against an Ag/AgCl reference electrode) or the partition coefficient of the quinones. After their reduction by DT-diaphorase the 1,4-naphthoquinone derivatives autoxidized at distinct rates, the extent of which was influenced by the nature of the substituents. Thus for the 1,4-naphthoquinone series the following order of rate of autoxidation was found: 5-hydroxy-1,4-naphthoquinone > 3-glutathionyl-1,4-naphthoquinone > 5-hydroxy-3-glutathionyl-1,4-naphthoquinone > 1,4-naphthoquinone > 2-hydroxy-1,4-naphthoquinone. For the 2-methyl-1,4-naphthoquinone (menadione) series the following order was observed: 5-hydroxy-2-methyl-1,4-naphthoquinone > 3-glutathionyl-5-hydroxy-2-methyl-1,4-naphthoquinone > 3-glutathionyl-2-methyl-1,4-naphthoquinone > 2-methyl-1,4-naphthoquinone > 3-hydroxy-2-methyl-1,4-naphthoquinone. The autoxidized naphthohydroquinone derivatives were re-reduced by DT-diaphorase, thus closing a cycle of enzymic reduction $\rightleftharpoons$ autoxidation. This was expressed as an excess of NADPH oxidized over the initial concentration of quinone present as well as H_2O_2 formation. These findings demonstrate that glutathionyl conjugates of 1,4-naphthoquinone and 2-methyl-1,4-naphthoquinone and those of their respective 5-hydroxy derivatives are able to act as substrates for DT-diaphorase and that they also autoxidize at rates higher than those for the unsubstituted parent compounds. These results are discussed in terms of the cellular role of DT-diaphorase in the reduction of hydroxy- or glutathionyl-substituted naphthoquinones as well as the further conjugation of these hydroquinones with glucuronide or sulphate within the cellular milieu, thereby facilitating their disposal from the cells.

INTRODUCTION

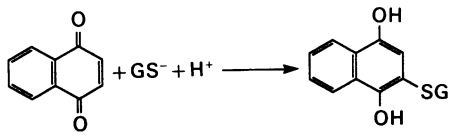
The cytotoxicity of several quinonoid compounds can be understood as comprising three main mechanisms (Gant & Cohen, 1987): (*a*) autoxidation of the semiquinones or hydroquinones following the enzymic reduction of the oxidized counterparts and within a process of cycling characteristics yielding primarily $O_2^{\cdot-}$. (*b*) Addition reactions with cellular nucleophiles such as GSH, DNA, RNA and proteins. (*c*) Inhibition of vital cellular functions such as DNA synthesis (Esterbauer *et al.*, 1987) and mitochondrial electron transport (Pristos *et al.*, 1982; Matwura *et al.*, 1983). The concerted participation of the above mechanisms can lead to modification of intracellular thiol balance and alteration of the intracellular Ca^{2+} homoeostasis, and, eventually, cell death (Thor *et al.*, 1982; Moore *et al.*, 1987).

Two processes can be envisaged in the reduction of quinones to either semiquinones or hydroquinones: on the one hand, the enzymic reduction of quinonoid compounds, and, on the other, the redox features of the addition chemistry of quinones. Firstly, quinones undergo one-electron reduction by a variety of flavoenzymes, including microsomal NADPH–cytochrome *P*-450 reductase, NADH–cytochrome b_5 reductase and mitochondrial NADH dehydrogenase, with concomitant formation of semiquinone species (Iyanagi & Yamazaki, 1970). DT-diaphorase [NAD(P)H:quinone oxidoreductase, EC 1.6.99.2], an enzyme present in most animal tissues, catalyses formally the two-electron reduction of quinones to hydroquinones (reaction 1) (Ernster, 1987):



(1)

where FP_{DT} represents DT-diaphorase flavoprotein. Secondly, the addition chemistry of quinones involves redox transitions that yield a reduced quinone conjugated to the nucleophile. Thus 1,4-naphthoquinones with sufficient electron density across the C-2=C-3 double bond can undergo 1,4-reductive addition with GSH to form the corresponding glutathionyl-hydroquinone conjugates (reaction 2) (Finley, 1974):



(2)

Abbreviations used: $E_{\frac{1}{2}}$, half-wave reduction potential; $E(Q/Q^{\cdot-})$, one-electron reduction potential.

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Both processes outlined above share the characteristic of yielding reduced quinonoid compounds either unconjugated (reaction 1) or bearing a glutathionyl substituent (reaction 2).

Hydroquinones autoxidize with transfer of an electron to molecular O_2 with formation of semiquinone and $O_2^{\cdot -}$. Glutathionyl-hydroquinone conjugates seem to autoxidize in a similar fashion with formation of glutathionyl-semiquinone transient species as detected by e.s.r. with a spin-trapping technique (Gant *et al.*, 1986; Takahashi *et al.*, 1987), as originally shown for the redox cycling of 3-glutathionyl-2-methyl-1,4-naphthoquinone (Wefers & Sies, 1983). The extent of autoxidation of semiquinones and hydroquinones or their glutathionyl derivatives is controlled by the reduction potentials of the hydroquinone/semiquinone and semiquinone/quinone couples, the nature of the substituents, solvent cage and solvation energy, pH and temperature.

The two-electron transfer from soluble reduced flavin to an acceptor has been shown in a non-enzymic system to be dependent upon the reduction potential of the acceptor molecules (Dubin & Wright, 1975). In the present study, this relationship was examined in an enzymic system using DT-diaphorase to catalyse the two-electron reduction of 1,4-naphthoquinones with hydroxy, methyl, methoxy and glutathionyl substituents. These substituents exert either slight or substantial changes in the reduction potential of the parent quinone, which was determined by h.p.l.c. with electrochemical detection by means of hydrodynamic voltamograms.

DT-diaphorase-catalysed reduction of the substituted 1,4-naphthoquinones was evaluated in terms of NADPH oxidation under anaerobic and aerobic conditions. The former approach reflected the actual affinity of the enzyme towards the different naphthoquinones tested. The latter approach included both the rate of enzymic reduction followed by autoxidation and the re-reduction of the autoxidized quinone by DT-diaphorase (enzymic reduction $\rightleftharpoons$ autoxidation). Accordingly, this was accompanied by NADPH oxidation in excess to the amount of quinone present as well as H_2O_2 formation.

MATERIALS AND METHODS

Chemicals and biochemicals

1,4-Naphthoquinone, 2-hydroxy-1,4-naphthoquinone, 5-hydroxy-2-methyl-1,4-naphthoquinone, *p*-hydroxyphenylacetic acid and H_2O_2 were from Aldrich-Chemie (Steinheim, Germany). 5-Hydroxy-1,4-naphthoquinone and scopoletin were from Sigma Chemical Co. (St. Louis, MO, U.S.A.). NADPH, GSH, *N*-acetyl-L-cysteine and horseradish peroxidase (grade I) were from Boehringer (Mannheim, Germany), and 2-methyl-1,4-naphthoquinone was from Merck (Darmstadt, Germany). 2,3-Dimethyl-1,4-naphthoquinone was a gift from Dr. M. Threadgill (University of Aston, Birmingham, U.K.), and 2,3-dimethoxy-1,4-naphthoquinone and 2-*N*-acetyl-L-cysteinyl-1,4-naphthoquinone were gifts from Dr. G. Cohen (School of Pharmacy, University of London, London, U.K.).

