Free Rad. Res., Vol. 35, pp. 145–158
Reprints available directly from the publisher
Photocopying permitted by license only

Concerted Action of DT-Diaphorase and Superoxide Dismutase in Preventing Redox Cycling of Naphthoquinones: An Evaluation

REX MUNDAY*

AgResearch, Ruakura Agricultural Research Centre, Private Bag 3123, Hamilton, New Zealand

Accepted by Prof. B. Halliwell

(Received 19 September 2000; In revised form 6 December 2000)

It has been suggested that the enzymes DT-diaphorase and superoxide dismutase act in concert to prevent redox cycling of naphthoquinones and thus protect against the toxic effects of such substances. Little is known, however, about the scope of this process or the conditions necessary for its operation. In the presence of low levels of DT-diaphorase, 2-methyl-1,4-naphthoquinone was found to undergo redox cycling. This was very effectively inhibited by SOD, and in the presence of both enzymes the hydroquinone was maintained in the reduced form. The inhibitory effect of the enzyme combination was overcome, however, at high concentrations of the quinone, or by small increases in pH. Furthermore, redox cycling was re-established by addition of haemoproteins such as cytochrome c and methaemoglobin. DT-diaphorase and SOD strongly inhibited redox cycling of 2,3-dimethyl- and 2,3-dimethoxy-1,4-naphthoquinone, but not that of 2-hydroxy-, 5-hydroxy- or 2-amino-1,4-naphthoquinone. Inhibition of redox cycling by a combination of DT-diaphorase and SOD is therefore not applicable to all naphthoquinone derivatives, and when it does occur, it may be overwhelmed at high quinone concentrations, and it may not operate under slightly alkaline conditions or in the presence of tissue components capable of initiating hydroquinone autoxidation.

Keywords: Naphthoquinones, redox cycling, DT-diaphorase, superoxide dismutase, protection against toxicity

INTRODUCTION

Naphthoquinone derivatives are widely distributed in nature, and many substances of this type are found as secondary metabolites of plants and fungi.[1] Folk medicines containing naphthoquinones have been used for many years[2,3] and the effectiveness of certain naphthoquinone derivatives as parasiticides and as anti-cancer agents has recently been demonstrated.[4–6] Naphthoquinones are also toxic. *In vitro*, harmful effects have been demonstrated in a variety of cell types,[7,8] while haemolytic anaemia and/or renal tubular necrosis has been observed after administration of such substances to animals or humans.[6,9–13] There is evidence that the therapeutic effects of naphthoquinones, as well as

* Tel.: +64 78 385 138. Fax: +64 78 385 012. E-mail: rex.munday@agresearch.co.nz.

their *in vitro* and *in vivo* toxicity, may be due, at least in part, to their ability to undergo redox cycling in the presence of biological reducing agents with concomitant generation of "active oxygen" species.[14–16]

The reduction of naphthoquinones may proceed either by a one-electron or a two-electron process. One-electron reduction of a naphthoquinone yields the corresponding semiquinone. Semiquinones are very unstable substances that rapidly autoxidise, regenerating the quinone and forming "active oxygen" species. One-electron reduction of naphthoquinones is generally regarded as an activation reaction, leading inevitably to toxic change.[14–16] Two-electron reduction of naphthoquinones leads to the formation of hydroquinones. Hydroquinones are also very labile substances, which readily autoxidise with formation of "active oxygen" species.[15,17–19] Two-electron reduction may therefore also lead to activation. However, an alternative reaction of hydroquinones involves conjugation with sulphate or glucuronic acid, forming products which cannot undergo redox cycling and which are readily excreted in the urine, thus leading to detoxification.[20] Whether or not the two-electron reduction of a particular naphthoquinone leads to activation or detoxification therefore depends upon the relative rates of autoxidation and conjugation of the hydroquinone that is formed. It would be anticipated that detoxification would be promoted by factors that stabilise the naphthohydroquinone and prevent its autoxidation.

The primary mediator of the two-electron reduction of quinones in animals is the enzyme DT-diaphorase (NAD(P)H:[quinone acceptor] oxidoreductase, EC 1.6.99.2). At relatively low levels of activity, DT-diaphorase promotes the redox cycling of naphthoquinones *in vitro*. At higher levels, however, the enzyme inhibits the autoxidation of some, but not all, naphthohydroquinones, so that the rate of redox cycling is decreased.[21] It is also known[19,22–25] that the autoxidation of some naphthohydroquinones is inhibited by superoxide dismutase (SOD), and it has been suggested[15,23,26,27] that the complementary action of DT-diaphorase and SOD, by preventing redox cycling and maintaining the hydroquinone in the reduced form, could be important in quinone detoxification. Although this is a very plausible suggestion, the factors that control inhibition of hydroquinone autoxidation by these enzymes have not been determined, and its applicability to naphthoquinones of different structural types has not been explored.

The experiments described in the present report, the stabilisation of 2-methyl-1,4-naphthohydroquinone (menadiol, 2-MeNHQ) and the inhibition of redox cycling of 2-methyl-1,4-naphthoquinone (menadione, 2-MeNQ) by a combination of DT-diaphorase and SOD has been explored, and factors that may negate the effectiveness of these processes have been identified. Furthermore, the effect of the two enzymes on the rates of redox cycling of five other naphthoquinone derivatives, of differing structural types, has been investigated.

MATERIALS AND METHODS

2-MeNQ was purchased from Sigma and 2-hydroxy- and 5-hydroxy-1,4-naphthoquinone from Aldrich. These substances were recrystallised from ethanol before use. 2-MeNHQ was synthesised by the method of Fieser,[28] 2,3-dimethyl-1,4-naphthoquinone by the method of Jacobsen and Torssell[29] and 2,3-dimethoxy-1,4-naphthoquinone by the method of Fieser and Brown.[30]

Rat haemoglobin was purified as described previously.[31] DT-diaphorase was obtained from rat liver by the method of Lind *et al.*[32] and its activity determined using dichlorophenol indophenol as the electron donor.[33]

SOD, from bovine erythrocytes, was purchased from Sigma. Its specific activity, as determined by the method of McCord and Fridovich,[34] was 2,500 U/mg. NADH and CAT (from bovine liver) were Boehringer products.

All other reagents were purchased from Sigma. Oxygen uptake was measured on a Yellow Springs Instruments Biological Oxygen Monitor, Model 5300, at 25 °C. Except where stated, reactions were conducted in 0.05 M phosphate buffer, pH 7.4, containing 50 μM DTPA, 0.25 M sucrose and 1 mM NADH. The reaction was initiated by addition of 2-MeNHQ or quinone. 2-MeNHQ was added as a solution in a mixture of ethanol and 0.1 N hydrochloric acid (1:1, v/v), the solution being maintained in an atmosphere of nitrogen before use in order to prevent spontaneous oxidation. All quinones were added as solutions in ethanol.

RESULTS

Inhibition of 2-MeNHQ Autoxidation by DT-Diaphorase and SOD

Pure 2-MeNHQ autoxidised rapidly at pH 7.4. During the autoxidation of a solution containing 150 μmoles/l of the hydroquinone, 147 ± 5 μmoles/l of oxygen (mean ± S.E.M, $n = 5$), were consumed, with 71 ± 4 μmoles/l of oxygen being returned to solution after addition of catalase. In the presence of DT-diaphorase, the rate of autoxidation of the hydroquinone was decreased. The degree of inhibition of the autoxidation reaction increased with increasing concentration of the enzyme, with a maximum effect of 68% being recorded (Figure 1). In the presence of SOD, a lag phase in oxygen uptake was recorded, which was followed by a linear phase of oxygen consumption. The relation between the concentration of SOD and the rate of the linear phase of oxygen uptake is shown in Figure 1. With SOD, the maximum inhibition of autoxidation, at high levels of enzyme, was 76%. In the presence of both DT-diaphorase and SOD, autoxidation of the hydroquinone was very strongly inhibited, and even at relatively low levels of the enzymes the rate of autoxidation was decreased by more than 99% (Figure 1).

