Bactericidal Agents Generated by the Peroxidase-catalyzed Oxidation of para-Hydroquinones*

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For the three Gram-negative bacteria, Pseudomonas fluorescens, Escherichia coli, and Erwinia amylovora, p-benzoquinone was the principal bactericidal agent formed in vitro during the oxidation of hydroquinone by horseradish peroxidase, whereas no toxicity could be associated with either phenolic or oxygen-free radicals. Even the continuous generation of p-benzoquinone during the simultaneous reduction of p-benzoquinone by xanthine oxidase and reoxidation of hydroquinone by peroxidase was no more toxic than p-benzoquinone alone. Anaerobiosis had no effect on the toxicity of either p-benzoquinone or the peroxidase reaction and the generation of superoxide and hydroxyl radicals catalyzed by xanthine oxidase was not bactericidal. Substitutions on the p-benzoquinone ring decreased quinone toxicity in rough proportion to the decrease in quinone redox potential, suggesting that strong oxidizing potentials are important for such quinone toxicity.

The oxidation of phenolic compounds by plant peroxidases (donorhydrogen peroxide oxidoreductase, EC 1.11.1.7) has long been known to increase bactericidal activity (1–3), but the precise nature of the killing agent or agents produced is obscured by the large array of possible reactions. Unlike mammalian peroxidases, which oxidize halides to form toxic hypohalous acids, plant peroxidases oxidize a wide range of phenolics to produce phenolic free radicals (semiquinones). These free radicals may dismutate to form quinones and hydroquinones, polymerize into more complex phenolics, or reduce oxygen to yield superoxide and possibly other activated oxygen species; all of these secondary products may undergo further reactions. The bactericidal activity resulting from oxidation of some phenolics lasts for only a few minutes (1, 2), which has led to the hypothesis that semiquinones may be toxic agents in plant disease resistance (4–6). Semiquinones are also believed to be involved in the cytotoxic mechanisms of several quinone-based anti-tumor agents in mammalian systems (7–9). However, the quinone products are known to be toxic (10) and can also be relatively unstable due to spontaneous secondary autoxidation to less toxic humic substances.

In the present study, bacterial survival was determined after short term exposure to a variety of reactions involving hydroquinone, horseradish peroxidase, and their reaction products to determine the principal bactericidal agents produced. Peroxidase oxidizes hydroquinone to a semiquinone anion, which rapidly dismutates to yield p-benzoquinone and hydroquinone. The chemistry of this reaction is simple in comparison to most other phenolics because p-benzoquinone is the principal product of the reaction and is a stable species. Most of the bactericidal assays utilized Pseudomonas fluorescens, a Gram-negative soil and water saprophyte that has been widely used as a comparison organism in plant pathology due to its taxonomic relationship with a broad range of plant pathogenic pseudomonads (11). Experiments were also carried out using Escherichia coli and Erwinia amylovora. The latter bacterium causes fire blight in pears, and hydroquinone and its β-glucoside, arbutin, are implicated in the resistance of pear trees to infection by Er. amylovora (12). The results indicate that p-benzoquinone, and not any free radical intermediate, is the chief toxic agent produced during the peroxidase-catalyzed oxidation of hydroquinone.

**MATERIALS AND METHODS**

Methyl-p-hydroquinone, trimethyl-p-hydroquinone, monochloro-p-hydroquinone, and tetrachloro-p-benzoquinone were purchased from Aldrich. The 2,6- and 2,5-dichloro-p-benzoquinone isomers were a gift from Dr. Charles Yocum, University of Michigan. Hydroquinone, EDTA, diethylenetriaminepentaacetic acid, and all proteins were purchased from Sigma, while Heps was obtained from U.S. Biochemical Corp.

para-Benzoinone (practical grade) was obtained from Eastman Kodak and initially purified by steam distillation. The yellow crystals were collected by vacuum filtration, washed with cold water, and resublimed under vacuum. Stock solutions of p-benzoquinone (10 mM) in distilled water were stored at −20 °C until use in the bactericidal assays. There was no detectable difference in toxicity between freshly dissolved and frozen p-benzoquinone solutions. The quinones of the various substituted p-hydroquinones were obtained by oxidation as described by Rich (13). Hydrogen peroxide (30%) was assayed spectrally (ΔE260 = 43.6 m2 M−1 cm−1; Ref. 14). It contains 5 mg/ml of both tartaric acid and sodium stannate as stabilizers (15), which were removed by steam distillation for several experiments. However, no differences were observed between steam-distilled and untreated hydrogen peroxide, so undistilled hydrogen peroxide was used in the studies reported here.

