Reductive Activation of Mitomycin C and Mitomycin C Metabolites Catalyzed by NADPH-Cytochrome P-450 Reductase and Xanthine Oxidase*

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Under anaerobic conditions and with proper electron donors, NADPH-cytochrome P-450 reductase (EC 1.6.2.4) and xanthine oxidase (EC 1.2.3.2) similarly reductively metabolized mitomycin C. Reversed phase high performance liquid chromatography was used to separate, detect, and isolate several metabolites. Three metabolites were identified by mass spectrometry and thin layer chromatography as 1,2-cis- and trans-2,7-diamino-1-hydroxymitosene and 2,7-diaminomitosene. Three metabolites were phosphate-dependent, and two of them were identified to be 1,2-cis- and trans-2,7-diaminomitosene 1-phosphate. The amounts of the five identified metabolites generated during the reduction of mitomycin C varied with pH and nucleophile concentration. At pH 6.5, 2,7-diaminomitosene was essentially the only metabolite formed, whereas from pH 6.8 to 8.0, trans- and cis-2,7-diamino-1-hydroxymitosene increased in quantity as 2,7-diaminomitosene decreased. The disappearance of mitomycin C and the production of metabolites were enzyme and mitomycin C concentration-dependent. Substrate saturation was not reached for either enzyme up to 5 mM mitomycin C. Electron paramagnetic resonance studies demonstrated the formation of mitomycin C radical anion as an intermediate during enzymatic activation. Our results indicate that either enzyme catalyzed the initial activation of mitomycin C to a radical anion intermediate. Subsequent spontaneous reactions, including the elimination of methanol and the opening of the aziridine ring, generate one active center at C-1 which facilitates nucleophilic attack. Simultaneous generation of two reactive centers was not observed. All five primary metabolites were metabolized further by either flavoenzyme. The secondary metabolites exhibited similar changes in their absorbance spectra and were unlike the primary metabolites, suggesting that a second alkylating center other than C-1 was generated during secondary activation. We propose that secondary activation of monofunctionally bound mitomycin C is probably a main route for the bifunctional binding of mitomycin C to macromolecules and that the cytotoxic actions of mitomycin C result from multiple metabolic activations and reactions.

Since its discovery by Hata et al. (1), mitomycin C has been extensively studied for its antibacterial and anticancer activities. Recently, increasing clinical importance of this unique compound has generated more interest in defining its molecular mechanism of action. Schwartz (2) and Iyer and Szybalski (3) found MC1 was activated by biological reductive systems so that activated MC covalently bound and cross-linked DNA (3–5). From this, the hypotheses of reductive activation and bifunctional alkylation were proposed (3, 6). The key to both of these hypotheses is a two-electron reduction of MC to the hydroquinone form, followed by spontaneous loss of methanol and generation of reactive centers at C-1 and C-10 that are subject to nucleophilic attack. MC has been considered as the only known bioreductive alkylating agent. Recently, Tomasz and Lipman (7) succeeded in identifying some metabolites of MC produced from reduction by rat liver microsomes. Their work provided the first direct evidence of the alkylating characteristics of MC and confirmed portions of the earlier hypotheses (3, 6). They identified metabolites generated in the presence of inorganic phosphate as 1,2-cis- and trans-2,7-diaminomitosene 1-phosphates and 1,2-cis- and trans-2,7-diamino-1-hydroxymitosenes and were the first to discover 2,7-diaminomitosene (Fig. 1). The formation of these metabolites supported the hypothesis that the reduction of the heterocyclic quinone of MC induced the opening of the aziridine ring and generated a reactive center at C-1. The proposed second alkylating center C-10 was inert in their system.

In our laboratory, a sensitive reversed phase HPLC method allowed us to separate, detect, and isolate metabolites of MC (8). This method enabled us to examine the kinetics of MC metabolism by purified microsomal NADPH-cytochrome P-450 reductase and bovine milk xanthine oxidase and to isolate the metabolites from these reactions. During these enzymatic reductions, we also studied free radical formation. A preliminary account of these studies was reported (9).