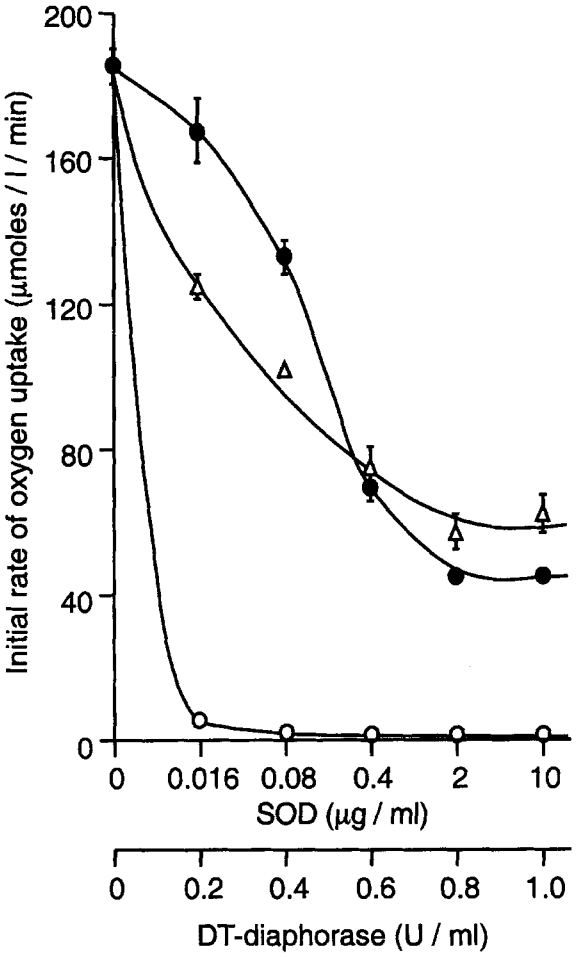

FIGURE 1 Effect of DT-diaphorase, alone and in combination, on the rate of autoxidation of 2-MeNHQ. Rates of autoxidation were measured as rates of oxygen uptake. Reactions were conducted at 25 °C in 0.05 M phosphate buffer containing 1 mM NADH, 50 μM DTPA and 0.25 M sucrose. The concentration of 2-MeNHQ was 150 μM. Results shown are the means of three separate determinations. Δ–Δ, DT-diaphorase alone; •–•, SOD alone; ○–○, DT-diaphorase and SOD together.

Redox Cycling by 2-MeNQ in the Presence of DT-Diaphorase

In the presence of NADH and a low concentration of DT-diaphorase (0.05 U/ml), 2-MeNQ underwent redox cycling, eventually consuming all the oxygen in the solution (Figure 2). With more enzyme (0.3 U/ml), the rate of oxygen uptake was higher, and all the oxygen in solution was again consumed. At 1 U/ml of DT-diaphorase,

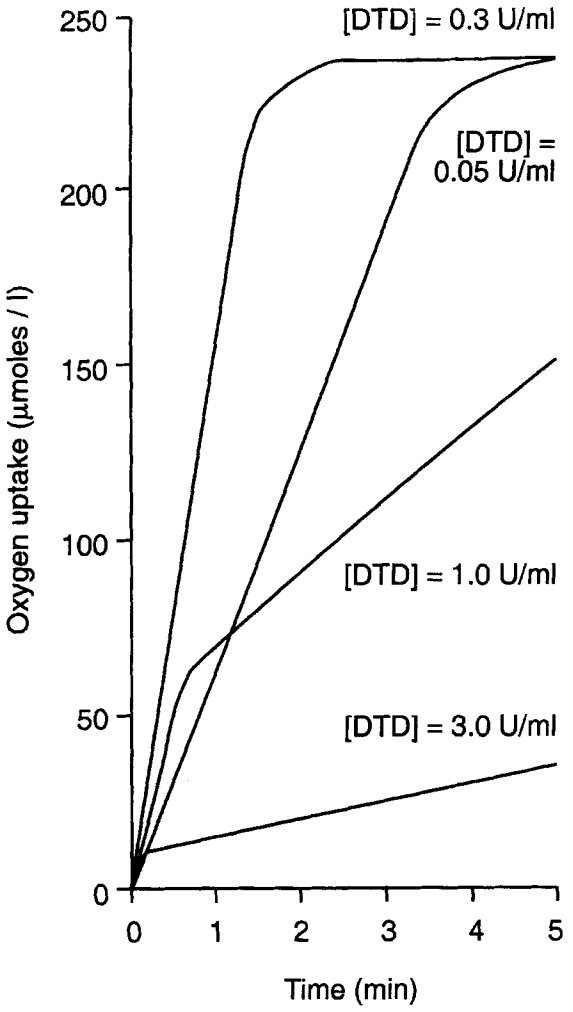

FIGURE 2 Redox cycling by 2-MeNQ in the presence of DT-diaphorase. Oxygen uptake was measured at 25 °C in 0.05 M phosphate buffer containing 1 mM NADH, 50 μM DTPA, 0.25 M sucrose and DT-diaphorase at the concentrations indicated. After equilibration, the reaction was initiated by addition of 2-MeNQ to a concentration of 150 μM.

however, the initial rate of oxygen uptake was lower than that at 0.3 U/ml, and after approximately 25% of the oxygen had been taken up, the rate abruptly declined, giving a slow, linear phase of oxygen uptake. At 3 U/ml, the inhibition of oxygen uptake was even more pronounced, with only a very brief period of rapid uptake, followed by a very slow phase of oxygen utilisation (Figure 2). This low rate continued unchanged for more than 25 minutes (data not shown).

Inhibition by SOD of Redox Cycling of 2-MeNQ in the Presence of DT-Diaphorase

The initial rate of oxygen uptake recorded with 2-MeNQ in the presence of NADH and 0.3 U/ml DT-diaphorase was decreased in the presence of SOD at a concentration of 0.01 μg/ml. Furthermore, after approximately 70% of the oxygen in solution had been consumed, the rate of oxygen consumption abruptly slowed (Figure 3).