Xanthine oxidase (Grade I) was measured at 450 nm (3.78 × 10^6 M−1 cm−1; Ref. 16); whereas the oxidation of xanthine was monitored by the rate of urate formation (ΔE260 = 1.1 × 10^6 M−1 cm−1; Ref. 17).

**P. fluorescens** was obtained from Carolina Biological Supply and grown on plain nutrient agar (BBL, Microbiology Systems, Inc.). Cultures were preserved by storing agar slants under sterile mineral oil at room temperature and revived by overnight growth in 15 ml of nutrient broth (BBL) for use as inocula. Liquid cultures for the bactericidal assays were inoculated with 0.2 ml of the overnight broth culture into 125-ml flasks containing 15 ml of nutrient broth and incubated at 30 °C on a tilted rotating arm for 4 h. Cells were centrifuged (3000 × g for 10 min), washed once with 10 ml of 100 mM Na-Hepes, pH 7.0, and resuspended in the same buffer at a titer of 2–3 × 10^7 bacteria/ml.

Control experiments showed that early log phase bacteria were more susceptible to p-benzoquinone toxicity than

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1 The abbreviation used is: Heps, N-2-hydroxyethylpiperazine-N'2-ethanesulfonic acid.
late log and stationary phase cultures, so only early log phase cultures were used in the experiments reported here.

_**E. coli**, ATCC 29682, was grown in nutrient broth as described for _P. fluorescens_. _Er. amylovora_, strains E8 and E9, were obtained from Dr. R. N. Goodman, University of Missouri. E8 is a capsule-minus mutant derived from E9. Cultures were grown on GYE medium (2.0% nutrient broth, 1% glucose, and 0.5% yeast extract, adjusted to pH 7.0, with NaOH before autoclaving) for 5 h on a rotating arm at 30 °C. The dilutions from bactericidal assays were grown on plain nutrient agar because GYE agar yielded large mucoid colonies that overgrew other colonies, obscuring possible bactericidal effects.

Two types of bacterial assays were conducted: concentration series experiments, where the bacteria were exposed to the putative toxic agent in a 1.0-ml volume for either 10 or 30 min, and time course experiments, where 1.0-ml aliquots were withdrawn from a 10-ml volume at 0, 1, 3, 6, and 9 min after adding the toxic agent. In both cases, the assays were quenched with 10 μl of 10 mM ascorbic acid containing 5% (v/v) β-mercaptoethanol and survival was determined as described above. Assays were incubated at 25 °C and buffered with 50 mM Na+-Hepes, pH 7.0.

Viable counts were made using a modified microdrop technique (18). Plastic Petri plates were marked on the bottom to form 10 sectors. Once the quenching solution was added, the aliquots were quickly diluted through a series of three 10-fold dilutions in 50 mM K+-phosphate buffer, pH 7.0, 5 mM NaCl, and 0.5 mM MgSO4. Late in the study, KCl was found to contribute to p-benzoquinone toxicity in _E. coli_. Dilution blanks with distilled water were used in subsequent experiments and are identified as such in the results. Starting with the lowest dilution, at least three 10-μl drops of each dilution were placed in the previously marked sectors on the Petri plate. The plates were stored upright at room temperature for 1 h to allow the drops to dry completely and then incubated at 30 °C. Because the toxic agents often had a bacteriostatic effect, the plates were counted each day for 3 days. Only sectors containing between 4 and 40 colonies were counted.