3-Glutathionyl-1,4-naphthoquinone conjugate was prepared by mixing a 3-fold molar excess of the quinone (in ethanol) with GSH (in water). The mixture was kept overnight at 4 °C, and the precipitate was collected, dried and washed repeatedly with chloroform to remove excess quinone. The purity of this preparation was assessed by h.p.l.c., and elemental analysis was: C, 50.9; H, 4.6; N, 9.1; O, 28.4; S, 6.9% (calc. for $C_{20}H_{22}N_3O_8S$: C, 51.7; H, 4.8; N, 9.0; O, 27.6; S, 6.9%). 3-Glutathionyl-5-hydroxy-1,4-naphthoquinone conjugate was prepared by mixing a 2-fold molar excess of the hydroxyquinone (in ethanol) with GSH (in water) and the mixture was kept overnight at 4 °C, the precipitate was treated as for the case of the 3-glutathionyl-1,4-naphthoquinone conjugate. 3-*N*-Acetyl-L-cysteinyl-2-methyl-1,4-naphthoquinone, 3-glutathionyl-2-methyl-1,4-naphthoquinone and 3-glutathionyl-5-hydroxy-2-methyl-1,4-naphthoquinone were synthesized and recrystallized as previously described (Nickerson *et al.*, 1963). 2,3-Diglutathionyl-1,4-naphthoquinone was prepared by mixing 1,4-naphthoquinone (in ethanol) with GSH (in water) at a molar ratio of 1:2.1, the mixture was kept overnight at 4 °C, and the precipitate was collected, dried and washed with ethanol to remove unconjugated quinone. 3-Hydroxy-2-methyl-1,4-naphthoquinone (pthiocol) was prepared as previously described (Fieser, 1940).

Purified DT-diaphorase, isolated from rat liver as described by Höjeberg *et al.* (1981), was provided by Dr. C. Lind (Arrhenius Laboratory, University of Stockholm, Stockholm, Sweden) and had a specific activity of 2450 μmol of NADH oxidized/min per mg of protein (at the Sephacryl S-200 purification step) with menadione as electron acceptor.

Standard assay conditions

The standard reaction mixture consisted of 20 μM-quinonoid compound (quinones were dissolved in ethanol, final concentration 2%, w/v; glutathionyl conjugates were dissolved in 0.1 M-potassium phosphate buffer, pH 7.4) and 200 μM-NADPH in 0.25 M-sucrose/0.1 M-potassium phosphate buffer, pH 7.55; the reaction was started by the addition of 45.2 ng of DT-diaphorase/ml (suspended in 0.1% bovine serum albumin before dilution to maintain its catalytic activity) to the reaction mixture. Assay temperature was 37 °C.

Anaerobic conditions were established by purging all reagents and buffers with high-purity Ar for more than 5 min. Reactions were carried out in capped cuvettes with a stream of Ar gas gently bubbling the surface of the mixture throughout the experiment. The cuvette holder was equipped with an electronic stirrer (model 200; Rank Bros., Cambridge, U.K.) to ensure a homogeneous gas distribution in the reaction mixture.

Spectrophotometric assays

NADPH oxidation was monitored at 340 nm (ϵ 6.22 $mM^{-1} \cdot cm^{-1}$) with a Varian DMS 100 u.v.–visible spectrophotometer (Varian AB, Solna, Sweden). H_2O_2 formation was determined fluorimetrically coupled to either *p*-hydroxyphenylacetic acid dimerization ($\lambda_{exc.}$ 315 nm; $\lambda_{em.}$ 410 nm) (Danner *et al.*, 1973; Hinsberg *et al.*, 1983) or scopoletin oxidation ($\lambda_{exc.}$ 360 nm; $\lambda_{em.}$ 460 nm) (Boveris, 1984). The two methods gave comparable results. Fluorescence was measured with a Shimadzu RF-540 spectrofluorimeter (Shimadzu Corporation, Kyoto, Japan). Partition coefficients between octan-1-ol and water were determined as described by Hodnett *et al.* (1983).

H.p.l.c. with electrochemical detection

Quinonoid compounds were analysed with electrochemical detection against an Ag/AgCl reference

Table 1. Retention times of substituted 1,4-naphthoquinones analysed by h.p.l.c. with electrochemical detection

Assay conditions were as described in the Materials and methods section.

1,4-Naphthoquinone substituents	Retention time (min)	
	35% propan-2-ol	35% methanol
None	6.8	
2-Hydroxy-	2.1	
5-Hydroxy-	6.3	
5-Hydroxy-2-methyl-	36.0	
2-Methyl-	31.0	
2,3-Dimethyl-	27.4	
2,3-Dimethoxy-	4.7	
3-Glutathionyl-		16.0
2,3-Diglutathionyl-		5.4
2-*N*-Acetylcysteinyl-		16.5
3-Glutathionyl-2-methyl-		38.0
3-*N*-Acetylcysteinyl-2-methyl-		26.0
3-Glutathionyl-5-hydroxy-		16.0
3-Glutathionyl-5-hydroxy-2-methyl-		46.0

electrode as previously described (Brunmark & Cadenas, 1987). Two isocratic mobile phases were utilized: (*a*) 35% (v/v) propan-2-ol/50 mM-sodium phosphate buffer, pH 6.5, and (*b*) 35% (v/v) methanol/0.2 mM-Tris/HCl buffer, pH 7.0. The former mobile phase was utilized for the analysis of un-, methyl-, methoxy- and hydroxy-substituted 1,4-naphthoquinones. The latter mobile phase was utilized for the determination of glutathionyl-substituted 1,4-naphthoquinone derivatives. The quinonoid compounds measured with either mobile phase as well as the observed retention times are listed in Table 1. Both mobile phases were filtered and degassed by vacuum and continuously purged with He in a closed system. Values for the half-wave reduction potential ($E_{\frac{1}{2}}$) of the 1,4-naphthoquinones studied were obtained from the respective hydrodynamic voltamograms.

RESULTS AND DISCUSSION

Effect of substituents on the half-wave reduction potentials of 1,4-naphthoquinones

The $E_{\frac{1}{2}}$ values of the 1,4-naphthoquinone derivatives studied were determined by means of hydrodynamic voltamograms against an Ag/AgCl reference electrode.

The introduction of methyl groups at C-2 and C-3 of 1,4-naphthoquinone is known to decrease the $E_{\frac{1}{2}}$ values in an additive manner (Zuman, 1967). It was similarly found here that the introduction of one methyl group at C-2 decreased the $E_{\frac{1}{2}}$ value by 45 mV (Table 2), and the introduction of two methyl groups at C-2 and C-3 decreased the $E_{\frac{1}{2}}$ value of the parent 1,4-naphthoquinone by about 120 mV (Brunmark *et al.*, 1988).

Table 2 illustrates the effects exerted by different substituents on the $E_{\frac{1}{2}}$ values of the two parent quinones studied here: 1,4-naphthoquinone and 2-methyl-1,4-naphthoquinone.

Hydroxy substitutents. The effect of hydroxy substituents on the $E_{\frac{1}{2}}$ values of 1,4-naphthoquinone or 2-methyl-1,4-naphthoquinone varied according to whether the hydroxy group was at C-2 (in the quinone ring) or C-5 (in the adjacent benzene ring).