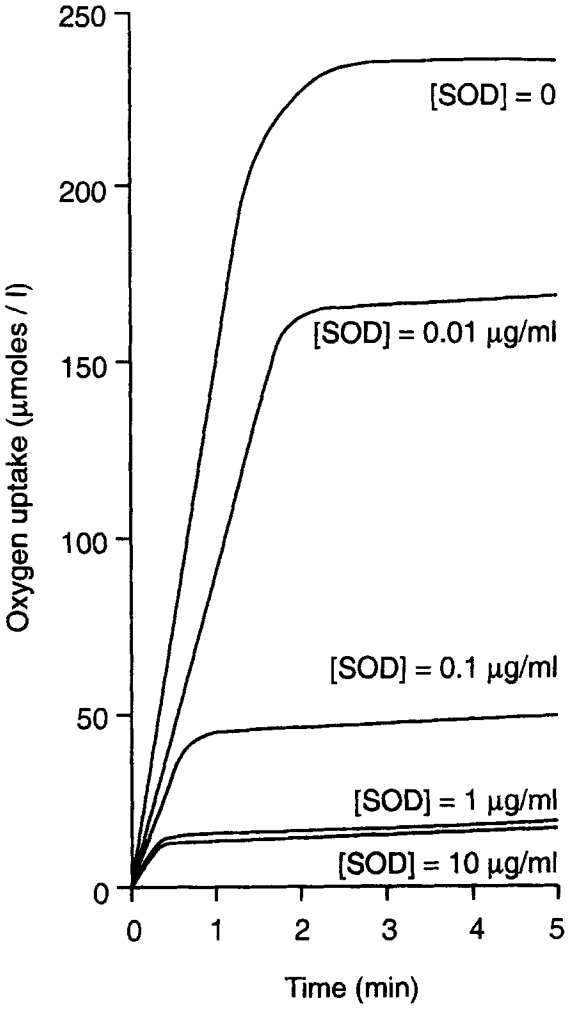

FIGURE 3 Inhibition by SOD of redox cycling of 2-MeNQ in the presence of DT-diaphorase. Oxygen uptake was measured at 25 °C in 0.05 M phosphate buffer containing 1 mM NADH, 50 μM DTPA, 0.25 M sucrose, 0.3 U/ml DT-diaphorase and SOD at the concentrations indicated. After equilibration, the reaction was initiated by addition of 2-MeNQ to a concentration of 150 μM.

The initial rate was even lower in the presence of 0.1 μg/ml of SOD, and only 20% of the oxygen in solution was consumed before the slow phase of oxygen consumption set in. At 1 μg/ml of the enzyme, redox cycling of 2-MeNQ was almost completely inhibited; little further effect was seen at a SOD concentration of 10 μg/ml (Figure 3).

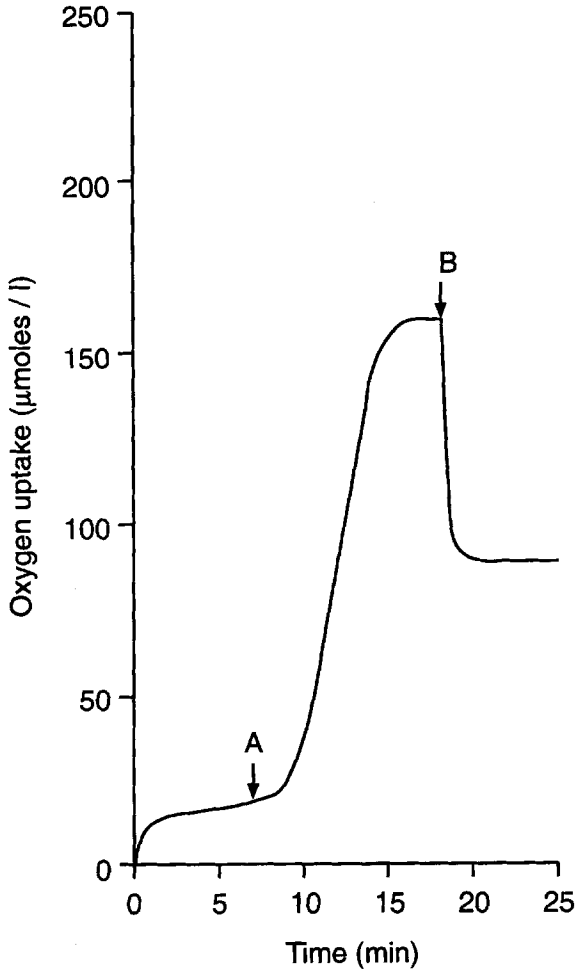

FIGURE 4 Effect of addition of dicoumarol on oxygen uptake in a solution containing 2-MeNQ, NADH, DT-diaphorase and SOD. Oxygen uptake was measured at 25 °C in 0.05 M phosphate buffer containing 1 mM NADH, 50 μM DTPA, 0.25 M sucrose, 0.3 U/ml DT-diaphorase and 10 μg/ml SOD. After equilibration, the reaction was initiated by addition of 2-MeNQ to a concentration of 150 μM. At point A, dicoumarol (30 μg) was added. At point B, catalase (10 μg) was added.

Effect of Dicoumarol on the Inhibited Reaction

In the presence of NADH, 0.3 U/ml DT-diaphorase and 10 μg/ml SOD, little redox cycling of 2-MeNQ was recorded. As in the previous experiment, a very brief rapid phase of oxygen uptake was recorded, followed by a slow phase. Addition of dicoumarol, however, led to rapid oxygen uptake after a brief lag phase. The amount of oxygen consumed showed a 1:1 stoichiometry with the amount of 2-MeNQ employed, and approximately 50% of the oxygen consumed was returned to solution after addition of catalase (Figure 4).

Effect of Further Aliquots of 2-MeNQ on the Inhibited Reaction

As before, addition of 2-MeNQ (150 μM) to a solution containing NADH, DT-diaphorase (0.3 U/ml) and SOD (10 μg/ml) induced little oxygen uptake. Addition of a further aliquot of 2-MeNQ, however, provoked substantial oxygen uptake, the extent of which was proportional to the amount of quinone added (Figure 5).

Effect of pH on Inhibition of Redox Cycling of 2-MeNQ by DT-Diaphorase and SOD

No decrease in the activities of SOD and DT-diaphorase was recorded when the pH of the assay system was changed from pH 7.4 to pH 8.0 (data not shown). However, the degree of inhibition of redox cycling of 2-MeNQ in the presence of these enzymes was much less at pH 7.7 than at pH 7.4, and at pH 8, very rapid cycling occurred, with total utilisation of the oxygen in solution (Figure 6).

Effect of Quinone Concentration on Inhibition of Redox Cycling of 2-MeNQ by DT-Diaphorase and SOD

As before, with DT-diaphorase at 0.3 U/ml and SOD at 10 μg/ml, redox cycling of 150 μM

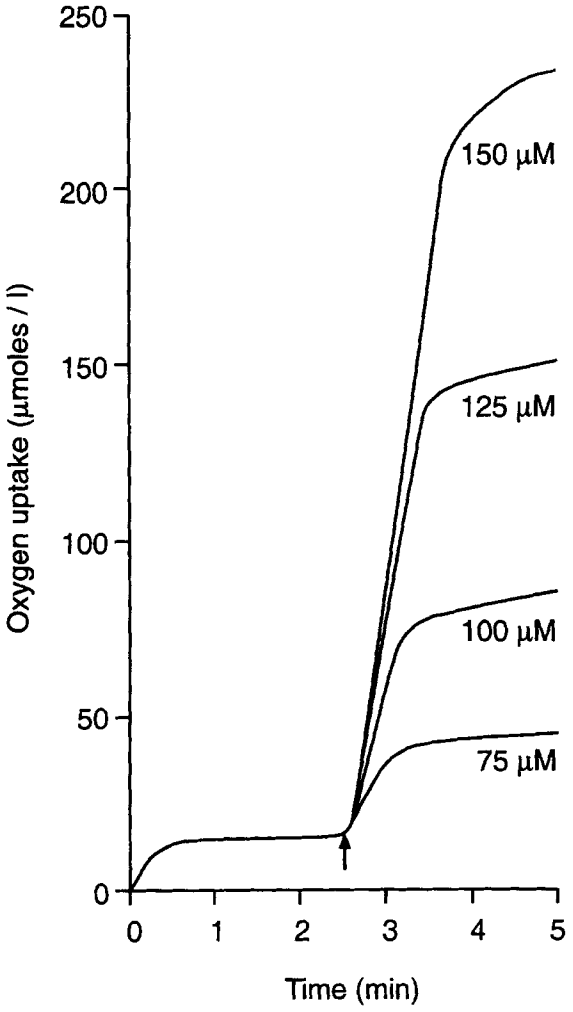

FIGURE 5 Effect of addition of further aliquots of 2-MeNQ on oxygen uptake in a solution containing 2-MeNQ, NADH, DT-diaphorase and SOD. Oxygen uptake was measured under the same conditions as those shown in the legend to Figure 4. At the point indicated, further aliquots of 2-MeNQ were added to give the concentrations indicated.