Anaerobic bactericidal assays with _P. fluorescens_ were conducted using 5 ml of solution in a test tube (16 × 100 mm) fitted with a rubber septum. The septum contained a cannula for bubbling, a short escape needle, and a second cannula for removing aliquots for plating. Commercial nitrogen was freed of residual oxygen by passage over hot copper turnings, rehumidified, and then bubbled through the assay solution for 12 min prior to and throughout the assay. Individual components of the assay were also bubbled with nitrogen prior to use and then added with Hamilton syringes. Because _P. fluorescens_ is a strict aerobic, dilution and plating were necessarily done aerobically. To overcome this, anaerobic experiments were also conducted with _E. coli_ in a Coy anaerobic chamber (Ann Arbor, MI), with all solutions and cultures stored inside the chamber for several days to allow equilibration with the anaerobic atmosphere. The bacterial suspension and enzyme solutions were thoroughly flushed with nitrogen and then placed in the chamber just prior to use. Assays, dilutions, plating, and growth were all carried out in the chamber. _E. coli_ was plated on trypticase soy agar for the anaerobic experiments because it gave better growth anaerobically than nutrient agar.

**RESULTS**

Para-benzoquinone was as toxic to _P. fluorescens_ as the complete reaction mixture containing hydroquinone, equimolar hydrogen peroxide, and peroxidase over all concentrations tested (Fig. 1). Virtually identical results were obtained with _E. coli_ and strains E8 and E9 of _Er. amylovora_ (data not shown). The two _Erwinia_ strains were equally sensitive to p-benzoquinone and there was no difference between the toxicity of p-benzoquinone and the hydroquinone-peroxidase reaction. Individual components of the hydroquinone-peroxidase reaction were far less toxic when tested separately or pairwise than the complete reaction mixture. Hydrogen peroxide and hydroquinone were nontoxic at concentrations up to 0.5 mM, only slightly toxic at 1 mM, and fully lethal at 10 mM. In K+-phosphate buffer, a metal chelator such as EDTA or diethylenetriaminepentaacetic acid was required to prevent oxidation when both hydroquinone and hydrogen peroxide were incubated together. Ultraviolet spectral and thin layer chromatography studies showed that hydroquinone was quantitatively converted to p-benzoquinone in the peroxidase reaction within a minute with 0.1 μM peroxidase and that secondary reactions such as polymerization or hydroxylation were negligible over the time span of the bactericidal assays. Peroxidase with or without hydrogen peroxide was not bactericidal, so direct oxidation of the bacteria was not responsible for the observed toxicity.

Time course experiments revealed no differences in the kinetics of killing between p-benzoquinone and the hydroquinone-peroxidase reaction (Fig. 2). Following a short lag, there was an exponential decrease in survival from which apparent first order rate constants could be estimated. The first order rate constants were proportional to quinone concentration (Fig. 3) and yielded an apparent second order rate constant (_k2_) for p-benzoquinone toxicity. For _P. fluorescens_ in 50 mM Na+-Hepes buffer, pH 7.0, _k2_ was estimated to be 80 M⁻¹s⁻¹. _P. fluorescens_ was far more susceptible to p-benzoquinone in K⁺-phosphate buffer (_k2_ = 1.3 × 10⁷ M⁻¹s⁻¹), which was traced to the presence of KCl in the physiological saline used to suspend the bacteria before the assays. Adding either RBCl, KCl, KBr, or KI to Na+-Hepes buffer increased p-benzoquinone toxicity, whereas Na⁺ or Li⁺ halides were not effective (Fig. 4). Potassium phosphate or nitrate was ineffective, indicating that the nature of the anion was also important for the enhanced toxicity. If _P. fluorescens_ was preincubated with 100 mM KCl for 4 min, centrifuged, and then resuspended, the toxicity of p-benzoquinone in Na+-Hepes buffer was the same as with untreated bacteria. Buffers with pH values more alkaline than 7.0 increased the toxicity of p-benzoquinone. The toxicity of the hydroquinone-peroxidase reaction was identical with that of p-benzoquinone in either K⁺-phosphate buffer or in Na+-Hepes buffer with KCl added. For _E. coli_, _k2_ was estimated to be 340 M⁻¹s⁻¹ in the presence of 50 mM KCl and only 5 M⁻¹s⁻¹ in Na+-Hepes buffer alone, whereas with
Toxicity Due to Hydroquinone Oxidation

**FIG. 2.** Time courses of toxicity for the hydroquinone-peroxidase reaction and p-benzoquinones. *P. fluorescens* was exposed to 10 μM (●), 100 μM (○), 200 μM (■), or 300 μM (▲) quinone. Closed symbols refer to p-benzoquinone alone, whereas the open symbols with dashed lines refer to identical concentrations of hydroquinone, equimolar hydrogen peroxide, plus 0.1 μM peroxidase. Survival was determined with water dilution blanks.