In the former instance hydroxy substitution led to a marked decrease in the $E_{\frac{1}{2}}$ value of both 1,4-naphthoquinone and 2-methyl-1,4-naphthoquinone, by 280 and 275 mV respectively. A similar decrease upon hydroxy substitution of 1,4-naphthoquinone at C-2 was observed in the one-electron reduction potential [$E(Q/Q^{\cdot-})_7$] (calculated by pulse radiolysis) ($\Delta E^1_7 = -275$ mV) (data from Ian Wilson quoted in d'Arcy Doherty *et al.*, 1987) and in the half-wave reduction potential (calculated against a saturated calomel electrode) ($\Delta E_{\frac{1}{2}} = -280$ mV) (Hodnett *et al.*, 1983). In addition, the hydroxy substituent in the quinone ring raises the pK_a value with regard to the parent quinone [pK_a(1,4-naphthoquinone) 4.1; pK_a(2-hydroxy-1,4-naphthoquinone) 4.7] (Mukherjee, 1987).

Introduction of methoxy groups caused an effect similar to that of the hydroxy substituents vicinal to the carbonyl groups: the $E_{\frac{1}{2}}$ value of 2,3-dimethoxy-1,4-naphthoquinone was -300 mV, 120 mV more negative than that of the parent compound. A 119 mV more negative $E_{\frac{1}{2}}$ value (against saturated calomel electrode) was found with the monomethoxy derivative of 1,4-naphthoquinone in aqueous solutions (Hodnett *et al.*, 1983). The decrease in the $E_{\frac{1}{2}}$ value is interpreted as mesomeric or inductive effects introduced by the hydroxy or the methoxy substituents (Flaig *et al.*, 1968).

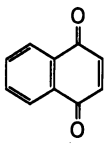
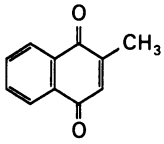
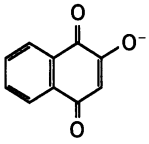
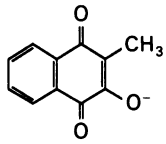
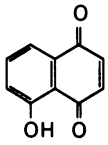
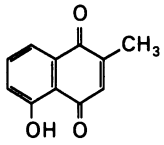
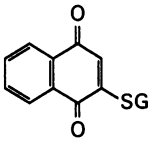
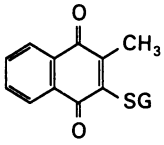
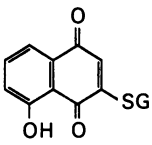
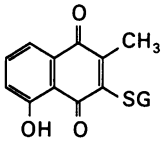
In the latter instance hydroxy substitution in the adjacent benzene ring caused an increase in the $E_{\frac{1}{2}}$ values of both 1,4-naphthoquinone and 2-methyl-1,4-naphthoquinone, by 40 and 25 mV respectively. This is in agreement with previous reports on the first and second electron reduction potentials (calculated by cyclic voltammetry) of 5-hydroxy-1,4-naphthoquinone in a non-polar medium (Ashnagar *et al.*, 1984) and in aqueous medium (Mukherjee, 1987). The increase in $E_{\frac{1}{2}}$ value is interpreted as a polar effect caused by the hydroxy group in the adjacent benzene ring. In contrast with 2-hydroxy-1,4-naphthoquinone, a hydroxy substituent in the benzene ring lowers the pK_a value with respect to the parent compound [pK_a(1,4-naphthoquinone) 4.1; pK_a(5-hydroxy-1,4-naphthoquinone) 3.65] (Mukherjee, 1987).

Hydroxyquinones tend to form strong intramolecular hydrogen-bonding, which leads to stabilization of the semiquinone transient species involving displacement over to the left of the disproportionation reaction $2\,Q^{\cdot-} \rightleftharpoons Q + Q^{2-}$ (Dodd & Mukherjee, 1984; Ashnagar *et al.*, 1984).

Glutathionyl substituents. Of the 1,4-naphthoquinones examined in this study, four were able to undergo 1,4-reductive addition with thiols such as GSH: 1,4-naphthoquinone (illustrated in reaction 2) and 2-methyl-1,4-naphthoquinone and their respective 5-hydroxy derivatives. Glutathionyl substitution of 1,4-naphthoquinone additively decreased the $E_{\frac{1}{2}}$ value from -180 mV to -225 mV for the monoconjugate 3-glutathionyl-1,4-naphthoquinone (Table 2) and to -265 mV for the diconjugate 2,3-diglutathionyl-1,4-naphthoqinone (not shown). The glutathionyl substitution of 2-methyl-1,4-naphthoquinone decreased the $E_{\frac{1}{2}}$ value by 40 mV. The nucleophilic addition of *N*-acetyl-L-cysteine to 1,4-naphthoquinone and 2-methyl-1,4-naphthoquinone resulted in more pronounced decreases in the $E_{\frac{1}{2}}$ value (75

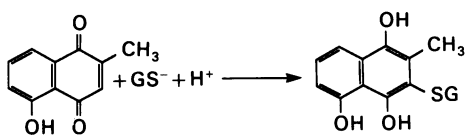
Table 2. Effect of substitution on $E_{\frac{1}{2}}$ of 1,4-naphthoquinones

Values were obtained against an Ag/AgCl reference electrode.

	$E_{\frac{1}{2}}$ (mV)	$E_{\frac{1}{2}}$ (mV)
	−180	 −225
Hydroxy-substituted in quinone ring		
	−460	 −500
Hydroxy-substituted in benzene ring		
	−140	 −200
Glutathionyl-substituted		
	−225	 −265
Hydroxy- and glutathionyl-substituted		
	−195	 −220

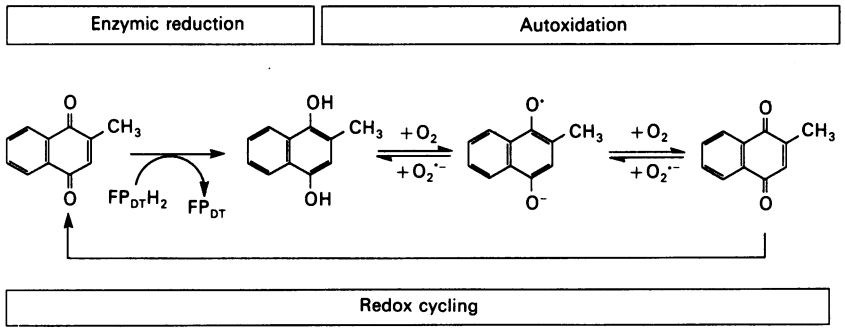
and 85 mV respectively; not shown) than those with GSH. The decrease in $E_{\frac{1}{2}}$ value caused by the thiol addition could be interpreted as a mesomeric effect similar to that produced by hydroxy and methoxy substituents. Small changes in the one-electron reduction potential [$(E(Q/Q^{\cdot-})_7$] of 2-methyl-1,4-naphthoquinone [$E(Q/Q^{\cdot-})_7 = -203$ mV] were observed upon GSH addition [$E(GS\text{-}Q/GS\text{-}Q^{\cdot-})_7 = -192$ mV] ($\Delta E^1_7 = -11$ mV) (Wilson *et al.*, 1986), in agreement with the relatively weak electron-withdrawing properties of thioether substituents, which have Hammett constants typically of the order $\sigma_p \approx 0$ and $\sigma_m \approx 0.1$–0.2 (Wilson *et al.*, 1986).