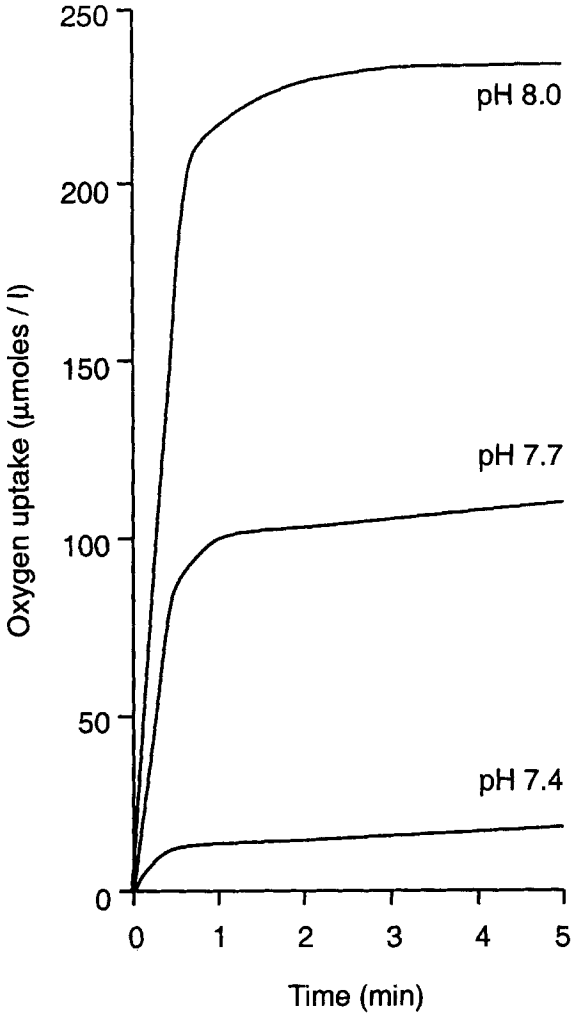

FIGURE 6 Effect of pH on inhibition of redox cycling of 2-MeNQ by DT-diaphorase and SOD. Oxygen uptake was measured under the same conditions as those shown in the legend to Figure 4 except that the buffer pH was varied as indicated.

2-MeNQ was strongly inhibited (Figure 7A). The effectiveness of inhibition decreased with increasing quinone concentration, however, and at 300 μM 2-MeNQ, rapid cycling occurred (Figure 7A). By increasing the diaphorase concentration to 3 U/ml, good inhibition of the redox cycling of 2-MeNQ at 400 μM could be achieved (Figure 7B). However, even small increments in quinone concentration decreased the effectiveness of inhibition, and very rapid oxygen uptake was recorded at a 2-MeNQ concentration of 550 μM. Increasing the SOD concentration to 50 μg/ml did not improve the degree of inhibition at high 2-MeNQ concentrations (data not shown).

Effect of Cytochrome c and Cytochrome Oxidase on the Inhibited Reaction

Addition of cytochrome c to the inhibited reaction during the slow phase of oxygen uptake

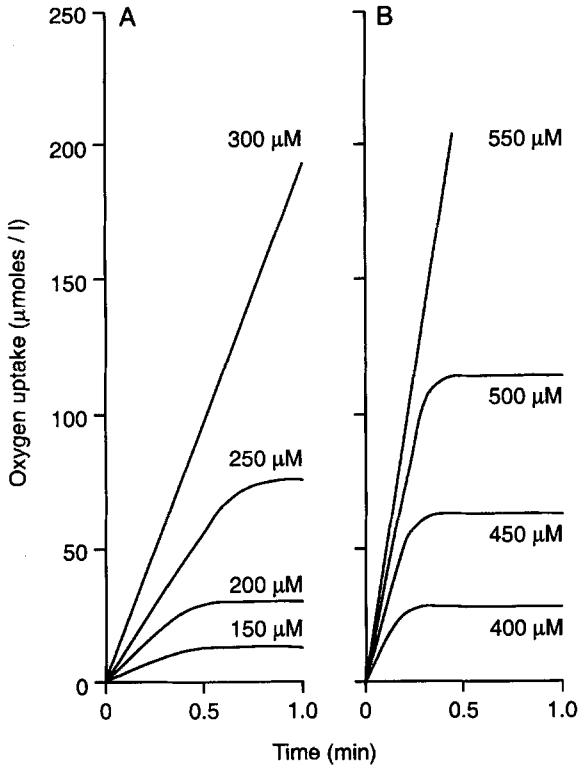

FIGURE 7 Effect of quinone concentration on inhibition of redox cycling of 2-MeNQ by DT-diaphorase and SOD. Oxygen uptake was measured at 25 °C in 0.05 M phosphate buffer containing 1 mM NADH, 50 μM DTPA, 0.25 M sucrose, 10 μg/ml SOD and DT-diaphorase. In A, the diaphorase concentration was 0.3 U/ml. In B, it was 3 U/ml. The reaction was initiated by addition of 2-MeNQ to the concentrations shown.

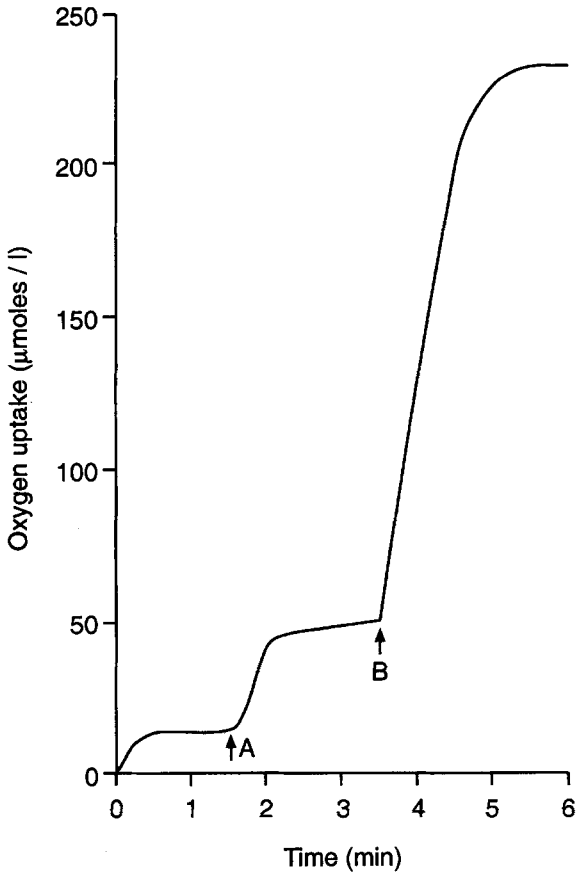

FIGURE 8 Effect of cytochrome c and cytochrome oxidase on oxygen uptake in a solution containing 2-MeNQ, NADH, DT-diaphorase and SOD. Oxygen uptake was measured under the same conditions as those shown in the legend to Figure 4. At point A, cytochrome c (final concentration 50 μM) was added. At point B, cytochrome oxidase (final concentration 5 I.U./ml) was added.

induced a brief period of rapid oxygen utilisation, which soon subsided. At this point, all the cytochrome c was in the reduced form, as indicated by change in optical density at 550 nm (data not shown). Addition of cytochrome oxidase at this point provoked rapid oxygen uptake, with total utilisation of oxygen in solution (Figure 8).