**FIG. 3.** Rate of p-benzoquinone toxicity versus quinone concentration. Apparent first order rate constants (k) were estimated from the log-linear phase of experiments similar to those shown in Fig. 2 and plotted as a function of p-benzoquinone concentration. Closed symbols refer to p-benzoquinone alone, whereas open symbols refer to equimolar concentrations of hydroquinone and hydrogen peroxide plus 0.1 μM peroxidase. ●, *P. fluorescens* in 50 mM Na+-Hepes buffer, pH 7.0, with water dilution blanks; ○, *Er. amylovora* in the same buffer; and ■, *P. fluorescens* in 50 mM K+-phosphate buffer, pH 7.0, using K+-phosphate dilution blanks; ▲, *E. coli* in Na+-Hepes buffer; and ◆, *E. coli* in the same buffer with 50 mM KCl.

*Er. amylovora* in Na+-Hepes buffer, k2 was 1.0 × 10^3 M^-1 s^-1 and was not affected by either KCl or K+-phosphate buffer.

Chloro- and methyl- substitutions to the p-benzoquinone ring decreased quinone toxicity with *P. fluorescens* (Fig. 5). A 30-min exposure was used because many of the quinones had so little toxicity. Chlorinated p-benzoquinones were only slightly less toxic than p-benzoquinone, with toxicity decreasing as more ring positions became substituted. However, the addition of methyl groups to p-benzoquinone led to a rapid loss of toxicity. Thymoquinone (2-isopropyl-5-methyl-p-benzoquinone) and 2,5-dihydroxy-p-benzoquinone were nontoxic. No differences in toxicity were observed in the complete peroxidase reaction with the monomethyl-, trimethyl-, tetramethyl-, monochloro-, and tetrachloro-p-hydroquinones when compared to the equivalent p-benzoquinones (data not shown). The remaining substituted hydroquinones were not assayed in the peroxidase reaction.
A variety of attempts to increase semiquinone formation failed to increase toxicity over that which could be accounted for by p-benzoquinone alone. Addition of equimolar hydroquinone with p-benzoquinone to favor the formation of semiquinone by xanthine oxidase and peroxidase did not affect the toxicity of p-benzoquinone. However, the amount of semiquinone formed would be small because the equilibrium constant is only 1 \times 10^{-8} at pH 7.0 (19). A convenient reaction to extend the period of semiquinone formation involved the reduction of p-benzoquinone by xanthine and xanthine oxidase (16, 20). If xanthine and xanthine oxidase are added to a mixture of p-benzoquinone, peroxidase, and hydrogen peroxide, semiquinone can be generated by a cycle involving reduction of p-benzoquinone by xanthine oxidase and oxidation of hydroquinone by peroxidase. Continuous generation of semiquinone did not increase the toxicity of p-benzoquinone, methyl-p-benzoquinone, or 2,6-dimethyl-p-benzoquinone (Fig. 6). The xanthine oxidase-peroxidase reaction in the absence of any quinone was not toxic, even in the presence of 100 \mu M ferric EDTA or in phosphate buffer.

Anaerobiosis had no effect on the toxicity of p-benzoquinone or the xanthine oxidase-peroxidase reaction for either P. fluorescens (Fig. 6) or E. coli (Fig. 7). P. fluorescens is a strict aerobe but tolerates brief exposures to anoxia, allowing the bubbling of the reaction with nitrogen during the assay period. When hydrogen peroxide is present in the xanthine oxidase-peroxidase assay, complete anaerobiosis cannot be achieved because bacterial catalases will convert some of the hydrogen peroxide into oxygen (although less than 3% of the added hydrogen peroxide was consumed by this route by P. fluorescens during the assay period). However, the xanthine oxidase-peroxidase reaction was no more toxic than adding p-benzoquinone alone under anaerobic conditions. In addition, the dilution and plating of P. fluorescens had to be done aerobically, which might allow oxygen to contribute to p-benzoquinone toxicity during the postreaction period. E. coli, which can be grown under strict anaerobic conditions, was as sensitive to p-benzoquinone toxicity when exposed, plated, and grown anaerobically in a Coy chamber as when exposed aerobically. The xanthine oxidase-peroxidase reaction was also no more toxic to E. coli anaerobically than p-benzoquinone alone (Fig. 7).

