Inhibition of DNA Cross-linking by Mitomycin C by Peroxidase-mediated Oxidation of Mitomycin C Hydroquinone*

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Mitomycin C requires reductive activation to cross-link DNA and express anticancer activity. Reduction of mitomycin C (40 μM) by sodium borohydride (200 μM) in 20 mM Tris-HCl, 1 mM EDTA at 37 °C, pH 7.4, gives a 50–60% yield of the reactive intermediate mitomycin C hydroquinone. The hydroquinone decays with first order kinetics or pseudo first order kinetics with a t1/2 of ~15 s under these conditions. The cross-linking of T7 DNA in this system followed matching kinetics, with the conversion of mitomycin C hydroquinone to leuco-aziridinium species and not to mitomycin C semiquinone radical anion (MC.).

The mitomycin antibiotics, produced by various Streptomyces, give rise to electrophiles capable of cross-linking DNA following either one-electron or two-electron reduction by enzymatic or chemical systems (1). The one-electron reduction product, the mitomycin C semiquinone radical anion (MC•−),1 reacts with molecular oxygen (itself a stable diradical) at close to the diffusion controlled rate (1010–1011 M−1 s−1) to give the parent mitomycin C (MC) quinone and superoxide (O2−) (2).

Since very few cross-links are required to give rise to a lethal event (3), the regeneration of MC and the production of a superoxide anion, a species of low toxicity relative to a DNA cross-link, represents a detoxification step. Under physiological oxygen concentrations, the half-life of MC•− would be expected to be less than 0.1 ms. Therefore, in the presence of physiological concentrations of oxygen, only an extremely small proportion of the MC•− produced could be involved in the direct alkylation of biomolecules, and the yields of DNA cross-links via this pathway would be negligible. The two-electron reduced species, mitomycin C hydroquinone (MCH2), does not react rapidly with oxygen and, therefore, cross-links DNA in a manner largely independent of the concentration of oxygen. Thus, the degree of initial DNA damage by MC under aerobic conditions almost exclusively depends upon a two-electron reduction of MC•− to MCH2. Under very low oxygen concentrations, greater cellular damage would be expected, reflecting the rate of production of both MCH2 and MC•−. There is also evidence that most of the damage resulting from the production of MC•− under hypoxic conditions is due to disproportionation (4) or further reduction of MCH2 to MCH2 and subsequent formation of an alkylating species therefrom, and not due to the direct interaction of MCH2 with DNA. Such processes may also play a role in aerobic alkylations following one-electron reduction, but competition from the reaction of MC•− with O2 is likely to greatly reduce the interaction of MCH2 with further reducing molecules.

In biological systems MC can be reduced by a variety of enzymes, some favoring one- and some two-electron activation (5).

Solid tumors show resistance to therapeutic modalities such as radiation due to areas of hypoxia arising from poor tumor vascularization (6). Mitomycin antibiotics have been used as an adjunct to x-irradiation to selectively target the radiation-resistant hypoxic fraction of human tumors (7, 8). Variations in the toxicity of MC to cells have been attributed to differing levels of activating enzymes, export pumps, and DNA repair. Mitomycin C hydroquinone-oxidizing enzymes give rise to a new mechanism by which oxic/ hypoxic toxicity differentials and resistance can occur.

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A number of genes coding for resistance proteins in Streptomyces lavendulae have been cloned and expressed. One of these genes, mcrA, codes for a 54-kDa protein (MCRA) that appears to be a mitomycin C hydroquinone oxidase (13, 14). Expression of the cDNA for the bacterial resistance protein MCRA in CHO-K1/dhfr− cells resulted in profound resistance to MC in these cells under aerobic conditions, with little change in sensitivity to MC under hypoxia (14). The marked resistance to MC under aerobic conditions observed in MCRA-expressing CHO-K1/dhfr− cells resembles that produced in cell lines selected for resistance to MC under aerobic conditions (14). This finding suggests that a mechanism of resistance based upon the oxidation of MCH2 by a functional homologue of the MCRA protein may be operative. Since many peroxidases have high

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affinities for hydroxylated aromatic compounds and hydroquinones as oxidizable substrates, we compared the ability of various peroxidases to that of MCRA to oxidize MCH₂ and to inhibit DNA cross-linking. In this report we demonstrate that horseradish peroxidase (HRP), myeloperoxidase (MP), and lactoperoxidase (LP) oxidize MCH₂ in the presence of a source of hydrogen peroxide and prevents to various degrees the cross-linking of T₇ DNA by MCH₂.

