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Autoxidation of Naphthohydroquinones: Effects of pH, Naphthoquinones and Superoxide Dismutase

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The rates of autoxidation of a number of pure naphthohydroquinones have been determined, and the effects of pH, superoxide dismutase (SOD) and of the parent naphthoquinone on the oxidation rates have been investigated. Most compounds were slowly oxidised in acid solution with the rates increasing with increasing pH, although 2-hydroxy-, 2-hydroxy-3-methyl- and 2-amino-1,4-naphthohydroquinone were rapidly oxidised at pH 5 and the rates of oxidation of these substances were comparatively unresponsive to changes in pH. At pH 7.4, autoxidation rates decreased in the order 2,3-dichloro-1,4-naphthohydroquinone > 5-hydroxy > 2-bromo > 2-hydroxy-3-methyl > 2-amino > 2-hydroxy > 2-methoxy > 2,3-dimethoxy > 2,3-dimethyl > 2-methyl > unsubstituted hydroquinone. The autoxidation rates of the alkyl, alkoxy, hydroxy and amino derivatives were decreased in the presence of SOD, but this enzyme had no effect on the rate of autoxidation of the 2,3-dichloro and 2-bromo derivatives while that of the 5-hydroxy derivative was increased. The rates of autoxidation of all compounds except the halogen derivatives and 5-hydroxy-1,4-naphthohydroquinone were increased by addition of the parent naphthoquinone, and quinone addition partially or completely overcame the inhibitory effect of SOD. There is evidence that the reduction of quinones to hydroquinones *in vivo* may lead either to detoxification or to activation. This may be due to differences in the rate or mechanism of autoxidation of the hydroquinones that are formed, and the data gained in this study will provide a framework for testing this possibility.

Keywords: Rate of naphthohydroquinone autoxidation, mechanism and structure–activity relationships, effect of superoxide dismutase, effect of pH

INTRODUCTION

Quinones are widely distributed in nature as plant and fungal metabolites.[1] They are also present in vehicle emissions and cigarette smoke,[2,3] and are pollutants of both air and water.[4,5] Many quinones are widely used in folk medicine[6,7] and a number of substances of this type have been shown to be effective anti-cancer drugs.[8,9]

The toxicology of quinones has been extensively studied. These substances induce a wide range of toxic effects in animals and man, including haemolysis,[10–14] renal damage,[11,15]

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cataracts[16,17] and cancer.[18,19] They are largely excreted via the urine, as sulphate or glucuronide conjugates of the corresponding hydroquinones.[20,21]

The enzyme DT-diaphorase (NAD(P)H:[quinone acceptor] oxidoreductase, E.C. 1.6.99.2) plays an important role in the conversion of quinones to hydroquinones,[22–24] and, if conjugation and excretion ensues, this process would constitute a detoxification reaction. However, as discussed previously,[24–26] whether or not this actually occurs depends upon the properties of the hydroquinone that is formed. Hydroquinones are unstable substances, which undergo autoxidation at physiological pH, re-forming the quinone with concomitant formation of "active oxygen" species.[25,27,28] If the autoxidation of the hydroquinone is comparatively slow under physiological conditions, conjugation may occur before oxidation. In this situation, the quinone would be detoxified and excreted, and increasing cellular levels of DT-diaphorase would be expected to decrease the toxicity of the quinone. If, however, the rate of autoxidation of the hydroquinone is fast, only a small proportion of the hydroquinone may be conjugated before oxidation occurs. Reduction by DT-diaphorase would then constitute an activation reaction, with establishment of a redox cycle for production of toxic radical species. In this case, enhanced tissue concentrations of DT-diaphorase would be expected to increase quinone toxicity.

These alternative processes are illustrated by the opposite effects of increased tissue levels of DT-diaphorase on the toxicity of 2-methyl-1,4-naphthoquinone and 2-hydroxy-1,4-naphthoquinone to rats. Both substances cause haemolytic anaemia, but the severity of the haemolysis induced by 2-methyl-1,4-naphthoquinone was decreased in rats with elevated tissue levels of DT diaphorase,[29] while that caused by the 2-hydroxy derivative was increased.[30] *In vitro* studies have shown that the hydroquinone derived from 2-hydroxy-1,4-naphthoquinone is a very unstable substance, which undergoes autoxidation very much faster than the hydroquinone formed from the methyl derivative.[26,31]