Hydroxy and glutathionyl substituents. The nucleophilic addition of GSH to the 5-hydroxy derivatives of 1,4-naphthoquinone and 2-methyl-1,4-naphthoquinone (reaction 3):



(3)

resulted in compounds with $E_{\frac{1}{2}}$ values similar to that of 1,4-naphthoquinone and 2-methyl-1,4-naphthoquinone, the difference amounting to −15 and +5 mV respect-



Scheme 1. Scheme illustrating the two-electron reduction of menadione by DT-diaphorase followed by autoxidation of the hydroquinone and re-reduction of the autoxidation product by the enzyme

Abbreviation: FP_{DT}, DT-diaphorase flavoprotein.

ively. This may be interpreted as the counterbalance of the rise in $E_{\frac{1}{2}}$ value caused by the hydroxy substituent at C-5 (see above) and the decrease in $E_{\frac{1}{2}}$ value caused by the glutathionyl substituent.

The electron-donating properties of the hydroxy substituent in the quinone ring increases the electron density at C-3, thereby preventing the nucleophilic addition of GSH to 2-hydroxy-1,4-naphthoquinone. However, 3-glutathionyl-2-hydroxy-1,4-naphthoquinone was obtained during the cleavage of the epoxide ring of 2,3-epoxy-1,4-naphthoquinone upon nucleophilic addition of GSH.

DT-diaphorase-catalysed reduction of substituted 1,4-naphthoquinones under anaerobic conditions

The two-electron reduction of quinones catalysed by the flavoprotein DT-diaphorase takes place within a ping-pong-type mechanism (Hosoda *et al.*, 1974). Initially, a hydride transfer occurs from either NADPH or NADH to the oxidized flavoprotein, yielding the reduced form of the flavin; electron transfer from the reduced flavin to the quinone acceptor is formally thought to take place in two one-electron transfer steps; the semiquinone radical, which would be expected as intermediate, may not be easily removed from the active site of the enzyme and will be reduced to its hydroquinone by a second one-electron transfer reaction (Iyanagi, 1987). This is supported by the fact that a rigid complex is formed between the enzyme and the quinone (Hosoda *et al.*, 1974), thereby immobilizing the semiquinone species at the active site.

The time course of NADPH oxidation linked to the DT-diaphorase-catalysed reduction of quinones under aerobic systems involves two processes: on the one hand, the initial reduction of the quinone by DT-diaphorase, and, on the other, the autoxidation of the hydroquinone product, which, in turn, can be re-reduced by the enzyme, thereby closing a cycle of enzymic reduction $\rightleftharpoons$ autoxidation, as illustrated in Scheme 1.

Of note, the autoxidation reactions involving the sequence $QH_2 \rightleftharpoons Q^{\cdot-} \rightleftharpoons Q$ are written as reversible processes, for their equilibrium is determined by the innate properties of the quinonoid compound and environmental factors.

As hydroquinones have the potential to undergo autoxidation and thus again become suitable substrates for DT-diaphorase within a system of cycling characteristics described in Scheme 1, the actual rate of NADPH oxidation was determined under anaerobic conditions, thereby preventing autoxidation reactions.

Effect of methyl and methoxy substituents. The introduction of a methyl group to 1,4-naphthoquinone increased the specific activity of DT-diaphorase by approx. 85% from 1252 to 2323 nmol of NADPH oxidized/min per μg of DT-diaphorase (Table 3). 2,3-Dimethyl substitution of 1,4-naphthoquinone did not change the specific activity, and 2,3-dimethoxy

Table 3. NADPH oxidation during the DT-diaphorase-catalysed reduction of substituted 1,4-naphthoquinones and partition coefficients

Assay conditions were as described in the Materials and methods section.

1,4-Naphthoquinone	NADPH oxidation (nmol/min per μg of DT-diaphorase)	Partition coefficient (log *P*)
1,4-Naphthoquinone series		
1,4-Naphthoquinone	1252	1.76
3-Glutathionyl-1,4-naphthoquinone	907	
5-Hydroxy-1,4-naphthoquinone	896	1.80
3-Glutathionyl-5-hydroxy-1,4-naphthoquinone	310	
2,3-Diglutathionyl-1,4-naphthoquinone	155	
2-Hydroxy-1,4-naphthoquinone	13	
2-Methyl-1,4-naphthoquinone series		
2-Methyl-1,4-naphthoquinone	2323	2.24
5-Hydroxy-2-methyl-1,4-naphthoquinone	1312	2.53
3-Glutathionyl-2-methyl-1,4-naphthoquinone	1208	
3-Glutathionyl-5-hydroxy-2-methyl-1,4-naphthoquinone	619	
3-*N*-Acetyl-L-cysteinyl-2-methyl-1,4-naphthoquinone	518	
2,3-Dimethyl-1,4-naphthoquinone	1252	2.27
2,3-Dimethoxy-1,4-naphthoquinone	1075	1.67

substitution decreased the rate of enzymic reduction by approx. 15%. Because the latter compounds possess similar $E_{\frac{1}{2}}$ values (−300 mV), it seems reasonable to conclude that the rate of their reduction by DT-diaphorase is not determined primarily by the redox potential of the quinone.

Effect of hydroxy substituents. Under anaerobic conditions the rate of reduction of hydroxyquinones (in terms of NADPH oxidation) by DT-diaphorase varied whether the hydroxy substituent was at C-2 or C-5 (Table 3). Substitution of 1,4-naphthoquinone at C-2 (2-hydroxy-1,4-naphthoquinone or lawsone) dramatically decreased the specific activity of DT-diaphorase to approx. 1% (13 nmol of NADPH oxidized/min per μg of DT-diaphorase) of that of the parent quinone (1252 nmol of NADPH oxidized/min per μg of DT-diaphorase), whereas hydroxy substitution at C-5 (i.e. 5-hydroxy-1,4-naphthoquinone or juglone) resulted in a decrease in specific activity of approx. 29%: 896 nmol of NADPH oxidized/min per μg of DT-diaphorase. Introduction of a hydroxy group at C-5 in 2-methyl-1,4-naphthoquinone (5-hydroxy-2-methyl-1,4-naphthoquinone or plumbagin) decreased the specific activity in comparison with the parent quinone by approx. 44%: 1312 nmol of NADPH/min per μg of DT-diaphorase.

Effect of glutathionyl substitution. Another aspect of certain quinonoid compounds is their ability to undergo thiol-mediated 1,4-reductive addition with biologically relevant molecules such as GSH (reaction 2) (Finley, 1974). Here we have examined the possibility that thiol–quinone conjugates could also act as substrates for DT-diaphorase, which upon reduction of the conjugate would close a redox cycle of enzymic reduction $\rightleftharpoons$ autoxidation.

In all cases glutathionyl conjugates were reduced by DT-diaphorase at the expense of reducing equivalents from NADPH (Table 3). The initial rate of NADPH oxidation for 3-glutathionyl-1,4-naphthoquinone was decreased to 907 nmol of NADPH oxidized/min per μg of DT-diaphorase compared with the unconjugated quinone (1252 nmol of NADPH oxidized/min per μg of DT-diaphorase), and diglutathionyl conjugation further decreased the initial rate to approx. 12% of the parent quinone (155 nmol of NADPH oxidized/min per μg of DT-diaphorase). Glutathionyl substitution of 2-methyl-1,4-naphthoquinone resulted in an approx. 48% decrease in specific activity from 2323 to 1208 nmol of NADPH oxidized/min per μg of DT-diaphorase, and conjugation with *N*-acetyl-L-cysteine decreased activity by approx. 77%. This lower activity could be rationalized in terms of steric hindrance at the active site of the enzyme created by the bulky glutathionyl substituent. Although the rate of reduction of glutathionyl-quinone conjugates was in every case lower than that of the parent compounds, these results clearly show that the enzyme is capable of reducing the glutathionyl-quinone conjugates efficiently.