**Experimental Procedures**

**Chemicals**—T₇ DNA and other chemicals and enzymes were purchased from Sigma, except where specified. Peroxidase purity was assessed by determining the Rz value (the Rz value equals the ratio of the absorbance at the hemoglobin group–Soret band λ₃₄₅₅ to that of the protein band λ₄₈₅₃) for the various enzyme preparations; for HRP (affinity-purified) the Rz (A₄₈₅₃/A₃₄₅₅) value was equal to that reported for the pure protein (15), for LP the Rz value (A₄₁₂/A₂₈₀) was 89% of that reported for the pure protein (16), and for MP the Rz value (A₄₈₂/A₂₈₀) was 60% of that reported for the pure protein (17). Hoescht 33258 was obtained from Molecular Probes, Inc. (Eugene, OR). Purified MCRA was prepared by David H. Sherman, and MC was supplied by Bristol Myers-Squibb, Inc. (Wallington, CT). Stock solutions of MC (2.8 mM) and sodium borohydride (NaBH₄) (20 mM) were made in isopropanol, which was used because it is an excellent hydroxide radical scavenger. Theoretically, the indirect generation of hydroxide radicals as a consequence of the redox cycling of MC, the reduction of oxygen by NaBH₄, or the action of potentially protective peroxidases could interfere with the DNA cross-linking assay by introducing strand breaks. Therefore, since the objective was to examine DNA–DNA cross-linking by MC/NaBH₄ and enzymatic inhibition of cross-links, the prevention of radical nicking was essential to these measurements. The final concentration of isopropanol in the reaction mixture was 2.4%, which was more than sufficient to prevent radical nicking in this system (18).

**DNA Cross-linking and Nicking**—DNA cross-linking kinetics and the extent of DNA cross-linking were determined using a DNA renaturation assay (14, 19). The assay is based upon the observation that, under conditions of neutral pH, upon snap cooling thermally denatured covalently cross-linked T₇ DNA rapidly renatures since the strands are held in register, yielding a highly fluorescent complex with H33258, whereas T₇ DNA containing no cross-links does not. T₇ DNA at a concentration of 100 μg/ml in 20 mM Tris–HCl buffer containing 1 mM EDTA and 40 μM MC at pH 7.4 was reacted with NaBH₄. The cross-linking reaction was initiated by the addition of 1% by volume of freshly prepared 20 mM NaBH₄ solution in isopropanol, which was prepared by a 25-fold dilution of a 0.5 M solution of NaBH₄ in 2-Methoxyethanol ether with dry isopropanol. The final concentration of NaBH₄ in the reaction mixture was 200 μM. Small (15 μl) aliquots were removed at various time intervals, diluted 100-fold with 5 mM Tris–HCl, 0.5 mM EDTA buffer, pH 8.0, containing 0.1 μg/ml H33258, and assayed for DNA cross-links. The 100-fold dilution of DNA and alkylating agent effectively prevents significant further reaction. The concentrations of reagents were chosen to give a maximum of ~30–50% of the DNA being cross-linked (i.e. at least one cross-link in 30–50% of the molecules). The potential nicking by radicals or other species under the reaction conditions employed was ascertained by measuring the decrease in the fluorescence of the complex of H33258 and the stably pre-cross-linked DNA following a heat/chill cycle (19, 20). The pre-cross-linked T₇ DNA was prepared by treating 0.5 mg/ml DNA with 0.2 mM 1,2-bis(methylsulfonyl)-1-(2-chloroethyl)hydrazine in 10 mM Tris–HCl, 1 mM EDTA buffer, pH 8.0, for 24 h at 37°C. Fluorescence measurements were performed using a Hoefer Scientific Instruments TRO 100 fluorometer.

**Decomposition of MC by HPLC—** HPLC measurements of the loss and regeneration of MC were performed in 20 mM Tris–HCl buffer containing 1 mM EDTA, 40 μM MC, and 200 μM NaBH₄ at pH 7.4 using a modification of the method of Kumar et al. (4).