In order to gain further information on the possible association between hydroquinone stability and *in vivo* toxic effects, information on the relative rates of autoxidation of other hydroquinones would be useful. Little information is presently available, however, on the relative rates of autoxidation of pure hydroquinones, and the information that is available[32–35] has been gathered over a wide range of reaction conditions, making comparison difficult. Furthermore, metal contamination of buffers was not controlled in the earlier experiments, which could have led to artefactually low oxidation rates.[27] In other studies, the autoxidation of a number of hydroquinones generated by reduction of the quinone by DT-diaphorase has been described,[31,36–41] but interpretation of these results is complicated by differences in rates of reduction of quinones by the enzyme[38] and by the fact that the autoxidation of some, but not all, hydroquinones is inhibited by DT-diaphorase.[26]

Information on factors that can influence the rate of hydroquinone autoxidation would also be valuable. The rate of oxidation of hydroquinones is strongly influenced by pH, with rates increasing with increasing alkalinity.[31,33] It has been shown that superoxide dismutase (SOD) can either increase or decrease the rate of hydroquinone autoxidation[28,31,35–37,41–43] while addition of quinone to a solution of hydroquinone may stimulate the oxidation of the latter.[26,34]

In the present experiments, the rates of autoxidation of 11 pure naphthohydroquinones have been compared under uniform reaction conditions and the effects of pH, SOD and quinone on the oxidation rates have been investigated.

MATERIALS AND METHODS

Chemicals

1,4-Naphthoquinone and 1,4-naphthohydroquinone were purchased from Aldrich. They were

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recrystallised (from benzene and 5 M hydrochloric acid, respectively) before use. 2-Methyl-, 2-hydroxy- and 5-hydroxy-1,4-naphthoquinone were purchased from Sigma, and the 2,3-dichloro derivative from Aldrich. The following naphthoquinone and naphthohydroquinone derivatives were synthesised by the methods indicated: 2-methyl, hydroquinone;[44] 2,3-dimethyl, quinone,[45] hydroquinone;[46] 2-methoxy, quinone,[47] hydroquinone;[47] 2,3-dimethoxy, quinone,[48] hydroquinone;[46] 2-hydroxy, hydroquinone;[49] 2-amino, quinone,[50] hydroquinone;[51] 2-hydroxy-3-methyl, quinone,[44] hydroquinone;[47] 2,3-dichloro, hydroquinone;[52] 2-bromo, quinone,[53] hydroquinone;[54] 5-hydroxy, hydroquinone.[55] The hydroquinones were stored desiccated at −20°C. SOD, from bovine erythrocytes, was from Sigma. Catalase, from bovine liver, was from Boehringer.

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Measurement of Hydroquinone Autoxidation

Reactions at varying pH were conducted in 50 mM phosphate–citrate buffers, pH 5–8. All other studies employed 50 mM phosphate, pH 7.4. All buffers contained 50 μM DTPA. Hydroquinones were dissolved in acetone, diluted 1 : 10 with 0.01 N hydrochloric acid and the solution maintained in an atmosphere of nitrogen in order to prevent spontaneous oxidation. Autoxidation rates were measured as rates of oxygen uptake using a Yellow Springs Instruments Oxygen Monitor, Model 53, at 25°C. In some instances, particularly at low pH, a lag phase was observed in the autoxidation reaction. This was followed by a linear phase of reaction. The quoted rates are those of the linear phase of the autoxidation.

RESULTS

The effect of pH on the autoxidation rates of 1,4-naphthohydroquinone and its 2-methyl and 2,3-dimethyl derivatives is shown in Figure 1. These compounds were stable at pH 6.5 or below and although the autoxidation rates increased with increasing pH, the rates of oxidation of these compounds were comparatively low even at pH 8. The 2-methoxy, 2,3-dimethoxy, 2,3-dichloro, 2-bromo and 5-hydroxy derivatives were all stable at pH 5 (Figure 2), but the rate of autoxidation increased very rapidly with increasing pH and

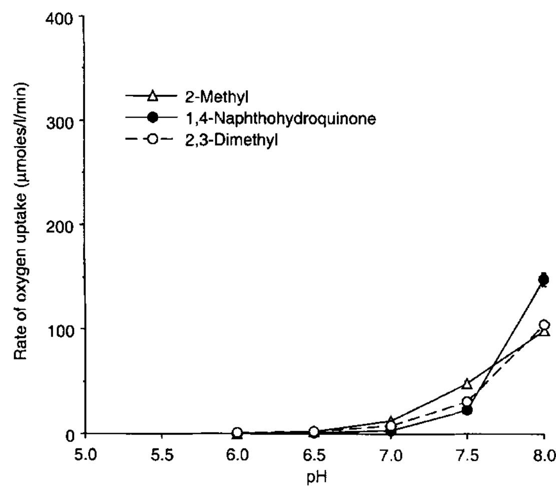

FIGURE 1 Effect of pH on the rates of autoxidation of 1,4-naphthohydroquinone and 2-methyl- and 2,3-dimethyl-1,4-naphthohydroquinone. Rates were measured as rates of oxygen uptake at 25°C. The initial concentration of hydroquinone was 50 μM.