Xanthine oxidase was not inactivated during the xanthine oxidase-peroxidase reaction because xanthine oxidation was linear over the 9-min assay period. Semiquinone formation could be monitored spectrally by the reduction of cytochrome c, because cytochrome c is reduced almost exclusively at physiological pH values by the semiquinone anion at a rate much faster than semiquinone dismutation (19, 21). The rate of cytochrome c reduction by hydroquinone is negligible at pH 7.0 (21). Addition of any of the three benzoquinones stimulated the rate of xanthine oxidation and cytochrome c reduction (Table I), but completely inhibited oxygen consumption as measured with a Clark oxygen electrode. Oxygen consumption resumed once the quinones were fully reduced.

Fig. 6. Toxicity due to continuous formation of semiquinone by xanthine oxidase and peroxidase. Each assay contained the following: 100 \mu M hydrogen peroxide, 100 \mu M EDTA, 100 \mu M xanthine, 0.1 \mu M peroxidase, 0.033 \mu M xanthine oxidase, 50 mM KCl, and 50 mM Na+-Hepes, pH 7.0. P. fluorescens was exposed to the complete assay plus 40 \mu M p-benzoquinone (b), 40 \mu M methyl-p-benzoquinone (c), 2,6-dimethyl-p-benzoquinone (x), or minus quinone (x). □ represents the complete p-benzoquinone assay run while bubbling nitrogen through the solution, whereas ■ and ○ represent the toxicity of 40 \mu M p-benzoquinone with only 50 mM KCl added run aerobically or anaerobically, respectively. Neither of the methyl-substituted quinones had any toxicity when tested alone. The reaction was initiated by adding, in rapid succession, hydrogen peroxide, xanthine oxidase, and p-benzoquinone to the otherwise complete reaction. Survival was determined with distilled water dilution blanks.

Fig. 7. Effects of anaerobiosis on p-benzoquinone toxicity with E. coli. E. coli, grown aerobically, was exposed to 20 \mu M (●), 40 \mu M (●), or 60 \mu M p-benzoquinone (●) either aerobically (open symbols) or anaerobically (closed symbols). E. coli was also exposed to the xanthine oxidase-peroxidase reaction described in Fig. 6 (dashed lines) anaerobically using the same three p-benzoquinone concentrations.
oxidase, which implied that a maximum of 54 μM semiquinone was produced. At steady state, hydroquinone was reoxidized by peroxidase as fast as it was formed via reduction of p-benzoquinone by xanthine oxidase and dismutation of semiquinone. The first order rate constant \( k_{HRP} \) for the two-electron oxidation of hydroquinone catalyzed by 0.1 μM peroxidase was experimentally determined to be 0.31 s\(^{-1}\). Assuming that the rate of xanthine oxidation (0.05 μM s\(^{-1}\)) equals the rate of p-benzoquinone reduction, the steady state concentration of hydroquinone was then 0.16 μM. The steady state concentration of semiquinone can be calculated from the relationship

\[
\frac{d[q^-]}{dt} = v_{x0} + 2k_{HRP} [QH_2] - 2k_d [q^-]^2 = 0
\]

where \( v_{x0} \) is the rate of univalent reduction of p-benzoquinone by xanthine oxidase, estimated in Table I to be 0.068 μM s\(^{-1}\), \( 2k_{HRP} [QH_2] \) is the rate of univalent oxidation of hydroquinone by peroxidase and \( 2k_d [q^-]^2 \) is the rate of semiquinone dismutation with \( k_d \) equal to 8 × 10\(^7\) M\(^{-1}\) s\(^{-1}\) (22). Rearranging the above equation gives

\[
[q^-] = \left[ \frac{v_{x0} + 2k_{HRP} [QH_2]}{2k_d} \right]^{1/2}
\]

which yields a steady state semiquinone concentration of 0.032 μM.