Effect of Methaemoglobin and Haemoglobin on the Inhibited Reaction

Addition of methaemoglobin to the inhibited reaction led to very rapid and complete oxygen utilisation. The rate of oxygen uptake was decreased by approximately 50% in the presence of catalase (Figure 9). Similar results were obtained in the presence of purified rat oxyhaemoglobin (data not shown).

Effect of DT-Diaphorase and SOD on Redox Cycling by Various Naphthoquinones

In the absence of SOD, oxygen uptake was recorded when various naphthoquinones were incubated with NADH and DT-diaphorase. The rate of oxygen uptake was highly dependent

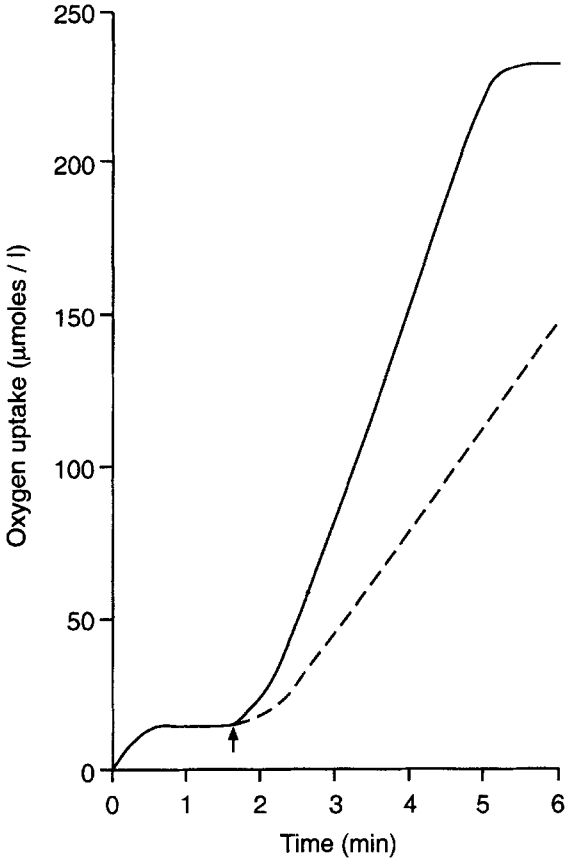

FIGURE 9 Effect of methaemoglobin on oxygen uptake in a solution containing 2-MeNQ, NADH, DT-diaphorase and SOD. Oxygen uptake was measured under the same conditions as those shown in the legend to Figure 4. At the point shown, methaemoglobin (final concentration 25 μM) was added. The broken line indicates uptake in the presence of catalase (10 μg/ml, added with the methaemoglobin).

upon the concentration of DT-diaphorase. In many instances, an initial rapid rate of oxygen uptake was recorded, followed by a prolonged linear phase of oxygen uptake. The rates of the latter phase of the reaction are plotted against DT-diaphorase concentration in Figure 10. With 2-MeNQ, rapid oxygen uptake was recorded at low levels of enzyme, but the rate diminished at high levels of DT-diaphorase. A similar pattern of oxygen uptake was recorded with 2,3-dimethyl-1,4-naphthoquinone, while a plateau was reached at high levels of enzyme with 2,3-dimethoxy-1,4-naphthoquinone. With 2-hydroxy-, 2-amino- and 5-hydroxy-1,4-naphthoquinone, however, the rate of oxygen uptake increased almost linearly with increasing DT-diaphorase concentration. SOD strongly inhibited the redox cycling of 2-MeNQ and the 2,3-dimethyl and 2,3-dimethoxy derivatives. Incomplete inhibition was recorded with 2-amino- and 2-hydroxy-1,4-naphthoquinone, while SOD had no effect on the rate of redox cycling of 5-hydroxy-1,4-naphthoquinone.

Relative Rates of Reduction of Naphthoquinones by DT-Diaphorase

The rates of reduction of 2-MeNQ, 2,3-dimethyl-, 2,3-dimethoxy, 2-hydroxy-, 2-amino- and 5-hydroxy-1,4-naphthoquinone are shown in Table I. 2-MeNQ was reduced the fastest, followed by 2,3-dimethyl and 5-hydroxy-1,4-naphthoquinone. The reduction of the 2,3-dimethoxy derivative was lower, but considerably faster than that of 2-amino-1,4-naphthoquinone. The rate of reduction of 2-hydroxy-1,4-naphthoquinone by DT-diaphorase was very low.

DISCUSSION

The mechanism of hydroquinone autoxidation is complex.[22,35,36] Ionisation of the hydroquinone (QH_2) (Reaction I) is a necessary prerequisite for the reaction, and oxidation is initiated by reaction of the anion with molecular oxygen (Reaction II), forming superoxide and the semiquinone ($Q^{-\bullet}$). Autoxidation of the latter via Reaction III yields the quinone (Q). More semiquinone may be generated in the comproportionation reaction between the quinone and hydroquinone anion (Reaction IV), while superoxide generated in Reactions II and III initiates a radical chain reaction by oxidising the hydroquinone anion, with formation of the semiquinone and hydrogen peroxide (Reaction V).