Effect of glutathionyl and hydroxy substitution. Glutathionyl and hydroxy substitution of 1,4-naphthoquinone and 2-methyl-1,4-naphthoquinone (i.e. 3-glutathionyl-5-hydroxy-1,4-naphthoquinone and 3-glutathionyl-5-hydroxy-2-methyl-1,4-naphthoquinone; reaction 3) decreased the specific activity to 310 and 619 nmol of NADPH oxidized/min per μg of DT-diaphorase respectively (Table 3) of the parent quinones (1252 and 2323 nmol of NADPH oxidized/min per μg of DT-diaphorase respectively).

Partition coefficients. At variance with a previous report by Powis & Appel (1980), in which the activity of one-electron transfer flavoproteins correlated with the reduction potential of the electron acceptor, our results do not support such a correlation. Another factor that might influence the rate of reduction of quinones by DT-diaphorase is a measure of the hydrophobicity provided that the active site of the enzyme is lined by hydrophobic groups.

The partition coefficients determined for 1,4-naphthoquinone and 2-methyl-1,4-naphthoquinone were nearly identical with those reported in the literature (Hodnett *et al.*, 1983) and are listed in Table 3. Methyl and 2,3-dimethyl substitution of 1,4-naphthoquinone significantly increased the partition coefficient by 0.5 log unit, whereas 2,3-dimethoxy substitution slightly decreased the coefficient. Substitution of 1,4-naphthoquinone with a hydroxy group at C-2 (2-hydroxy-1,4-naphthoquinone) significantly decreased the partition coefficient, whereas substitution at C-5 (5-hydroxy-1,4-naphthoquinone) resulted in a slight increase. Hydroxy substitution of 2-methyl-1,4-naphthoquinone at C-5 (5-hydroxy-2-methyl-1,4-naphthoquinone) increased the partition coefficient by 0.3 log unit. Comparison of the partition coefficients with the initial rates of DT-diaphorase-catalysed reduction of these quinones showed no direct correlation between the two parameters (Table 3).

Autoxidation following the reduction of substituted 1,4-naphthoquinones by DT-diaphorase

In the absence of O_2 NADPH oxidation was stoichiometrically related to the concentration of quinone present ([NADPH] oxidized/[quinone] reduced ~ 1), after which NADPH oxidation was halted (not shown). In the presence of O_2 semiquinones and hydroquinones have the potential to undergo non-enzymic autoxidation, and thus again become suitable substrates for DT-diaphorase within a system of cycling characteristics and expressed as ([NADPH] oxidized/[quinone] reduced > 1) (Scheme 1). Initial rates of NADPH oxidation under aerobic conditions (not shown) were slightly faster than those under anaerobic conditions (Table 3), indicative of the participation of redox cycling processes. Figs. 1–3 illustrate the time courses of NADPH oxidation and H_2O_2 formation for 1,4-naphthoquinone and 2-methyl-1,4-naphthoquinone (menadione) and their respective hydroxy, glutathionyl and glutathionyl hydroxy adducts under aerobic conditions.

The rates of autoxidation were defined as the rate of NADPH oxidation or the rate of H_2O_2 formation 1 min after the addition of DT-diaphorase, as distinct from the initial rates, and are listed in Table 4.

Effect of methyl substitution. Low rates of both NADPH oxidation and H_2O_2 formation were found for 1,4-naphthoquinone and 2-methyl-1,4-naphthoquinone (66 and 44 nmol of NADPH oxidized/min per μg of DT-diaphorase respectively) (Table 4). The initial rates of NADPH oxidation of 2,3-dimethyl-1,4-naphthoquinone and 2,3-dimethoxy-1,4-naphthoquinone differed substantially (44 and 885 nmol of NADPH oxidized/min

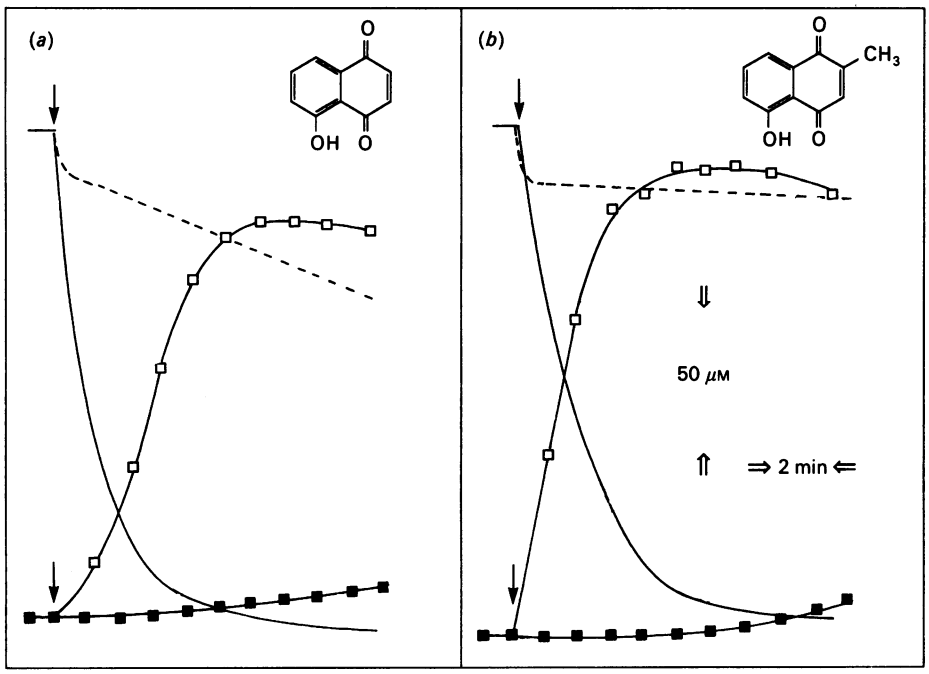


Fig. 1. Effect of hydroxy substituents on the rate of enzymic reduction by DT-diaphorase of 1,4-naphthoquinone and 2-methyl-1,4-naphthoquinone and the rate of hydroquinone autoxidation

Assay conditions: 20 μM-quinonoid compound and 200 μM-NADPH in 0.25 M- or 0.1 M-potassium phosphate buffer, pH 7.55, were supplemented with 45.2 ng of DT-diaphorase/ml to initiate the reaction (indicated by the arrow). (*a*) Time courses of NADPH oxidation (---- and ——) and H_2O_2 formation (■ and □) for 1,4-naphthoquinone and 5-hydroxy-1,4-naphthoquinone respectively. (*b*) Time courses of NADPH oxidation (---- and ——) and H_2O_2 formation (■ and □) for 2-methyl-1,4-naphthoquinone and 5-hydroxy-2-methyl-1,4-naphthoquinone respectively.

per μg of DT-diaphorase respectively) (Table 4), whereas the $E_{\frac{1}{2}}$ values of both quinonoid compounds were similar (−300 mV). This might be attributed to the mesomeric effect of the electron-donating properties of methoxy groups, which would contribute to an enhanced autoxidation.