**DISCUSSION**

In the peroxidase-catalyzed oxidation of hydroquinone, p-benzoquinone appears to be the principal toxic agent for *P. fluorescens*, *E. coli*, and *Er. amylovora*. Earlier reports that found roughly equivalent toxicity of hydroquinone when compared with p-benzoquinone in bactericidal assays (12, 23) did not control for the autoxidation of hydroquinone, which readily occurs in the presence of trace metals (24). The use of buffers having low metal contamination (Hepes) or of metal chelators in phosphate buffer drastically reduces the toxicity of hydroquinone in the presence of hydrogen peroxide. However, metal chelators also affect the permeability of the outer envelope of Gram-negative bacteria (25) and, as such, can affect p-benzoquinone toxicity (26).

Among the substituted p-benzoquinones tested, toxicity showed no relationship with octanol-water partition coefficients, but was correlated with the redox potential of the hydroquinone-quinone couple (Table II). The relationship between redox potential and toxicity does not exactly account for the chlorinated quinones which are somewhat better oxidizing agents but slightly less toxic than p-benzoquinone. Although fully substituted quinone rings are usually found to be nontoxic (10), tetrachloro-p-benzoquinone was highly toxic to *P. fluorescens*, which may be due to the ability of chlorine to be readily displaced from quinone rings (31). Geiger (32) reported that the mono- and dimethyl-p-benzoquinones were more toxic than p-benzoquinone to a wide range of bacteria, whereas the trimethyl- and tetramethyl-p-benzoquinones were nontoxic. In Geiger’s study, the quinones were added directly to the growth medium, so if p-benzoquinone reacted more rapidly with components in the medium than the substituted quinones, the apparent toxicity of the substituted quinones might be increased relative to p-benzoquinone.

In the present study, no toxicity could be attributed to the extracellular generation of free radicals. No system that generated semiquinone was more toxic than p-benzoquinone alone—even the continuous regeneration of semiquinone by the xanthine oxidase-peroxidase reaction. The xanthine oxidase reaction also produces superoxide anion and the presence of a suitable catalyst such as iron-EDTA will lead to the generation of hydroxyl radical (33). Oxygen-free radicals did not account for the toxicity observed in the present assays, because 1) the xanthine oxidase reaction was nontoxic in the absence of added quinone, even if run in phosphate buffer plus 100 μM iron-EDTA, and 2) anaerobiosis could not be shown to affect p-benzoquinone toxicity, either alone or in

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**Table I**

<table>
<thead>
<tr>
<th>Quinone added</th>
<th>Xanthine oxidation</th>
<th>-Peroxidase</th>
<th>+ Peroxidase</th>
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<tr>
<td></td>
<td>( \mu M/min )</td>
<td>( \mu M/min )</td>
<td>( \mu M/min )</td>
</tr>
<tr>
<td>Minus quinone</td>
<td>2.3</td>
<td>0.7</td>
<td>15</td>
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<tr>
<td>p-Benzilquinone</td>
<td>3.6</td>
<td>4.1</td>
<td>68</td>
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<td>Methyl-p-benzoquinone</td>
<td>2.8</td>
<td>2.1</td>
<td>38</td>
</tr>
<tr>
<td>2,6-Dimethyl-p-benzoquinone</td>
<td>2.8</td>
<td>1.4</td>
<td>25</td>
</tr>
</tbody>
</table>