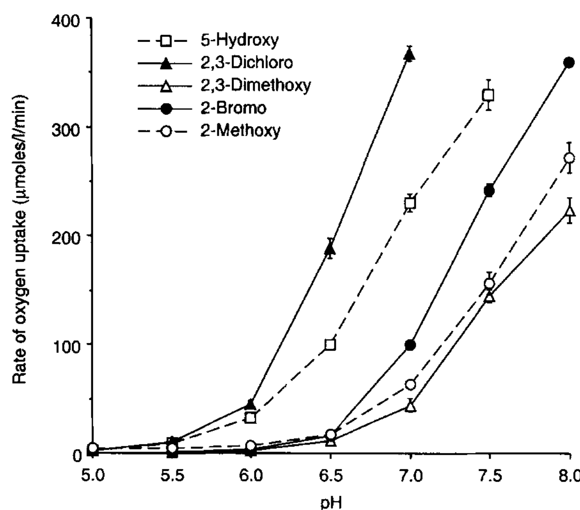

FIGURE 2 Effect of pH on the rates of autoxidation of 5-hydroxy-, 2,3-dichloro-, 2,3-dimethoxy-, 2-bromo- and 2-methoxy-1,4-naphthohydroquinone. Conditions were as described in the legend to Figure 1.

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under alkaline conditions these substances, particularly the halogenated derivatives and 5-hydroxy-1,4-naphthohydroquinone, were very rapidly oxidised. In contrast, 2-hydroxy-, 2-hydroxy-3-methyl and 2-amino-1,4-naphthohydroquinone showed a comparatively small increase in oxidation rate with increasing pH, and these substances underwent appreciable oxidation even at pH 5 (Figure 3).

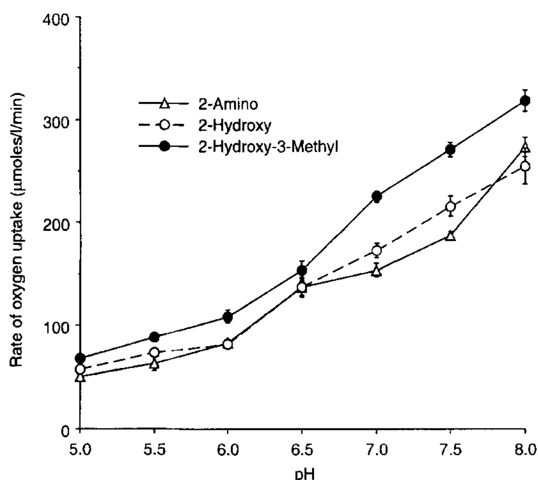

FIGURE 3 Effect of pH on the rates of autoxidation of 2-hydroxy-, 2-hydroxy-3-methyl- and 2-amino-1,4-naphthohydroquinone. Conditions were as described in the legend to Figure 1.

The relative rates of oxidation of the various compounds at pH 7.4 are shown in Table I. Large variations in rate were recorded, with the fastest compound (2,3-dichloro-1,4-naphthohydroquinone) oxidising nearly 28 times faster than the slowest (1,4-naphthohydroquinone). At the completion of the reaction, between 0.95 and 1.01 mol of oxygen were consumed for every mole of hydroquinone oxidised. Addition of catalase at this time led to the return of between 44% and 48% of the oxygen consumed (data not shown).

SOD inhibited the autoxidation of the alkoxy, amino and hydroxy derivatives. The enzyme was without effect on the rate of oxidation of 1,4-naphthohydroquinone and the 2,3-dichloro and 2-bromo derivatives while that of 5-hydroxy-1,4-naphthohydroquinone was increased (Table I). Addition of the parent naphthoquinone increased the rate of autoxidation of all the compounds except the 2,3-dichloro, 2-bromo and 5-hydroxy derivatives. The effect was particularly marked with 1,4-naphthohydroquinone itself (Table I).