Effect of hydroxy substitution. Substitution of a hydroxy group at C-5 (5-hydroxy-1,4-naphthoquinone and 5-hydroxy-2-methyl-1,4-naphthoquinone) dramatically increased the rates of NADPH oxidation (24- and 31-fold higher than the parent compounds lacking a hydroxy substituent respectively), indicative of rapid redox cycling processes (Figs. 1*a* and 1*b*). Substitution of a hydroxy group at C-2 (2-hydroxy-1,4-naphthoquinone) showed little difference from the parent quinone (Table 4). The $E_{\frac{1}{2}}$ value of 2-hydroxy-1,4-naphthoquinone (−460 mV) indicates that it should autoxidize very rapidly, though in this case the rate-limiting step is likely to be the reduction of the quinone by DT-diaphorase, though the redox potential of this flavin remains to be determined.

On the assumption that DT-diaphorase is capable of reducing all the quinonoid compounds studied to their respective hydroquinones, one would expect that the more negative the $E_{\frac{1}{2}}$ value of the quinone the more rapid the electron transfer from the hydroquinone to molecular O_2. On this basis the present autoxidation data are not consistent with this view, for 5-hydroxy-1,4-naphthoquinone and 5-hydroxy-2-methyl-1,4-naphthoquinone, which have more positive $E_{\frac{1}{2}}$ values than their parent compounds, autoxidize at far higher rates. The common substituent in these two quinones is a hydroxy group at C-5. According to Ashnagar *et al.* (1984) and Mukherjee (1987), substantial stabilization of the semiquinone forms of 5-hydroxy-1,4-naphthoquinone and 5-hydroxy-2-methyl-1,4-naphthoquinone results from hydrogen-bonding between the hydroxy group at C-5 and the adjacent carbonyl group (–OH···O=C<). As outlined above, the reduction of quinones by DT-diaphorase takes place by a ping-pong-type mechanism, where the semiquinone species is retained in a 'rigid' enzyme–quinone complex and is released as hydroquinone after a second one-electron transfer reaction (Hosoda *et al.*, 1974; Iyanagi, 1987). As it is not known under what conditions the semiquinone intermediate can be retained in the active site of DT-diaphorase, 5-hydroxy-1,4-naphthoquinone and 5-hydroxy-2-methyl-1,4-naphthoquinone, after the first one-electron reduction to more stable semiquinones, may be 'prematurely' released from the active site before the second successive electron transfer takes place.

Effect of glutathionyl substitution. With respect to the relatively low rate of autoxidation of 1,4-naphthoquinone and 2-methyl-1,4-naphthoquinone, their monoglutathionyl conjugates (3-glutathionyl-1,4-naphthoquinone and 3-glutathionyl-2-methyl-1,4-naphthoquinone) gained a striking ability to redox cycle, resulting in the depletion of NADPH within 10–12 min (Figs. 2*a* and 2*b*). This demonstrates the participation of glutathionyl conjugates in an enzymic reduction ⇌ autoxidation redox cycle mediated by DT-diaphorase. The redox cycling was mirrored by the large amount of

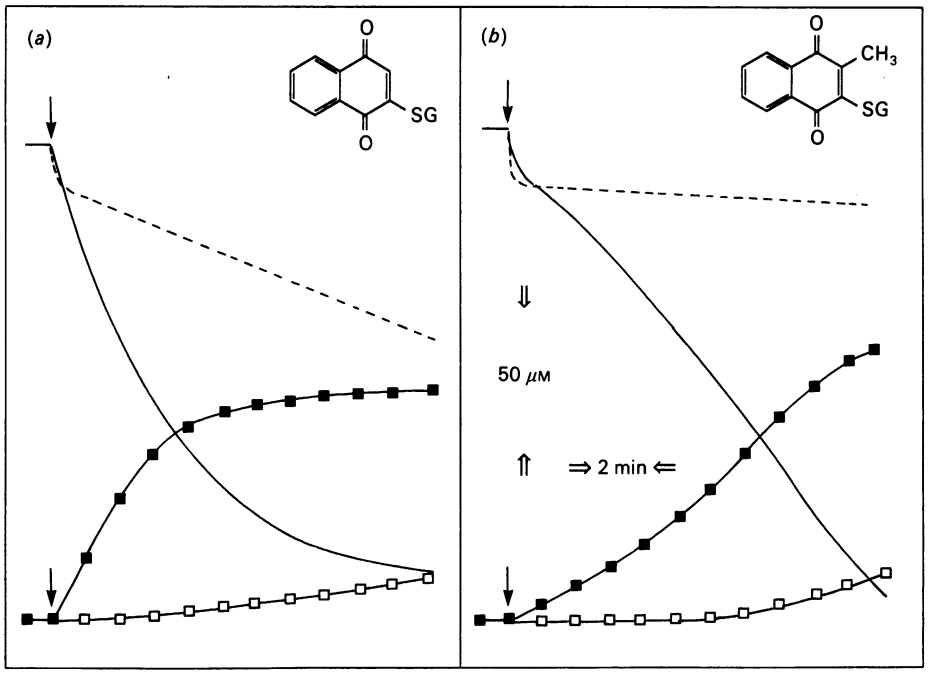


Fig. 2. Effect of glutathionyl substitution on the rate of enzymic reduction by DT-diaphorase of 1,4-naphthoquinone and 2-methyl-1,4-naphthoquinone and the rate of hydroquinone autoxidation

Assay conditions were as described in Fig. 1 legend and the Materials and methods section. The arrow indicates the addition of 45.2 ng of DT-diaphorase/ml to initiate the reaction. (*a*) Time courses of NADPH oxidation (---- and ——) and H_2O_2 formation (□ and ■) for 1,4-naphthoquinone and 3-glutathionyl-1,4-naphthoquinone respectively. (*b*) Time courses of NADPH oxidation (---- and ——) and H_2O_2 formation (□ and ■) for 2-methyl-1,4-naphthoquinone and 3-glutathionyl-2-methyl-1,4-naphthoquinone respectively.

H_2O_2 formed. Diglutathionyl conjugation of 1,4-naphthoquinone resulted in almost complete lack of autoxidation (Table 4), probably due to the inefficient reduction of this compound by the enzyme (Table 3). It is worth noting that the electrochemical properties of the glutathionyl derivatives differ very little from those of the parent quinones (Wilson *et al.*, 1986); however, their rate of autoxidation (following reduction by DT-diaphorase) is 6–12-fold higher than that for the parent quinones. It is to be assumed that factors other than the reduction potential are controlling the extent of autoxidation of glutathionyl-hydroquinone conjugates.

Effect of glutathionyl and hydroxy substitution. Glutathionyl and hydroxy (at C-5) substitution also increased the rates of autoxidation but not in an additive manner (Fig. 3). Although the autoxidation rate for 3-glutathionyl-5-hydroxy-1,4-naphthoquinone was approx. 5-fold greater than that for 1,4-naphthoquinone (Fig. 3*a*) and the autoxidation rate for 3-glutathionyl-5-hydroxy-2-methyl-1,4-naphthoquinone was approx. 10-fold greater than that for 2-methyl-1,4-naphthoquinone (Fig. 3*b*), they were substantially lower than the respective rates for 5-hydroxy-1,4-naphthoquinone (Fig. 1*a*) and 5-hydroxy-2-methyl-1,4-naphthoquinone (Fig. 1*b* and Table 4).