**Spectroscopic Measurement of the Formation of Mitosene**—All spectroscopic determinations were performed using a Beckman model 25 spectrophotometer at 575 nm. In these studies, MC and NaBH₄ were used at increased final concentrations of 200 and 500 μM, respectively, because of the low absorbances of MC and the MC-derived species being measured at 575 nm. Stock solutions of 10 mM MC and 50 mM NaBH₄ in isopropanol were employed for these studies. All cuvettes were freshly acid-washed (H₂SO₄/HNO₃) and stored submerged in distilled H₂O prior to use. This procedure minimized problems due to bubble formation and adhesion to the faces of the cuvettes.

**Decomposition Kinetics of NaBH₄**—The decomposition kinetics of NaBH₄ in 20 mM Tris–HCl buffer containing 1 mM EDTA and 20 μg/ml phenol red at pH 7.4 were determined by following the change in absorbance of phenol red at 560 nm, which varies linearly with very small molar changes in the consumption or generation of hydrogen ions (21). The measurements were based upon the alkalization of the medium from the consumption of 1 mol of protons/mol of NaBH₄ during its decomposition at pH values close to neutrality, as represented in the following reaction.

\[
\text{NaBH}_4 + 3\text{H}_2\text{O} + \text{H}^+ \rightarrow \text{Na}^+ + \text{H}_3\text{BO}_3^- + 4\text{H}_2\text{O} \\
\text{REACTION 1}
\]

The alkalization occurs because at pH values close to neutrality boric acid (pKₐ 10.2) is essentially undissociated. The reaction was...
The yields of MC as a result of the oxidation of MCH₂ by various oxidizing components as determined by HPLC

| Reaction mixture | Yield of MC from MCH₂ |
|------------------|----------------------|
| MC (40 μM) + NaBH₄ (200 μM) | 0% |
| MC + NaBH₄ + MCR (1 μM) | 92% |
| MC + NaBH₄ + (MCR added at t = 30 s and 30 °C) | 40% |
| MC + NaBH₄ + HRP (2 units/ml) | 69% |
| MC + NaBH₄ + HRP + H₂O₂ (100 μM) | 66% |
| MC + NaBH₄ + (HRP + H₂O₂ added at t = 30 s and 30 °C) | 33% |
| MC + NaBH₄ + H₂O₂ | 2% |
| MC + NaBH₄ + HRP + catalase (250 units/ml) | 34% |
| MC + NaBH₄ + LP (20 units/ml) + H₂O₂ | 37% |
| MC + NaBH₄ + MP (3.5 units/ml) + H₂O₂ | 10% |

*a* Half-reaction time point based on change in absorbance at 575 nm and at 30 °C.
*b* One unit will form 1.0 mg of purpurogallin from pyrogallol in 20 s at pH 6.0 and 20 °C.
*c* One unit will decompose 1.0 μmol of H₂O₂/min at pH 7.0 and 25 °C using an initial H₂O₂ concentration of 10.3 mm.
*d* One unit will produce a change in absorbance at 470 nm of 1.0 A/min at pH 7.0 and 25 °C using guaiacol as a substrate.

Visible spectral scans of MC before and at various times after the addition of NaBH₄ were performed, and a rapid bleaching followed by a rise in absorbance beyond the original level was observed, centered around 575 nm. At 37 °C, the bleaching at 575 nm was maximal within 3–5 s (Fig. 1). This bleaching was followed by a progressive increase in absorption at this wavelength to approximately twice the absorption of the initial MC solution and appeared to follow first order or pseudo first order kinetics with a 1/2 value of 14.8 ± 2.8 s at 37 °C and pH 7.4. This finding is consistent with the “pulse-like” generation of MCH₂. HPLC studies indicated that only about one half of the initial amount of MC was reduced under these conditions despite the 5-fold molar excess of NaBH₄. The short 1/2 of NaBH₄ due to its rapid reaction with the vast molar excess of H₂O provides a possible explanation for the limited reduction of the available MC. Since only ~50% of the MC was reduced, the initial reduction product(s) have little absorption at 575 nm compared with MC and the mitosenes products subsequently generated have ~2-3-fold greater absorption at 575 nm than MC.