Addition of quinone partially overcame the inhibitory effect of SOD on the autoxidation of the 2-methyl, 2,3-dimethyl, 2-hydroxy, 2-hydroxy-3-methyl, 2-methoxy and 2-amino derivatives

TABLE I Relative rates of 1,4-napthohydroquinone autooxidation at pH 7.4, and effects of SOD and quinone. Rates were measured as rates of oxygen uptake (μmol/l/min) at 25°C. The initial concentration of hydroquinone was 50 μM. When added, the concentration of SOD was 10 μg/ml, and that of the parent napthoquinone was 50 μM. Results shown are the means and SEM of at least 3 separate determinations

Naphthohydroquinone derivative	Control	+SOD*	+Quinone*	+ SOD + Quinone†
2-Methyl	23.8 ± 0.8	3.80 ± 0.15 (−84%)	32.0 ± 1.1 (+34%)	9.43 ± 0.31 (+147%)
2,3-Dimethyl	28.6 ± 1.1	0.72 ± 0.06 (−97%)	43.5 ± 1.1 (+52%)	2.73 ± 0.11 (+279%)
2-Hydroxy	175 ± 4	0.88 ± 0.13 (−99%)	206 ± 11 (+17%)	1.24 ± 0.02 (+41%)
2-Hydroxy-3-methyl	221 ± 7	1.45 ± 0.12 (−99%)	256 ± 7 (+16%)	2.32 ± 0.08 (+53%)
2-Methoxy	120 ± 5	20.4 ± 0.9 (−83%)	152 ± 3 (+27%)	64.4 ± 3.5 (+216%)
2,3-Dimethoxy	67.3 ± 5.2	23.0 ± 0.8 (−66%)	110 ± 6 (+63%)	75.9 ± 2.7 (+230%)
2-Amino	184 ± 7	11.3 ± 0.4 (−94%)	212 ± 6 (+16%)	41.3 ± 1.2 (+265%)
2-Bromo	290 ± 25	324 ± 16 (NS)	258 ± 7 (NS)	290 ± 2 (NS)
2,3-Dichloro	352 ± 5	351 ± 12 (NS)	343 ± 3 (NS)	350 ± 11 (NS)
5-Hydroxy	313 ± 6	357 ± 10 (+14%)	289 ± 9 (NS)	331 ± 12 (NS)
1,4-Unsubstituted	12.6 ± 0.3	13.1 ± 0.3 (NS)	32.6 ± 0.3 (+159%)	47.6 ± 0.9 (+263%)

*Figures in brackets refer to the percentage change compared to the control rate. NS = not significant. All other values are significantly different at $P < 0.01$.
†Figures in brackets refer to the percentage change compared to the rate in the present of SOD. NS = not significant. All other values are significantly different at $P < 0.05$.

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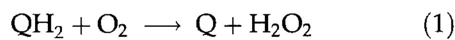
and completely negated the effect of this enzyme on the autoxidation of 2,3-dimethoxy-1,4-naphthohydroquinone (Table I).

DISCUSSION

The overall reaction of hydroquinone (QH_2) autoxidation[34,35] is described by Reaction (1). This stoichiometry was observed with all the hydroquinones of the present experiments, with an uptake of close to 1 mol of oxygen for every mole of hydroquinone oxidised. The formation of hydrogen peroxide during the oxidation is shown by the observation that oxygen was returned to solution after addition of catalase, which destroys hydrogen peroxide according to Reaction (2). The amount of oxygen released by catalase in all cases was almost 50% of that utilised, as expected from the stoichiometry of this reaction:

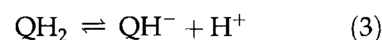


$$QH_2 + O_2 \longrightarrow Q + H_2O_2 \quad (1)$$

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

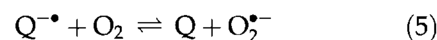
The rates of autoxidation of the various hydroquinones were strongly dependent upon structure. At pH 7.4, the rates decreased in the order 2,3-dichloro > 5-hydroxy > 2-bromo > 2-hydroxy-3-methyl > 2-amino > 2-hydroxy > 2-methoxy > 2,3-dimethoxy > 2,3-dimethyl > 2-methyl > unsubstituted naphthohydroquinone. These results are consistent with earlier reports of a relatively low rate of oxidation of 2-methyl-1,4-hydroquinone[25,26,33,38] and fast oxidation of the 2-hydroxy and 5-hydroxy derivatives.[25,26] It should be noted, however, that although the rates of oxidation varied, all the hydroquinones were unstable at neutral pH, and even the slowest compound underwent more than 50% oxidation in less than 2 min.