The situation with the glutathionyl conjugates with hydroxy substitution at C-5 is somewhat more complicated. In contrast with the monoglutathionyl derivatives of 1,4-naphthoquinone and 2-methyl-1,4-naphthoquinone, the autoxidation of the glutathionyl derivatives of 5-hydroxy-1,4-naphthoquinone and 5-hydroxy-2-methyl-1,4-naphthoquinone was decreased to values similar to those for derivatives without hydroxy substituent at C-5. The possible implications for the involvement of stabilization of the semiquinone form by hydrogen-bonding between the hydroxy group at C-5 and the adjacent carbonyl group as discussed above may or may not be applicable for these derivatives bearing a glutathionyl substituent. Alternatively, glutathionyl substitution of these quinones may decrease the specific activity by steric hindrance at the active site.

The effect of substituents on the hydroquinone autoxidation can be summarized for both series, 1,4-naphthoquinone and 2-methyl-1,4-naphthoquinone, as follows: (*a*) 1,4-naphthoquinone series: 5-hydroxy-1,4-naphthoquinone > 3-glutathionyl-1,4-naphthoquinone > 3-glutathionyl-5-hydroxy-1,4-naphthoquinone > 1,4-naphthoquinone > 2-hydroxy-1,4-naphthoquinone; (*b*) 2-methyl-1,4-naphthoquinone series: 5-hydroxy-2-methyl-1,4-naphthoquinone > 3-glutathionyl-5-hydroxy-2-methyl-1,4-naphthoquinone > 3-glutathionyl-2-methyl-1,4-naphthoquinone > 2-methyl-1,4-naphthoquinone > 3-hydroxy-2-methyl-1,4-naphthoquinone (values for the last compound obtained by A. Brunmark, unpublished work).

It is to be noted that there is a lack of stoichiometry between the rates of NADPH oxidation and H_2O_2 formation during hydroquinone autoxidation. At present we cannot provide an explanation for this discrepancy, though we have considered the following possibilities: (*a*) an additional NADPH oxidation by a mechanism other than that illustrated in Scheme 1; (*b*) an underestimation of the H_2O_2 values measured.

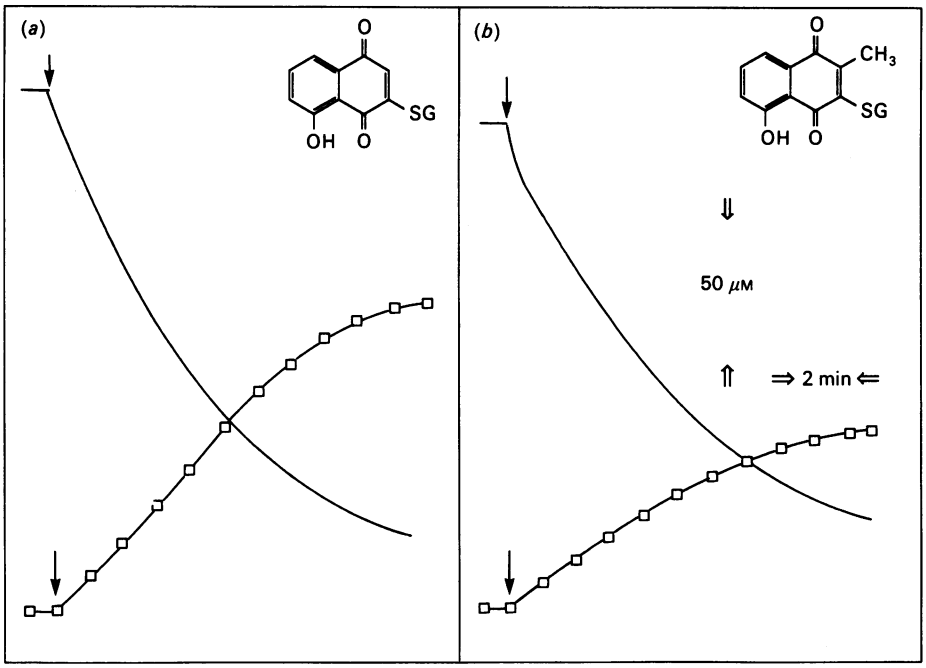


Fig. 3. Effect of glutathionyl and hydroxy substitution on the rate of enzymic reduction of 1,4-naphthoquinone and 2-methyl-1,4-naphthoquinone and the rate of hydroquinone autoxidation

Assay conditions were as described in Fig. 1 legend and in the Materials and methods section. The arrow indicates the addition of 45.2 ng of DT-diaphorase/ml to initiate the reaction. Time courses of NADPH oxidation (——) and H_2O_2 formation (□) respectively for (*a*) 3-glutathionyl-5-hydroxy-1,4-naphthoquinone and (*b*) 3-glutathionyl-5-hydroxy-2-methyl-1,4-naphthoquinone. Compare with traces for the unsubstituted parent compounds in Figs. 1 and 2.

Table 4. Autoxidation of 1,4-naphthohydroquinone derivatives after the reduction of the oxidized counterpart by DT-diaphorase

Assay conditions were as described in the Materials and methods section. Abbreviation: N.D., not detected.

	NADPH oxidation (nmol/min per μg of DT-diaphorase)	H_2O_2 formation (nmol/min of DT-diaphorase)
1,4-Naphthoquinone series		
2,3-Diglutathionyl-1,4-naphthoquinone	22	N.D.
2-Hydroxy-1,4-naphthoquinone	58	6
1,4-Naphthoquinone	66	22
3-Glutathionyl-5-hydroxy-1,4-naphthoquinone	354	111
3-Glutathionyl-1,4-naphthoquinone	774	420
5-Hydroxy-1,4-naphthoquinone	1548	1172
2-Methyl-1,4-naphthoquinone series		
2-Methyl-1,4-naphthoquinone	44	22
3-*N*-Acetyl-L-cysteinyl-2-methyl-1,4-naphthoquinone	44	N.D.
3-Glutathionyl-2-methyl-1,4-naphthoquinone	265	199
3-Glutathionyl-2-methyl-5-hydroxy-1,4-naphthoquinone	531	199
5-Hydroxy-2-methyl-1,4-naphthoquinone	1349	1084
2,3-Dimethyl-1,4-naphthoquinone	44	22
2,3-Dimethoxy-1,4-naphthoquinone	885	80

(*a*) The former process could be discussed in terms of a non-enzymic oxidation of NADPH by either quinones or reactive species generated during autoxidation. Hydride transfer between NADH and substituted 1,4-benzoquinones has been described to proceed with rate constants of 0.23 to 79 $M^{-1} \cdot s^{-1}$, being higher for those quinones bearing substituents with electron-withdrawing properties than for those with electron-donating properties (Carlson & Miller, 1985). These rates are negligible and unlikely to contribute to NADPH oxidation; accordingly, controls carried out in the absence of DT-diaphorase showed no oxidation of NADPH by 1,4-naphthoquinones (results not shown). NADPH may be oxidized by $O_2^{\cdot -}$ provided that the hydroquinones formed autoxidize in one-electron transfer steps. This consideration seems also unlikely because, on the one hand, the rate of reaction of NADH with $O_2^{\cdot -}$ is slow (27 $M^{-1} \cdot s^{-1}$; Land & Swallow, 1971) and, on the other, the product of the reaction is H_2O_2 ($NADPH + H^+ + O_2^{\cdot -} \rightarrow NADP^+ + H_2O_2$). In control experiments 100 μM-H_2O_2 did not oxidize NADPH (results not shown).