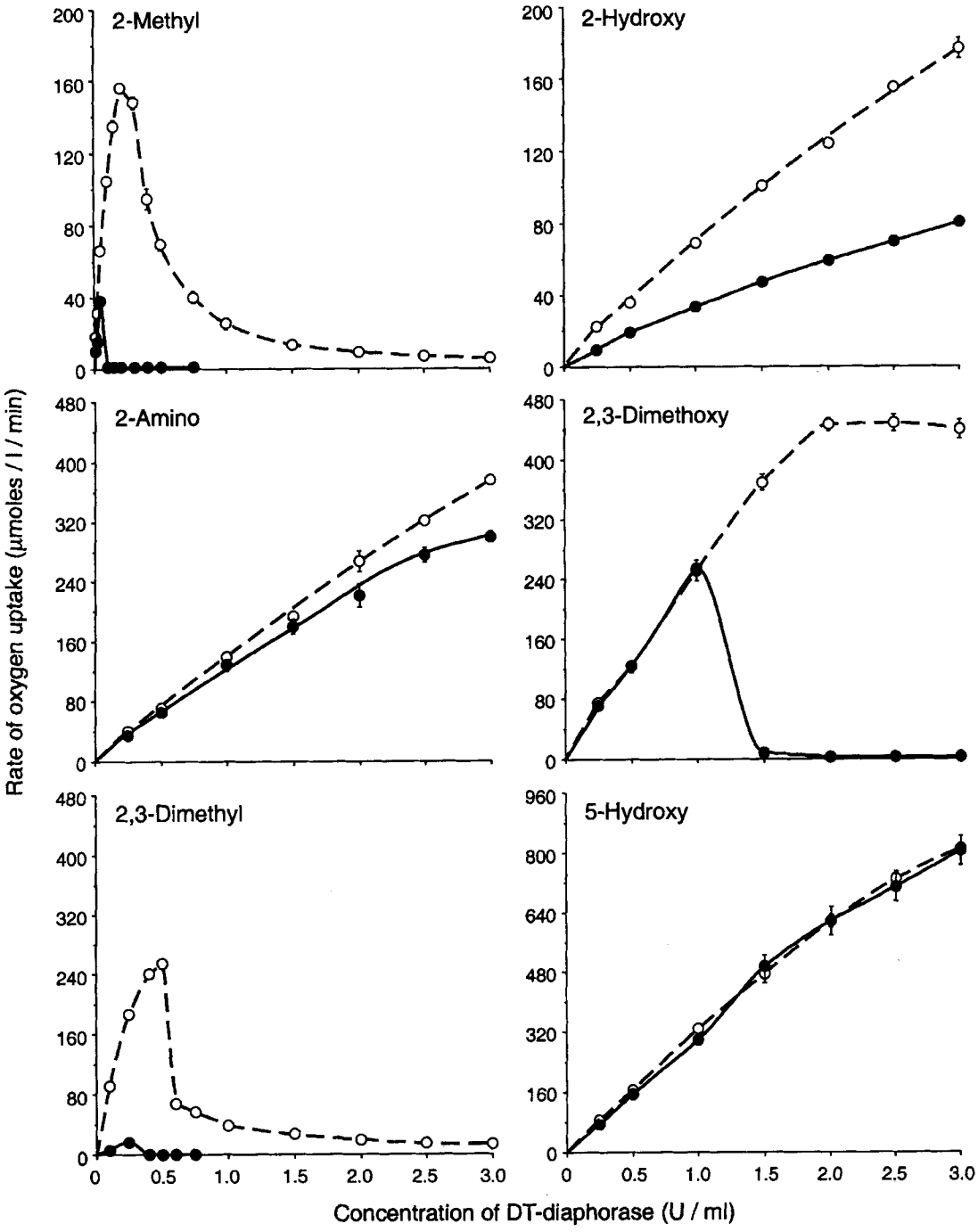

FIGURE 10 Effect of DT-diaphorase and SOD on redox cycling by various naphthoquinones. Oxygen uptake was measured under the same conditions as those shown in the legend to Figure 4, using various concentrations of DT-diaphorase as indicated. The concentration of quinone in all cases was 150 μM. The broken line indicates control rates, the unbroken line rates in the presence of 10 μg/ml SOD. Data shown are the means of four separate determinations.

TABLE I Rates of reduction of naphthoquinone derivatives by DT-diaphorase

Naphthoquinone derivative	Rate of reduction by DT-diaphorase (μmoles/l/min)
2-Methyl	10.17 ± 0.14
2,3-Dimethyl	6.76 ± 0.09
2,3-Dimethoxy	3.24 ± 0.08
2-Amino	1.20 ± 0.08
2-Hydroxy	0.12 ± 0.01
5-Hydroxy	6.77 ± 0.28

Rates of reduction of the naphthoquinones were measured as initial rates of reduction of cytochrome c (μmoles/l/min) in the presence of 0.003 U/ml DT-diaphorase. Results shown are the means and SEM of five separate determinations.

$$QH_2 \rightleftharpoons QH^- + H^+ \quad (I)$$

$$QH^- + O_2 \longrightarrow Q^{-\bullet} + O_2^{\bullet-} + H^+ \quad (II)$$

$$Q^{-\bullet} + O_2 \rightleftharpoons Q + O_2^{\bullet-} \quad (III)$$

$$Q + QH^- \rightleftharpoons 2Q^{-\bullet} + H^+ \quad (IV)$$

$$QH^- + O_2^{\bullet-} + H^+ \longrightarrow Q^{-\bullet} + H_2O_2 \quad (V)$$

The overall reaction is described by Reaction VI:

$$QH_2 + O_2 \longrightarrow Q + H_2O_2 \quad (VI)$$

In the present experiments, the stoichiometry of oxygen uptake by 2-MeNHQ was in accord with Reaction VI. As expected from this stoichiometry, approximately 50% of the oxygen consumed was returned to solution after addition of catalase, consistent with destruction of hydrogen peroxide via Reaction VII.

$$2H_2O_2 \longrightarrow 2H_2O + O_2 \quad (VII)$$

The rate of oxidation of 2-MeNHQ was decreased in the presence of SOD, reflecting elimination of Reaction V through destruction of superoxide via Reaction VIII.

$$2O_2^{\bullet-} + 2H^+ \longrightarrow H_2O_2 + O_2 \quad (VIII)$$

As in other superoxide-driven autoxidation reactions,[37–39] autoxidation of 2-MeNHQ in the presence of SOD showed an initial lag phase, with a subsequent increase in rate. Initially, no quinone will be present in solution, and in the presence of SOD the rate of autoxidation will be limited by the rate of the initiation reaction (Reaction I). With time, however, the amount of quinone available to participate in Reaction IV will increase, and an alternative pathway for oxidation will be established, thus leading to an increase in rate.

The rate of autoxidation of 2-MeNHQ was also decreased by DT-diaphorase, reflecting reduction of quinone formed in Reaction III back to the hydroquinone. Such reduction, by competing with comproportionation (Reaction IV) will lower the concentration of semiquinone in solution and thus decrease the overall rate of oxidation.

The inhibitory effect of both DT-diaphorase and SOD in isolation increased with increasing enzyme concentration, and under the conditions of the present experiments, a plateau was reached, with maximum inhibitions of 68% and 76% respectively. Since DT-diaphorase and SOD act on different reactions of the autoxidation pathway, it would be expected that their effects would reinforce one another. This was shown to be the case, and even at low levels of both enzymes, the rate of autoxidation of 2-MeNHQ was decreased by more than 99%.

With 2-MeNQ and DT-diaphorase, the situation is rather different, since, initially at least, both the quinone and hydroquinone will simultaneously be present in solution, permitting semiquinone formation via Reaction IV. At low concentrations of the enzyme, redox cycling was observed, which continued until all the oxygen in solution had been consumed. At higher levels of DT-diaphorase, however, redox cycling of

2-MeNQ was inhibited, with an abrupt decrease in the rate of oxygen uptake being recorded. The concentration of quinone in solution will progressively decrease with increasing levels of DT-diaphorase, and the abrupt change in the rate of oxygen uptake presumably reflects the point at which the quinone concentration becomes insufficient for a significant contribution of Reaction IV to the oxidation process.

With a combination of DT-diaphorase and SOD, inhibition of redox cycling became much more effective. In the presence of both enzymes, the quinone was maintained in the reduced form, as shown by the oxygen uptake recorded after addition of dicoumarol, a specific inhibitor of DT-diaphorase.[33] The quantity of oxygen consumed was consistent with the presence of 2-MeNHQ, at a concentration equal to that of the 2-MeNQ added, in the solution.

The rate of autoxidation of naphthohydroquinones increases with increasing pH,[25] reflecting higher concentrations of the anion available for participation in Reaction II. The degree of inhibition of redox cycling of 2-MeNQ by DT-diaphorase and SOD was similarly found to be strongly dependent on pH. While almost complete inhibition was recorded at pH 7.4, a much smaller effect was seen at pH 7.7, and rapid redox cycling occurred at pH 8.0.

Addition of further aliquots of 2-MeNQ to the inhibited reaction also stimulated oxygen uptake, presumably by facilitating semiquinone formation via Reaction IV. The effectiveness of inhibition was also strongly dependent upon naphthoquinone concentration. At a DT-diaphorase concentration of 0.3 U/ml with 2-MeNQ at 150 μM, strong inhibition was recorded. Rapid redox cycling occurred, however, when the quinone concentration was doubled. A 10-fold increase in DT-diaphorase concentration permitted inhibition of redox cycling of 2-MeNQ at a concentration of 400 μM, but under these conditions, only a relatively small increase in quinone concentration was sufficient to provoke very rapid cycling.