The rate of production of mitosenes was highly temperature-sensitive with the 1/2 at 30 °C being ~30 s compared to ~15 s at 37 °C (Fig. 2A). Addition of MCR prior to the reduction of MC by NaBH₄ blocked these spectral changes under aerobic conditions (Fig. 2B). MC is believed to react following reduction as shown in Fig. 3; the methoxy group at position 9a is lost subsequent to hydroquinone formation to give leuco-aziridinomitosene. Oxidation after the formation of leuco-aziridinomitosene cannot result in the regeneration of MC, which can only occur if enzymatic oxidation occurs at the level of MCH₂. Ad-
ditation of MCRA at the point of maximum bleaching (3-5 s after the NaBH₄) gives results almost equivalent to those obtained by the addition of MCRA prior to NaBH₄. The spectroscopic absorption is restored to close to the original MC level without the production of high absorbing mitosenes; the bleaching dip is reduced slightly in magnitude when MCRA is added prior to the NaBH₄, probably due to some MCRA mediated re-oxidation occurring during the reduction phase. The late addition of MCRA, i.e. at the maximum MCH₂ concentration, served to combat the argument that the MCRA merely prevented the initial reduction of MC.

HPLC analysis indicated that, when MCRA was initially present in the reaction mixture, essentially all of the MCH₂ was back-oxidized to MC and there was little or no net MC loss or mitosenes formed (Table I). Addition of MCRA or HRP/HzO₂ at a time point equivalent to the half-reaction point (~30 s at 30 °C and pH 7.4), as judged from the spectroscopic studies (Fig. 2A), reduced the quantity of MC being restored by one half, indicating that one half of the total MCH₂ generated must still be present at this time. This finding implies that the t½ values determined spectroscopically and by DNA cross-linking reflect those for the loss of MCH₂ itself and not a subsequent low absorbing species, i.e. the conversion of MCH₂ to leucocarzinomitosene, which appears to be the rate-determining step. The kinetics do not represent a rate-determining reduction of MC by NaBH₄ since the t½ of NaBH₄ is much shorter than that observed for the 575 nm spectral changes, nor can it represent the reaction of a low absorbing component formed subsequent to the loss of the methoxy group since MC regeneration would not be possible after this point (Fig. 3).

Peroxidase Oxidation of MCH₂—Spectroscopic experiments similar to those described above were performed in which peroxidases were substituted for MCRA. Addition of HRP at equivalent time points gave visible spectral traces that were surprisingly similar to those obtained with MCRA (Fig. 4A). Surprisingly, the addition of HzO₂ was not required for HRP to block the production of the high absorbing species (Fig. 4A), to prevent the net loss of MC as determined by HPLC, or to block the cross-linking of T7 DNA by MC (Tables I and II). Thus, like MCRA, HRP catalyzed the regeneration of MC from the bleached material, although the yield was somewhat less. When these experiments were repeated using LP and MP, both of these peroxidases were able to decrease the generation of mitosenes and affect the regeneration of MC from the bleached material to various degrees, but were much less effective than HRP and MCRA (Fig. 5 and Table I). The order of effectiveness was MCRA > HRP > LP > MP.

The absence of a requirement for HzO₂ to manifest the inhibitory effects of HRP coupled with the observation that catalase antagonized the effects of HRP (Fig. 4B and Tables I and II) strongly suggested the presence of an endogenous source of HzO₂ in the reaction mixture. For a peroxidase to fully protect from the generation of MCH₂, an equivalent quantity of HzO₂ would be required if a two-electron oxidation occurred.

Endogenous Source of Hydrogen Peroxide—There are three potential sources of HzO₂ in the reaction mixture that are initially obvious: (a) the dismutation of O₂ generated from the autoxidation of MC; (b) the one-electron oxidation of MCH₂ to MC* mediated by peroxidase/H₂O₂, resulting in a self-propagating chain reaction as a result of further HzO₂ generation via MC* autoxidation and O₂ dismutation; and (c) the reduction of O₂ by NaBH₄ to generate HzO₂ directly or via O₂⁻. Mechanism (a) could not supply sufficient HzO₂ to oxidize all of the generated MCH₂ if a two-electron oxidation were occurring since MCH₂ is the major product of MC reduction by NaBH₄ under these conditions and MC* is a minor product (14). Autoxidation of the minor product MC* would result in the net generation of 1 mol of HzO₂ for every 2 mol of MC* while the peroxidation of the major product MCH₂ would require 1 mol of HzO₂/mol of MCH₂. However, if mechanism (b) were also operative, mechanism (a) could supply sufficient HzO₂ to initiate the chain reaction. To explore the feasibility of mechanism (b), which is described in Scheme 1, we have examined the oxidation of the model MCH₂ analog hydroquinone (QH₂). Oxidation of QH₂ by HRP, MP, and LP was measured and shown to require a stoichiometric quantity of HzO₂; accordingly, the oxidations catalyzed by the peroxidases proceeded at an insignificant rate in the absence of HzO₂. The data for the oxidation of QH₂ by HRP in the presence of HzO₂ is shown in Fig. 6; LP and MP gave identical traces to those obtained with HRP (see Scheme I).³