(*b*) The latter process, that is a possible underestimation of the rates of H_2O_2 formation measured, could be due to a decomposition of H_2O_2 by contaminating transition-metal ions via a Fenton-type reaction. However, the inclusion of 100 μM-EDTA in reaction mixtures either increased slightly or had no effect on H_2O_2 formation (results not shown). Further, any interference of the naphthoquinones or NADPH on the method used to detect H_2O_2 can be excluded, for their initial concentration in the assay mixture for H_2O_2, after sampling and dilution, was 10–100-fold lower than that of *p*-

hydroxyphenylacetic acid (see the Materials and methods section).

Another observation was that the time course of H_2O_2 formation exhibited a lag phase, the duration of which varied with the naphthoquinone tested. However, we could not find a correlation between the duration of the lag phase and the physicochemical properties of the quinone, on the one hand, and its reduction by DT-diaphorase, on the other. The occurrence of a lag phase preceding H_2O_2 formation might indicate that reactions other than those involved in enzymic reduction $\rightleftharpoons$ autoxidation (Scheme 1) are also operative, but as yet remain undefined.

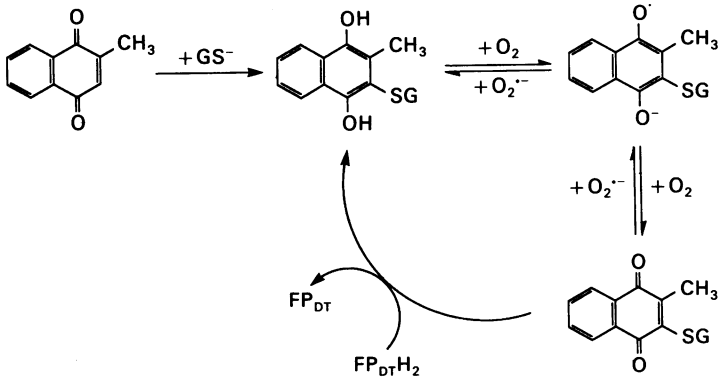
CONCLUSIONS AND COMMENTS

The present research permits us to draw the following conclusions. (*a*) In addition to the conventional quinone substrates (Ernster, 1987), DT-diaphorase reduces naphthoquinones bearing hydroxy, glutathionyl, methoxy and both hydroxy and glutathionyl substituents (the present work) as well as quinone epoxides (Brunmark *et al.*, 1987, 1988). (*b*) The rate of reduction by DT-diaphorase of hydroxy- (at C-5) and/or glutathionyl-substituted 1,4-naphthoquinones proceeds at considerable rates, though lower than those of the unsubstituted parent quinones. (*c*) The specific activity of DT-diaphorase does not correlate with either the $E_{\frac{1}{2}}$ value or the partition coefficient of the unsubstituted or substituted quinones studied here. (*d*) The DT-diaphorase-reduced unsubstituted and substituted naphthoquinones undergo autoxidation, the extent of which does not correlate with the $E_{\frac{1}{2}}$ values of the quinones either. The rates of autoxidation of hydroxy- and glutathionyl-substituted naphthoquinones are 6–31-fold higher than those of the unsubstituted parent compounds, depending on the position and number of substituents. This is unexpected, for the changes in the electrochemical properties of the quinones exerted by hydroxy substituents at C-5 or by glutathionyl substituents at C-2 or C-3 are not large enough to explain these substantially higher autoxidation rates.

In agreement with the data presented here, the mechanism of toxicity to isolated rat hepatocytes of 5-hydroxy-1,4-naphthoquinone and 2-hydroxy-1,4-naphthoquinone showed that the former was significantly more toxic than the latter (d'Arcy Doherty *et al.*, 1987). This could be accounted for by the ability of 5-hydroxy-1,4-naphthoquinone to redox cycle and undergo nucleophilic addition with GSH, whereas 2-hydroxy-1,4-naphthoquinone, because of its very low reduction potential, would be a very poor substrate for reduction to a semiquinone by one-electron transfer flavoproteins or to a hydroquinone by DT-diaphorase (Table 3). The degree of hydrophobicity of quinones would govern the extent of intracellular partitioning in either the lipid membrane milieu or the aqueous cytosolic milieu, and therefore determine their metabolic routes. The localization of several one-electron transfer flavoproteins in the microsomal fraction would favour the formation of semiquinone species and subsequent autoxidation reactions with $O_2^{\cdot-}$ formation. However, the relative participation of NADPH–cytochrome *P*-450 reductase and DT-diaphorase in quinone reduction requires considerably more information with regard to substrate specificity and the $k_{cat.}$ parameters for these enzymes in order to elucidate their relative roles in quinone detoxification and disposition (Prough *et al.*, 1987). Of note, in our experimental model, cross-oxidation reactions, i.e. between glutathionyl-hydroquinones and quinones to yield the respective semiquinones, are not possible because synthesized glutathionyl-quinone adducts were used.

Glutathionyl conjugation of quinones, hence altering the polarity of the quinone, would preferentially distribute the conjugates in the aqueous cytosolic fraction, where approx. 90% of the DT-diaphorase is located (Ernster, 1987). As shown in studies with perfused rat liver, isolated hepatocytes and microsomal fractions (for a discussion see Wefers & Sies, 1986) and in the present work, the glutathionyl conjugates are able to participate in DT-diaphorase-catalysed reduction and autoxidation reactions as summarized in Scheme 2. However, it remains to be investigated whether glutathionyl conjugation affects the ability of the hydroquinone conjugates to participate in the enzymic processes involving further conjugation with glucuronide or sulphate, thus facilitating their excretion as water-soluble products, as is found with certain quinones (Sato *et al.*, 1962; Lind *et al.*, 1978).

Some support for a role of DT-diaphorase in the detoxication of quinones is provided by the observations



Scheme 2. Proposed mechanism for the GSH-mediated nucleophilic addition to quinones followed by autoxidation and redox cycling supported by DT-diaphorase

Abbreviation: FP_{DT}, DT-diaphorase flavoprotein.

that dicoumarol, an inhibitor of DT-diaphorase (Ernster, 1987), potentiates the toxicity of quinones to hepatocytes (Thor *et al.*, 1982; d'Arcy Doherty *et al.*, 1984; Wefers & Sies, 1986) and that induction of the enzyme by various agents protects cultured cells against quinone toxicity (Atallah *et al.*, 1987). A major determinant in, for example, 2-methyl-1,4-naphthoquinone or menadione cytotoxicity would be the rates at which it undergoes either two-electron reduction by DT-diaphorase or 1,4-reductive addition to GSH, which is present at intracellular concentrations of 1–10 mM. Ross *et al.* (1985) have shown that the conjugation of 2-methyl-1,4-naphthoquinone with GSH *in vitro* with a quinone/GSH molar ratio of 1:12.5 was half-maximal at about 1–2 min. Thus there would be two competing reaction pathways that govern the metabolic fate of the quinonoid compound: on the one hand, its rapid two-electron reduction by DT-diaphorase (reaction 1), and, on the other, the reductive 1,4-addition with GSH (reaction 2), the autoxidation product of this reaction being a substrate for DT-diaphorase (see Scheme 2). The former process would be restrained by both the poor aqueous partitioning of the quinone and lower concentrations of DT-diaphorase associated with intracellular membranes; the latter process would be favoured by the high ratio of thiol to quinone, though restrained by the poor partitioning of the quinone in the aqueous phase.

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