The rate of oxidation of a hydroquinone by oxygen depends upon its oxidation–reduction potential; the lower the potential relative to that of oxygen, the faster the reaction.[46] Compounds with low potentials, as measured by half-wave potentials, would therefore be expected to oxidise faster than those with high potentials. However, the mechanism of hydroquinone autoxidation (Reactions (3)–(7)) is complex[28,35,42] and many other factors, particularly the one-electron reduction potentials of the hydroquinone/semiquinone and semiquinone/quinone couples, will influence the overall rate of oxidation:



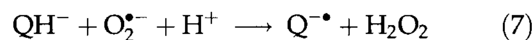
$$QH_2 \rightleftharpoons QH^- + H^+ \quad (3)$$

$$QH^- + O_2 \longrightarrow Q^{-\bullet} + O_2^{\bullet-} + H^+ \quad (4)$$

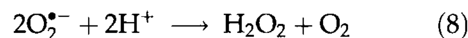


$$Q^{-\bullet} + O_2 \rightleftharpoons Q + O_2^{\bullet-} \quad (5)$$

$$Q + QH^- \rightleftharpoons 2Q^{-\bullet} + H^+ \quad (6)$$



$$QH^- + O_2^{\bullet-} + H^+ \longrightarrow Q^{-\bullet} + H_2O_2 \quad (7)$$



$$2O_2^{\bullet-} + 2H^+ \longrightarrow H_2O_2 + O_2 \quad (8)$$

Ionisation of the hydroquinone (Reaction (3)) is a prerequisite for autoxidation. The rate of autoxidation will therefore increase with increasing pH, since the concentration of anion available for reaction will increase. Furthermore, at a particular pH, the lower the pK_a of a hydroquinone, the faster will be its rate of oxidation. Oxidation is initiated by reaction of the anion of the hydroquinone (QH^-) with molecular oxygen (Reaction (4)). The semiquinone ($Q^{-\bullet}$) formed in this reaction and via comproportionation between the quinone (Q) and the hydroquinone (Reaction (6)) is oxidised by oxygen (Reaction (5)), and the superoxide formed in this process and in Reaction (4) oxidises more hydroquinone (Reaction (7)). If Reaction (7) is important to the overall rate of oxidation, SOD will decrease the rate of reaction through destruction of superoxide via Reaction (8). Conversely, if (5) is important, SOD will increase the rate of oxidation by driving the equilibrium of this reaction to the right. Since semiquinones react with molecular oxygen very much faster than the corresponding hydroquinones,[56–59] it would be expected that addition of quinone would increase the rate of hydroquinone

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oxidation, since the equilibrium of Reaction (6) would be driven to the right and the concentration of semiquinone thereby increased.

On the basis of their rates of oxidation and the effect of the various interventions, the hydroquinones investigated in the present study can be divided into 4 categories.

The first, which comprises 2-methyl-, 2,3-dimethyl-, 2-methoxy- and 2,3-dimethoxy-1,4-naphthohydroquinone, were slowly oxidised at low pH. The rate of autoxidation increased with increasing pH, but even at pH 7 the rates of oxidation of these compounds were comparatively low, as expected from their low degree of ionisation (< 0.5% at this pH) and their relatively high half-wave potentials (Table II). In accord with previous observations,[27,36] the autoxidation rates were markedly decreased by SOD, indicating an important role for superoxide as a radical chain initiator via Reaction (7). Addition of quinone increased the rate of oxidation, particularly with the 2,3-dimethoxy derivative. Furthermore, quinone addition partially or completely overcame the inhibition seen in the presence of SOD, indicating that the comproportionation reaction can provide an alternative pathway for oxidation when Reaction (7) is abolished, in accord with findings in related systems.[60]

TABLE II pK_a values, half-wave potentials and equilibrium constants for reaction of semiquinone with oxygen (Reaction (5)), for the 1,4-naphthohydroquinones

Naphthohydroquinone derivative	pK_a*	Half-wave potential (mV)†	Equilibrium constant of Reaction (5)‡
2-Methyl	9.6	+20	6.5
2,3-Dimethyl	10.0	−48	28
2-Hydroxy	8.7	−86	26 000
2-Hydroxy-3-methyl	8.9	−106	
2-Methoxy	9.4	−39	3.0
2,3-Dimethoxy	9.5	−30	28
2-Amino	9.6	−128	2800
2-Bromo	8.5	+103	
2,3-Dichloro	7.5	+98	9600
5-Hydroxy	6.6	+82	8900
1,4-Unsubstituted	9.4	+80	0.6