Oxygen uptake was stimulated by addition of cytochrome c, which readily oxidises hydroquinones to semiquinones.[33] Oxygen uptake was close to stoichiometric, and ceased when all the added cytochrome c had been reduced. Addition of cytochrome oxidase, however, by initiating re-oxidation of reduced cytochrome c by molecular oxygen, permitted rapid and complete oxygen uptake. Addition of methaemoglobin also stimulated oxygen uptake, but with this substance the oxidation was non-stoichiometric, and proceeded to complete oxygen utilisation. In this case, the haemoprotein must be acting catalytically, with initial one-electron reduction of the methaemoglobin (Reaction IX) being followed by further oxidation of the hydroquinone by oxyhaemoglobin (Reaction X). This mechanism is supported by the observed decrease in the rate of oxygen uptake in the presence of catalase and by the fact that oxyhaemoglobin also catalytically stimulated oxygen uptake.

$$QH^- + Hb^{III} + O_2 \longrightarrow Q^{\bullet -} + Hb^{II}O_2 + H^+ \quad (IX)$$

$$QH^- + Hb^{II}O_2 + H^+ \longrightarrow Q^{\bullet -} + Hb^{III} + H_2O_2 \quad (X)$$

In order to provide information on the general applicability of a combination of DT-diaphorase and SOD in preventing redox cycling of naphthoquinones, the behaviour of several other compounds of this type was compared with that of 2-MeNQ, determining the effect of increasing concentrations of DT-diaphorase alone on the rate of redox cycling of the quinone and the effect of DT-diaphorase in combination with SOD. The compounds selected were 2-hydroxy-, 2-amino-, 2,3-dimethoxy-, 2,3-dimethyl- and 5-hydroxy-1,4-naphthoquinone. These compounds were all reduced by DT-diaphorase, albeit at greatly dissimilar rates. With the exception of the 5-hydroxy derivative, the autoxidation of the hydroquinones derived from these substances is inhibited by SOD,[25] and, again with the exception of 5-hydroxy-1,4-naphthoquinone, comproportionation between quinone and hydroquinone is important in their autoxidation.[25]

The behaviour of 2,3-dimethyl-1,4-naphthoquinone was very similar to that of 2-MeNQ, consistent with the similar rates of reduction of these substances, their similar rates of autoxidation[25] and the powerful effect of SOD on the autoxidation of the corresponding hydroquinones.[19,24] Little inhibition of redox cycling of 2,3-dimethoxy-1,4-naphthoquinone was seen with DT-diaphorase alone, which may reflect the comparatively low rate of reduction of the quinone by DT-diaphorase and the high rate of autoxidation of the hydroquinone,[25] which would compromise maintenance of the hydroquinone in the reduced form. Redox cycling was, however, reduced to low levels in the presence of SOD, but much higher levels of DT-diaphorase were required for inhibition with this substance than with 2-MeNQ or the 2,3-dimethyl derivative. The behaviour of 2-hydroxy- and 2-amino-1,4-naphthoquinone was quite different. The rate of redox cycling increased with increasing activity of DT-diaphorase over the whole concentration range employed, and although some effect of SOD was recorded, the low level of redox cycling observed with the methyl, dimethyl and dimethoxy compounds was not achieved. Both these compounds are slowly reduced by DT-diaphorase while their hydroquinones undergo very rapid oxidation.[25] It would appear that even with high levels of diaphorase and in the presence of SOD, maintenance of the hydroquinone in the reduced form is not possible. Very rapid redox cycling was recorded with 5-hydroxy-1,4-naphthoquinone. The autoxidation of the hydroquinone derived from this substance is very rapid, reflecting extensive ionisation at neutral pH.[40] Since neither the comproportionation reaction nor oxidation of the hydroquinone by superoxide is important in the autoxidation reaction,[25] inhibition of redox cycling by DT-diaphorase and SOD is not to be expected. The exceptionally high rate of redox cycling seen with this compound may be explained in terms of its rapid reduction by DT-diaphorase and the extremely high rate of autoxidation of the hydroquinone that is formed.[25]

From these results, it is clear that a combination of DT-diaphorase and SOD inhibits the redox cycling of only certain naphthoquinone derivatives, and the combination of these enzymes does not provide a general protective mechanism against naphthoquinone toxicity. Whether or not redox cycling is inhibited is influenced by the chemical properties of the quinone and hydroquinone. The degree of ionisation of the hydroquinone and its oxidation potential are very important, since these determine the rate of autoxidation.[25] A high pKa and high oxidation potential, leading to a low autoxidation rate, would favour inhibition of redox cycling by the enzymes. The positions of the equilibria of the reaction between the semiquinone and oxygen and the reaction between the quinone and hydroquinone are also important, since they govern the importance of comproportionation and superoxide-driven radical chain reactions in the autoxidation mechanism.[25] If semiquinone formation and oxidation are favoured, inhibition is to be expected. Similarly, rapid reduction of the quinone by DT-diaphorase, leading to low steady-state concentrations of quinone, would promote inhibition.

Apart from the intrinsic properties of the quinone and hydroquinone, external factors can influence the degree of inhibition of redox cycling by SOD and DT-diaphorase. An increase in the rate of the initiation reaction by increasing pH or by addition of haemoproteins can by-pass inhibition by the enzymes, as can a second aliquot of the quinone. Furthermore, the degree of inhibition is very sensitive to quinone concentration, so if the concentration of quinone is high enough, redox cycling will inevitably ensue. The tolerable level of quinone can be increased by increasing the concentration of DT-diaphorase, an observation which is in accord with the protection given against the *in vivo* toxicity of 2-MeNQ by substances that increase tissue levels of DT-diaphorase.[41,42]

An understanding of the factors that control redox cycling of naphthoquinones is important in understanding structure–activity relationships in their toxic and therapeutic actions. From the results of the present study, it would be expected that the redox cycling of 2,3-dimethyl- and 2,3-dimethoxy-1,4-naphthoquinone would be inhibited by high levels of DT-diaphorase, and it would be anticipated that increased tissue activity of this enzyme would decrease the toxicity of these substances, as it does that of MeNQ.[41,42] Similarly, it would be expected that the toxicity of the 2-amino and 5-hydroxy derivatives would be increased by increasing tissue levels of DT-diaphorase in a similar manner to that described for 2-hydroxy-1,4-naphthoquinone.[42,43] Experiments on these possibilities are in progress.

References

[1] R.H. Thomson (1987) *Naturally occurring quinones. III: Recent advances*. Chapman and Hall, London.

[2] J.A. Duke (1985) *Handbook of medicinal herbs*. CRC Press, Boca Raton.

[3] J.M. Watt and M.G. Breyer-Brandwijk (1962) *The medicinal and poisonous plants of Southern and Eastern Africa*. Second edn. Livingstone, Edinburgh.

[4] A.T. Hudson (1988) Antimalarial hydroxynaphthoquinones. *Special Publications of the Royal Society of Chemistry*, **65**, 266–283.

[5] G. Powis (1987) Metabolism and reactions of quinone anticancer agents. *Pharmacology and Therapeutics*, **35**, 57–162.

[6] K.A. Margolin, S.A. Akman, L.A. Leong, R.J. Morgan, G. Somlo, J.W. Raschko, C. Ahn and J.H. Doroshow (1995) Phase I study of mitomycin c and menadione in advanced solid tumors. *Cancer Chemotherapy and Pharmacology*, **36**, 293–298.

[7] P.J. O'Brien (1991) Molecular mechanisms of quinone cytotoxicity. *Chemico-Biological Interactions*, **80**, 1–41.

[8] B.-Z. Ahn and D.-E. Sok (1996) Michael acceptors as a tool for anticancer drug design. *Current Pharmaceutical Design*, **2**, 247–262.