³ HRP (compound I) is the Fe³⁺O₂⁻ form of HRP and HRP (compound II) is the Fe⁷⁺O₂⁻ form of HRP.
The quantity of H$_2$O$_2$ trapped by HRP increased with detection under aerobic conditions only when the peroxidase/phenol red system was present during the decomposition of the H$_2$O$_2$. The H$_2$O$_2$ was found to remain after the decomposition of the NaBH$_4$ (Table III). Thus, H$_2$O$_2$ has a dynamic existence in this system, with H$_2$O$_2$ being generated from the reduction of O$_2$ by catalase.

The effects of various MCH$_2$ oxidizing components on the level of T7 DNA cross-linking by MC are shown in Table II. For each component, a reaction mixture was prepared and incubated for 15 min at 37°C. The percentage of cross-linking was determined by gel electrophoresis.

| Reaction mixture | Cross-linking % |
|------------------|----------------|
| T7 DNA + MC + NaBH$_4$ | 47.3 ± 1.2 |
| T7 DNA + MC + NaBH$_4$ + MCRA | 2.2 ± 1.5 |
| T7 DNA + MC + NaBH$_4$ + H$_2$O$_2$ (100 μM) | 7.5 ± 0.9 |
| T7 DNA + MC + NaBH$_4$ + H$_2$O$_2$ (100 μM) + H$_2$O$_2$ | 2.4 ± 1.2 |
| T7 DNA + MC + NaBH$_4$ | 44.4 ± 1.4 |
| T7 DNA + MC + NaBH$_4$ + H$_2$O$_2$ (100 μM) + H$_2$O$_2$ | 30.4 ± 4.1 |
| T7 DNA + MC + NaBH$_4$ + LP (4 units/ml) + H$_2$O$_2$ | 17.2 ± 1.0 |
| T7 DNA + MC + NaBH$_4$ + MP (3.5 units/ml) + H$_2$O$_2$ | 11.8 ± 0.7 |

* One unit will form 1.0 mg of purpuragallin from pyrogallol in 20 s at pH 6.0 and 20°C.
* One unit will decompose 1.0 μmol of H$_2$O$_2$ per min at pH 7.0 and 25°C using an initial H$_2$O$_2$ concentration of 10.3 mM.
* One unit will produce a change in absorbance at 470 nm of 1.0 A/min at pH 7.0 and 25°C using guaiacol as a substrate.

Effects of Peroxidases on DNA Cross-linking—Enzymes with the ability to rapidly oxidize MCH$_2$ back to MC before a significant proportion of the MCH$_2$ had undergone subsequent reactions would be expected to protect DNA against cross-linking in model systems. For this reason we compared the ability of HRP, LP, and MP to that of MCRA to protect T7 DNA from cross-linking by NaBH$_4$ reduced MC. All of the peroxidases were effective in blocking the cross-linking of T7 DNA by reduced MC, and relatively high levels of protection were produced by relatively low concentrations of some of these enzymes (Table II). Smaller differences were observed in the ability of peroxidases to block T7 DNA cross-linking by reduced MC than in their ability to inhibit the production of high absorbing mitosenes and the yields of MC as a result of the back oxidation of the reduced species. Matching kinetics were observed for DNA cross-linking by reduced MC and for the postreduction spectral changes in MC. Half-maximal effects were found to occur for both parameters in ~15 s at 37°C and pH values of 7.4 (Fig. 7) and in ~4 min at room temperature (~23°C) and pH 8.0.