*Data from Refs. [61–63].
†Data from Refs. [38,64,65]. Potentials are referred to the normal hydrogen electrode.
‡Calculated from one-electron reduction potentials by the method of Swallow.[66]

The second group of hydroquinones, comprising the 2-hydroxy, 2-hydroxy-3-methyl and 2-amino derivatives, underwent appreciable oxidation at pH 5. The reaction rate showed comparatively little change with increasing pH, indicating that degree of ionisation was of relatively little importance in determining the rate of oxidation of these substances. Although the pK_a values of the hydroxy derivatives are comparatively low (Table II), they would still be less than 0.02% ionised at pH 5. It is possible that the highly negative potentials of these substances (Table II) compensate for the low degree of ionisation. The oxidation of these substances is strongly dependent on superoxide-driven reactions, as indicated by > 90% inhibition of oxidation by SOD. The effect of added quinone was generally smaller than that seen with the Group 1 compounds, indicating that comproportionation is less important in the overall oxidation pathway. The effect of quinone in overcoming the inhibitory effect of SOD was also lower with the hydroxy derivatives than with the Group 1 compounds, but a pronounced effect was recorded with 2-amino-1,4-naphthohydroquinone.

The third group comprises 2-bromo-, 2,3-dichloro- and 5-hydroxy-1,4-naphthohydroquinone. These substances were stable at pH 5, but oxidation rates increased rapidly with increasing pH and at pH 7.4 or above they were higher than any of the other substances investigated in the present study. These compounds have low pK_a values (Table II) and their extensive ionisation must compensate for their high oxidation potentials (Table II). Addition of quinone had little effect on the rate of oxidation of these substances, indicating that comproportionation does not play an important role in the oxidation reaction. Superoxide dismutase had no significant effect on the rate of oxidation of the halogenated derivatives, but that of 5-hydroxy-1,4-naphthoquinone was increased, in accord with earlier findings.[36]

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This is consistent with the fact that the equilibrium of Reaction (5) lies far to the left for this compound (Table II).

1,4-Naphthohydroquinone is in a group of its own. The reaction rate was markedly increased by addition of quinone, indicating an important role for Reaction (6) in the oxidation. SOD was without effect, although an increase would have been expected because the equilibrium of Reaction (5) lies to the left (Table II). It is possible that at the beginning of the reaction, when the rate was measured, the concentration of quinone was too small for a measurable effect. In other studies,[28] it has been shown that SOD stimulates the reaction only when quinone is available to propagate the reaction via Reaction (6), consistent with the observation that SOD markedly increased the rate in the presence of added quinone.

The rates of naphthohydroquinone autoxidation are clearly very variable, as are the pathways by which the reaction proceeds. The hydroxy and amino derivatives underwent rapid autoxidation in a reaction in which superoxide was an important chain propagator, with comproportionation a less important pathway. The rates of oxidation of the alkyl and alkoxy derivatives were slower, with both chain propagation by superoxide and comproportionation being important in the reaction mechanism. Comproportionation was particularly important in the autoxidation of 1,4-naphthohydroquinone itself, but neither this process nor superoxide-driven reactions were significant in the very rapid oxidations of the halogen derivatives and 5-hydroxy-1,4-naphthoquinone, with which the reactions of hydroquinone and semiquinone with oxygen appear to predominate in the autoxidation reaction.

The results of the present experiments provide, for the first time, a comparison of the rates and pathways of naphthohydroquinone autoxidation under standard reaction conditions. It is possible that both the rate and mechanism of hydroquinone autoxidation are important in determining whether or not hydroquinone formation is a detoxification process for quinones *in vivo*. The present data will provide a logical basis for selecting compounds for testing this hypothesis. It is known that 2,3-dimethyl-, 2-methoxy- and 2,3-dimethoxy-1,4-naphthoquinone are readily reduced by DT-diaphorase.[26] Since the behaviour of the hydroquinones formed from these substances was similar to that of the 2-methyl derivative, it would be predicted that their toxicity, like that of the 2-methyl derivative, would be decreased by increasing tissue levels of DT-diaphorase. Conversely, in view of the characteristics of the autoxidation of the hydroquinones derived from 2-hydroxy-3-methyl- and 2-amino-1,4-naphthoquinone, it would be expected that the toxicity of these substances would increase with increasing DT-diaphorase levels, as it does with 2-hydroxy-1,4-naphthoquinone.[30] Studies on these possibilities are in progress.

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