[9] R.K. Morrison, D.E. Brown, J.J. Oleson and D.A. Cooney (1970) Oral toxicology studies with lapachol. *Toxicology and Applied Pharmacology*, **17**, 1–11.

[10] R. Munday, B.L. Smith and E.A. Fowke (1991) Haemolytic activity and nephrotoxicity of 2-hydroxy-1,4-naphthoquinone in rats. *Journal of Applied Toxicology*, **11**, 85–90.

[11] R. Munday, E.A. Fowke, B.L. Smith and C.M. Munday (1994) Comparative toxicity of alkyl-1,4-naphthoquinones in rats: relationship to free radical production *in vitro*. *Free Radical Biology and Medicine*, **16**, 725–731.

[12] R. Munday, B.L. Smith and C.M. Munday (1995) Toxicity of 2,3-dialkyl-1,4-naphthoquinones in rats: comparison with cytotoxicity *in vitro*. *Free Radical Biology and Medicine*, **19**, 759–765.

[13] S.S. Lau (1995) Quinone-thioether-mediated nephrotoxicity. *Drug Metabolism Reviews*, **27**, 125–141.

[14] M. Comporti (1989) Three models of free radical-induced cell injury. *Chemico-Biological Interactions*, **72**, 1–56.

[15] A. Brunmark and E. Cadenas (1989) Redox and addition chemistry of quinoid compounds and its biological implications. *Free Radical Biology and Medicine*, **7**, 435–477.

[16] M.T. Smith, C.G. Evans, H. Thor and S. Orrenius (1985) In *Oxidative stress* (ed. H. Sies), Academic Press, London, pp. 91–113.

[17] G. Schenk, K.J. Vetter and R. Müller (1959) Polarographische Untersuchungen der Autoxidation von 1,2,4-Trioxynaphthalin. *Archiv der Pharmazie*, **292**, 62–70.

[18] J.P. Colpa-Boonstra and E.C. Slater (1958) The possible role of vitamin K in the respiratory chain. *Biochimica et Biophysica Acta*, **27**, 122–133.

[19] R. Munday (1997) Autoxidation of naphthohydroquinones: effects of metals, chelating agents, and superoxide dismutase. *Free Radical Biology and Medicine*, **22**, 689–695.

[20] K.W. Bock, W. Lilienblum, G. Fischer, G. Schirmer and B.S. Bock-Hennig (1987) The role of conjugation reactions in detoxication. *Archives of Toxicology*, **60**, 22–29.

[21] R. Munday (1997) Inhibition of naphthohydroquinone autoxidation by DT-diaphorase (NAD(P)H:[quinone acceptor] oxidoreductase). *Redox Report*, **3**, 189–196.

[22] T. Ishii and I. Fridovich (1990) Dual effects of superoxide dismutase on the autoxidation of 1,4-naphthohydroquinone. *Free Radical Biology and Medicine*, **8**, 21–24.

[23] K. Öllinger, G.D. Buffinton, L. Ernster and E. Cadenas (1990) Effect of superoxide dismutase on the autoxidation of substituted hydro- and semi-naphthoquinones. *Chemico-Biological Interactions*, **73**, 53–76.

[24] R. Munday (1999) Inhibition of 2,3-dimethyl-1,4-naphthohydroquinone autoxidation by copper and by superoxide dismutase. *Free Radical Biology and Medicine*, **26**, 1475–1479.

[25] R. Munday (2000) Autoxidation of naphthohydroquinones: effects of pH, naphthoquinones and superoxide dismutase. *Free Radical Research*, **32**, 245–253.

[26] J. Segura-Aguilar and C. Lind (1989) On the mechanism of the Mn^{3+}-induced neurotoxicity of dopamine: prevention of quinone-derived oxygen toxicity by DT-diaphorase and superoxide dismutase. *Chemico-Biological Interactions*, **72**, 309–324.

[27] L. Ernster (1998) DT-diaphorase: its structure, function, regulation and role in antioxidant defence and cancer chemotherapy. In: *Pathophysiology of lipid peroxides and related free radicals* (ed. K. Yagi), Japan Scientific Societies Press, Tokyo, pp. 149–168.

[28] L.F. Fieser (1940) Convenient procedures for the preparation of antihemorrhagic compounds. *Journal of Biological Chemistry*, **133**, 391–396.

[29] N. Jacobsen and K. Torssell (1972) Radikalische Alkylierung von Chinonen: Erzeugung von Radikalen in Redoxreaktionen. *Liebig's Annalen der Chemie*, **763**, 135–147.

[30] L.F. Fieser and R.H. Brown (1949) Synthesis of naphthoquinones for studies of the inhibition of enzyme systems. *Journal of the American Chemical Society*, **71**, 3609–3614.
[31] R. Munday (1995) *In vivo* toxicity of thiols: relationship to rate of one-electron oxidation by oxyhaemoglobin. *Methods in Enzymology*, **251**, 117–120.
[32] C. Lind, E. Cadenas, P. Hochstein and L. Ernster (1990) DT-diaphorase: purification, properties, and function. *Methods in Enzymology*, **186**, 287–301.
[33] L. Ernster, L. Danielson and M. Ljunggren (1962) DT-diaphorase I. Purification from the soluble fraction of rat-liver cytoplasm, and properties. *Biochimica et Biophysica Acta*, **58**, 171–188.
[34] J.M. McCord and I. Fridovich (1969) Superoxide dismutase. An enzymic function for erythrocuprein (hemocuprein). *Journal of Biological Chemistry*, **244**, 6049–6055.
[35] P. Eyer (1991) Effects of superoxide dismutase on the autoxidation of 1,4-hydroquinone. *Chemico-Biological Interactions*, **80**, 159–176.
[36] B. Bandy, J.A. Moon and J. Davison (1990) Multiple actions of superoxide dismutase: why can it both inhibit and stimulate reduction of oxygen by hydroquinones? *Free Radical Biology and Medicine*, **9**, 143–148.
[37] R. Munday (1988) Generation of superoxide radical, hydrogen peroxide and hydroxyl radical during the autoxidation of *N,N,N′,N′*-tetramethyl-*p*-phenylenediamine. *Chemico-Biological Interactions*, **65**, 133–143.
[38] C.C. Winterbourn, W.B. Cowden and H.C. Sutton (1989) Auto-oxidation of dialuric acid, divicine and isouramil. Superoxide dependent and independent mechanisms. *Biochemical Pharmacology*, **38**, 611–618.
[39] R. Munday and C.C. Winterbourn (1989) Reduced glutathione in combination with superoxide dismutase as an important biological antioxidant defence mechanism. *Biochemical Pharmacology*, **38**, 4349–4352.
[40] T. Mukherjee (1987) One-electron reduction of juglone (5-hydroxy-1,4-naphthoquinone): a pulse radiolysis study. *Radiation Physics and Chemistry*, **29**, 455–462.
[41] R. Munday, B.L. Smith and C.M. Munday (1998) Effects of butylated hydroxyanisole and dicoumarol on the toxicity of menadione to rats. *Chemico-Biological Interactions*, **108**, 155–170.
[42] R. Munday, B.L. Smith and C.M. Munday (1999) Effect of inducers of DT-diaphorase on the toxicity of 2-methyl and 2-hydroxy-1,4-naphthoquinone to rats. *Chemico-Biological Interactions*, **123**, 219–237.
[43] R. Munday, B.L. Smith and C.M. Munday (1999) Effects of butylated hydroxyanisole on the toxicity of 2-hydroxy-1,4-naphthoquinone to rats. *Chemico-Biological Interactions*, **117**, 241–256.