The primary reason isopropanol was used as the vehicle for both MC and NaBH$_4$ was to provide protection for the T7 DNA from potential oxygen-derived radical-mediated nicking. Previously, it had been shown that the 2.4% final concentration of isopropanol used in these experiments was more than sufficient to block radical nicking of DNA (18). However, to verify that the T7 DNA was not being nicked significantly relative to the level of cross-linking, we measured the net level of cross-linking of T7 DNA stably pre-cross-linked to ~30% (30% of the population of DNA molecules contained one or more cross-links) with another agent, 1,2-bis(methylsulfonyl)-1-(2-chloroethyl)hydrazine, after which the DNA was subjected to further cross-linking in the MC/NaBH$_4$/peroxidase systems. If an intact T7 DNA molecule contained just one cross-link, the strands would be held in register and the entire molecule after thermal denaturation would renature upon snap cooling. If just one strand break was introduced into the DNA molecule, a minimum of two cross-links, depending upon their position relative to the nick, would be required to fully renature the DNA after snap cooling. Therefore, the presence of significant nicking in relation to the number of cross-links would greatly attenuate the measured cross-linking signal. If a pre-cross-linked population with an X mole fraction of the molecules containing one or more cross-links was subjected to further cross-linking by a second agent to the extent that this would give a Y mole fraction of cross-links when used as a single agent, the net mole fraction cross-linked in the resultant population if no nicking occurred would be X + (1 − X)Y. This formula approximates the sum of X and Y only if X and Y are small. The true value is somewhat less than the sum because additional cross-links added to previously cross-linked molecules would not give rise to an increase in signal. If nicking was the primary reason, for example in the >90% decrease in the cross-linking signal observed when HRP/H$_2$O$_2$ was added to the MC/NaBH$_4$ system, a comparable reduction would be expected in the pre-existing cross-linking signal and the overall level of cross-linking would be greatly reduced. In our dual
systems, it would be optimum to be able to add MCH₂ directly and not to an artifactually reduced signal due to the introduction of a large number of nicks. Therefore, the protection seen by the peroxidases was largely due to a decrease in the number of cross-links and not to an artifactually reduced signal due to the introduction of a large number of nicks.

**DISCUSSION**

The instability of MCH₂ under normal physiological conditions complicates the study of enzymes that interact with this species. Ideally, to study the oxidation of MCH₂ by enzymatic systems, it would be optimum to be able to add MCH₂ directly to reaction systems or to be able to “pulse” generate the MCH₂ in solution. NaBH₄ appears to reduce MC to MCH₂ as the major product over a short temporal window under the chosen conditions (Fig. 1) relative to the rate of subsequent MCH₂ reactions and, therefore, appears to be a suitable reductant for studies of this kind. Since the kinetics and by-products of the reactions of NaBH₄ with MC, water, and oxygen are relevant to studies on the possible role of peroxidases in the mechanism of resistance to MC, we have examined these reactions. Of particular relevance is the transient generation of H₂O₂ during the aerobic decomposition of NaBH₄, which appears to be the rate-determining step. The kinetics do not represent a rate-determining reduction of MC by NaBH₄, since the t₁/₂ of NaBH₄ is much shorter than that observed for the spectral changes, nor can it represent the reaction of a low absorbing component formed subsequent to the loss of the methoxy group since MC regeneration would not be possible.

**TABLE III**

The production and consumption of H₂O₂ during the aerobic decomposition of 200 μM NaBH₄ in 20 mM Tris-HCl, 1 mM EDTA buffer, pH 7.4. The concentration values are the arithmetic means of three determinations ± the standard deviation.

| NaBH₄ | HRPα | N₂ purge | H₂O₂ | H₂O₂ trapped |
|-------|-------|----------|------|--------------|
| μM   | units/ml | nmol/ml |  nmol/ml |
| 200  | 0      | −        | −    | 0.0          |
| 200  | 10     | −        | −    | 3.2 ± 0.1  |
| 200  | 10     | +        | −    | 0.5 ± 0.2  |
| 200  | 20     | −        | −    | 5.1 ± 0.7  |
| 200  | 30     | −        | −    | 6.3 ± 0.9  |
| 200  | 40     | −        | −    | 6.7 ± 0.5  |
| 200  | 50     | −        | −    | 7.5 ± 0.8  |
| 200  | 50²    | −        | −    | 0.1 ± 0.1  |
| 10   | 20     | −        | −    | 20.4 ± 1.2 |
| 200  | 10⁴    | −        | 20  | 0.0         |
| 200  | 10⁴    | −        | 40  | 0.0         |
| 200  | 10⁴    | −        | 80  | 4.2 ± 0.9  |

a One unit will form 1.0 mg of purpurogallin from pyrogallol in 20 s at pH 6.0 and 20 °C.

HRP added after the decomposition of the NaBH₄.