**Table II**

<table>
<thead>
<tr>
<th>Compound</th>
<th>( LD_{50} )</th>
<th>( P )</th>
<th>( E_{pH}(QH_2) )</th>
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<tr>
<td></td>
<td>μM</td>
<td></td>
<td></td>
</tr>
<tr>
<td>p-Benzilquinone</td>
<td>10</td>
<td>1.3</td>
<td>+280</td>
</tr>
<tr>
<td>Chloro-</td>
<td>10</td>
<td>7.5</td>
<td>+300</td>
</tr>
<tr>
<td>2,5-Dichloro-</td>
<td>15</td>
<td>43</td>
<td>+305</td>
</tr>
<tr>
<td>2,6-Dichloro-</td>
<td>15</td>
<td>43</td>
<td>+305</td>
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<tr>
<td>Tetrachloro-</td>
<td>20</td>
<td>1100</td>
<td>+322</td>
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<tr>
<td>Methyl-</td>
<td>35</td>
<td>5.3</td>
<td>+224</td>
</tr>
<tr>
<td>2,6-Dimethyl-</td>
<td>170</td>
<td>17.3</td>
<td>+168</td>
</tr>
<tr>
<td>Trimethyl-</td>
<td>220</td>
<td>29.2</td>
<td>+109</td>
</tr>
<tr>
<td>Tetramethyl-</td>
<td>N.T.*</td>
<td>130.8</td>
<td>+46</td>
</tr>
<tr>
<td>2,5-Dihydroxy-</td>
<td>N.T.</td>
<td>0.06</td>
<td>+108</td>
</tr>
<tr>
<td>2-Methyl-6-isopropyl</td>
<td>N.T.</td>
<td>92</td>
<td>+169</td>
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</table>

* Not toxic.
the xanthine oxidase-peroxidase reaction.

While it is always possible that these results may be a peculiarity of the present assay system or test organisms, several explanations can be offered for the lack of toxicity of extracellular free radicals. The rapid dismutation of semiquionones limits their diffusion to well under a micrometer (27). If free radicals are generated intracellularly, their potential diffusion is further limited by nonspecific reactions with host cellular components. In yeast cells, the diffusion of primary oxygen radicals induced in situ by radiation is limited to about 3 nm (34). Extracellularly generated free radicals must penetrate the outer envelope of bacteria to react with critical intracellular components. Because the lipopolysaccharide of the outer envelope is negatively charged, anionic detergents and antibiotics are particularly ineffective bactericidal agents unless the external pH is lower than the pK, of the dissociable group (35). Hence, the outer wall should produce an electrostatic repulsion of anionic free radicals such as semiquinone and superoxide.

In contrast to the free radicals produced when hydroquinone is oxidized by peroxidase, the secondary product, p-benzoquinone, is stable, uncharged, moderately lipid-soluble, a strong oxidizing agent, and reacts rapidly with sulfhydryl groups. Schaechter and Santamassino (36) have shown that the sulfhydryl reagent, p-chloromercuribenzoate, will induce lysis in early log phase cultures of many bacterial species, including P. fluorescens, at about the minimal toxic concentration of p-benzoquinone (10 μM). Stationary phase cultures were much less sensitive to sulfhydryl reagents, which was also true of p-benzoquinone toxicity. Bernheim (37) found that HgCl₂ and oxidized mercaptoethanolamine inhibit the swelling of P. aeruginosa produced by K⁺-phosphate buffer. Intracellular K⁺ concentrations are regulated by bacteria as a function of the osmotic strength of the medium (38, 39). In the present study, KCl was found to have a synergistic effect on p-benzoquinone toxicity with P. fluorescens and E. coli, which suggests that an osmotic regulatory mechanism could be involved in p-benzoquinone toxicity.

A frequently proposed mechanism of quinone toxicity involves the intracellular reduction of quinones by various flavoproteins to form semiquinones which in turn reduce oxygen to superoxide and subsequently form other partially reduced oxygen species (40, 41). However, intracellular redox cycling of p-benzoquinone does not appear to be the cause of p-benzoquinone toxicity, because anaerobiosis had no effect on the bactericidal assays. P. fluorescens can slowly reduce p-benzoquinone (26), but this reaction is unlikely to be directly involved in toxicity. In dilute bacterial suspensions, a small fraction of the added p-benzoquinone (<5%) was rapidly reduced within the first minute, probably due to endogenous reductants. The presence of respiratory substrates such as malate or glucose extended the period of p-benzoquinone reduction, but these substrates had no effect on the toxicity of p-benzoquinone (26).

The production of free radicals during phenolic oxidation is often considered to be a sufficient explanation of toxicity. Free radicals have been suggested to be involved in plant disease resistance (4-6) and the mechanism of action of several anti-tumor quinones via intracellular reduction (7-9, 40). It should be remembered that some quinones can be quite toxic in their own right without invoking a free radical-based mechanism of toxicity.

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