**FIG. 5. Effects of LP and MP on mitosene formation.** Panel A, the effects of LP plus/minus H₂O₂ on the production of high absorbing (575 nm) mitosene species from MC (200 μM) following reduction by 500 μM NaBH₄ at 30 °C and pH 7.4. A, control; B, control plus H₂O₂ (100 μM); C, control plus LP (4 units/ml); D, control plus LP (20 units/ml); E, control plus LP (4 units/ml) plus H₂O₂; F, control plus LP (20 units/ml) plus H₂O₂. Panel B, the effects of MP plus/minus H₂O₂ on the production of high absorbing (575 nm) mitosene species from MC (200 μM) following reduction by 500 μM NaBH₄ at 30 °C and pH 7.4. A, control; B, control plus H₂O₂ (100 μM); C, control plus MP (3.5 units/ml) plus H₂O₂; D, control plus MP (3.5 units/ml).

**FIG. 6. Oxidation of hydroquinone by HRP.** HRP catalyzed step-wise stoichiometric oxidation of hydroquinone to benzoquinone by the addition of small aliquots of H₂O₂, as determined by the loss in absorbance at 288 nm.

Cross-linking experiments, the measured net cross-linking closely matched the calculated value for the combined cross-linking signal in the absence of significant nicking (Table IV). Therefore, the protection seen by the peroxidases was largely due to a decrease in the number of cross-links and not to an artifactually reduced signal due to the introduction of a large number of nicks.

HPLC analysis confirmed the regeneration of MC from the initial reduced species (MCH₂) by MCRA and the various peroxidases. Addition of MCRA at the half-reaction point, based upon the time course of the spectroscopic changes (−30 s at 30 °C and pH 7.4), resulted in the regeneration of MC being decreased by approximately one half, indicating that one half of the total MCH₂ generated must still be present at this time. This finding implies that the t₁/₂ values determined spectroscopically by DNA cross-linking reflect those for the loss of MCH₂ itself and not a subsequent low absorbing species, i.e., the conversion of MCH₂ to leuko-aziridinomitosene, which appears to be the rate-determining step. The kinetics do not represent a rate-determining reduction of MC by NaBH₄, since the t₁/₂ of NaBH₄ is much shorter than that observed for the spectral changes, nor can it represent the reaction of a low absorbing component formed subsequent to the loss of the methoxy group since MC regeneration would not be possible.
after this point.

The cross-linking of T7 DNA by NaBH₄ reduced MC appeared to follow first order kinetics and was extremely rapid under these conditions, with half maximal cross-linking occurring in ~15 s at 37 °C and pH 7.4, matching the kinetics seen for the production of high absorbing mitosenes in the spectroscopic studies (Fig. 7). This finding implies that the same rate-determining step of MCH₂ to leuco-aziridinomitosene was scopic studies (Fig. 7). This finding implies that the same

for the production of high absorbing mitosenes in the spectroscopic studies in these conditions, with half maximal cross-linking occurring in ~15 s at 37 °C and pH 7.4, matching the kinetics seen for the production of high absorbing mitosenes in the spectroscopic studies (Fig. 7). This finding implies that the same rate-determining step of MCH₂ to leuco-aziridinomitosene was limiting in both of these processes. If the enzymes were oxidizing MCH₂, their inclusion should block T7 DNA cross-linking in the MC/NaBH₄ system; this indeed occurred, with MCRA, HRP, LP, and MP strongly blocking the measured cross-linking signal (Table III).

Single-strand breaks could arise from radicals that theoretically could be generated by the interaction of components in the reaction mixture and appear to reduce the level of cross-linking of DNA that was measured. However, experiments involving the additional cross-linking of stably pre-cross-linked DNA in the presence and absence of protective systems were consistent with the absence of significant nicking and the inhibition of cross-link formation. Unlike MCRA, concentrations of HRP, MP, and LP that largely blocked the cross-linking of T7 DNA were not as effective in preventing the net loss of MC or the spectral changes in the cases of MP and LP. Therefore, significant quantities of products other than MC must be produced by the HRP-, LP-, and MP-mediated oxidation of MCH₂, or a large fraction of the protection from the cross-linking of DNA via these peroxidases arose from the preferred oxidation of species subsequent to MCH₂ formation but prior to cross-link formation. Preferential oxidation of the species formed after the loss of the methoxy group could restrict the molecule to monofunctional alkylaition that would not be detected in our system. Oxidation to species that may not be able to be reductively reactivated to a cross-linking or alkylating species may result in superior resistance to that resulting from the regeneration of MC. Thus, it seems feasible that plant peroxidases such as HRP may offer plant roots some protection from the toxic products of Streptomyces and other soil bacteria.

Variations in the sensitivity of neoplastic cells to MC have been attributed to differing activities of reductive activating enzymes, export pumps, and DNA repair enzymes (9–12). The existence of enzymes capable of oxidizing MCH₂ back to the MC prodrug or other inactive forms gives rise to another possible mechanism by which resistance to the mitomycin antibiotics and toxicity differentials between oxygenated and hypoxic tumor cells could arise. Recently, work from our laboratory has shown that MCRA expressed in CHO-K1/dhfr⁻ cells conferred profound resistance to MC under aerobic conditions only, resulting in a phenotype with an extremeoxic/hypoxic differential (14). High levels of resistance only under aerobic conditions resembles that produced in cell lines selected aerobically for MC resistance (see Ref. 14 for the appropriate references). These findings suggest that a mechanism of resistance based upon the oxidation of MCH₂ by proteins that are functional homologues of MCRA could be a mechanism contributing to resistance to MC.

It is of interest to note that one of a group of proteins cloned because of their ability to block the hypersensitivity of Fanconi anemia lymphoblastoid cells to MC was tentatively identified as a peroxidase based upon sequence analysis (23); moreover, this protein showed the appropriate induction after H₂O₂ exposure (24). It should be noted that the selective loss of two-electron reducing pathways responsible for the toxicity under aerobic conditions could result in similar resistance profile if the total two-electron flux was small compared with the total one-electron flux, resulting in an insignificant contribution of two-electron reducing mechanisms to the total toxicity under hypoxia. If some form of peroxidase activity were involved in the oxidative detoxification of MCH₂, a source of H₂O₂ would also be required. MC could supply this source of H₂O₂ under aerobic conditions as a consequence of one-electron reduction and redox cycling. If such a system were operative, the ratio of one- to two-electron reducing systems, and possibly their relative locations in the cell, could influence toxicity. Increasing the level of one-electron reduction, thereby fueling a MCH₂ peroxidase with H₂O₂, could be expected to alleviate toxicity under aerobic conditions. Consistent with this hypothesis is the observation that increased resistance to MC under aerobic conditions was found when NADH:cytochrome b₅ reductase was over expressed in the mitochondria of Chinese hamster ovary cells (11).

**Table IV**

Comparison of calculated net and measured net cross-linking of DNA stably pre-cross-linked with 1,2-bis(methylsulfonyl)-1-(2-chloroethyl)hydrazine and subjected to further cross-linking by NaBH₄ reduced MC.

| Reaction mixture | Measured cross-linking of T7 DNA | Measured net cross-linking of T7 DNA (no nicking) |
|------------------|-------------------------------|-----------------------------------------------|
| MC (40 μM), NaBH₄ (200 μM) | 47.3 ± 1.2 | 60.5 ± 4.2 |
| MC, NaBH₄, MCRA (1 μM) | 2.3 ± 1.5 | 34.5 ± 2.0 |
| MC, NaBH₄, HRP (2 units/ml), H₂O₂ (100 μM) | 2.4 ± 1.2 | 32.5 ± 2.3 |
| MC, NaBH₄, MP (3.5 units/ml), H₂O₂ | 11.8 ± 0.7 | 36.2 ± 1.9 |
| MC, NaBH₄, LP (4 units/ml), H₂O₂ | 17.2 ± 1.0 | 33.4 ± 1.2 |

Comparing the kinetics of mitosene formation and DNA cross-linking. The kinetics of T7 DNA cross-linking (graphical plot plus minus the standard deviation) compared with the changes in absorbance at 575 nm (spectral trace) under equivalent conditions (pH 7.4 and 37 °C).
Heme peroxidases occur in a variety of mammalian tissues and secretions, with very high levels being found in some of the lymphocyte cell lineages. Neutrophils contain the highest levels of any normal mammalian tissue (25) containing 2% (dry weight) myeloperoxidase sufficient to impart a faint green color to these cells. Chloromas, an extramedullary tumor of granulocytic lineage, contain extremely high levels of myeloperoxidase (6% dry weight) giving the tumor a characteristic green coloration (26). This level of myeloperoxidase equates to several hundred times the concentration of MP used in these model studies.

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Inhibition of DNA Cross-linking by Mitomycin C by Peroxidase-mediated Oxidation of Mitomycin C Hydroquinone

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