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† The abbreviations used are: MC, mitomycin C; HPLC, high performance liquid chromatography; cis-2d, 1,2-cis-2,7-diamino-1-hydroxymitosene; trans-2d, 1,2-trans-2,7-diamino-1-hydroxymitosene; cis-2d-phosphate, 1,2-cis-2,7-diaminomitosene 1-phosphate; trans-2d-phosphate, 1,2-trans-2,7-diaminomitosene 1-phosphate; AUFS, absorbance units at full scale.
of MC, cis-2d, trans-2d, and others were synthesized following the procedure of Stevens et al. (13). 2,7-Diaminomitosene was prepared according to Tomasz and Lipman (7). All purities were verified by semipreparative HPLC, and the purity of each substance was confirmed by analytical HPLC and TLC. The details of the resolution of the MC derivatives by HPLC and their identity verification by mass spectrometry have recently appeared (8).

**Enzyme Activity**

**Reduction of MC and Metabolites**—Unless otherwise stated, the enzyme reaction mixture contained 65 μg of xanthine oxidase/0.5 ml, 1.0 mM MC, 2 mM NADH, and 0.1 mM phosphate buffer at pH 7.8 or 5.0 mM of NADPH-cytochrome P-450 reductase/0.5 ml, 1.0 mM MC, 2 mM NADPH, and 0.1 mM phosphate buffer at pH 7.4. MC was first dissolved in dimethyl sulfoxide at a concentration of 50 mM and was then added to the reaction mixture at the concentration specified. All reaction mixtures contained 2% dimethyl sulfoxide, unless stated otherwise. Enzyme inhibition by 2% dimethyl sulfoxide was negligible, and dimethyl sulfoxide did not affect the production of metabolites. Anaerobic conditions were achieved by flushing the reaction mixture with nitrogen for 1–3 min in closed test tubes or anaerobic cuvettes. The reaction was initiated by the injection of enzymes (preflushed) or by mixing enzyme contained in the side arm when using the anaerobic cuvette. All reactions were carried out at 37 °C. Termination of the reaction after a 20-min incubation was achieved by exposing the reaction mixture vigorously to air and adding 0.25 ml of methanol. Aliquots of the terminated reaction solution were injected directly onto the HPLC column for analysis or isolation of metabolites. When MC metabolites were used as substrates, the same reaction conditions were kept except metabolites at a concentration of 0.8 mM were used to replace MC. Several metabolites such as cis-2d and trans-2d were kept in methanol, and when added to the aqueous reaction mixture, methanol was flushed out by a stream of N2. The concentration of each metabolite was determined by its absorbance at the following wavelengths and molar absorptivities: cis- and trans-2d, 11,400 at 310 nm; 2,7-diaminomitosene, 10,300 at 314 nm; 2d-phosphate, 13,100 at 310 nm (7, 13).

**Phosphatase Digestion**—Conditions reported by Block and Schlesinger (15) were used with modification. Substrate was replaced by unknown metabolites to be analyzed, and detection of products was conducted by HPLC, as described above.

**Absorbance Studies**

A Cary model 118 spectrophotometer (Varian Associates, Inc., Palo Alto, CA) was used to obtain the absorption spectra of MC and isolated metabolites and to study the absorption changes during the anaerobic reduction of MC by both NADPH-cytochrome P-450 reductase and xanthine oxidase. As the reduction of MC occurred, the major changes of absorbance in 0.1 M potassium phosphate were a decrease at 367 nm, an increase at 312 nm, and an increase in the region of 550–560 nm. Most metabolites have the 7-aminoiminomitosene chromophore and have maximum absorption at 312 nm and in the 550–560 nm region. The rate of total metabolite production was estimated by measuring the increase of absorbance at 554 nm. This wavelength was chosen over the more sensitive absorbance at 312 nm to avoid the interference by NADPH (or NADH) and MC which remained in the reaction mixture. The absorbance by MC at 554 nm was negligible. Absorption units were chosen to express the relative amounts of the production of metabolites.

**Mass Spectrometry**

A VG Micromass 30F mass spectrometer (V. G. Analytical, Altrincham, Great Britain) was used to obtain electron ionization mass spectra. Details are published elsewhere (8). Field desorption mass spectrometry was performed by the Middle Atlantic Mass Spectrometry Laboratory, a National Science Foundation shared instrumentation facility.

**Electron Paramagnetic Resonance Spectra**

EPR spectra were obtained on a Varian E-109 (Palo Alto, CA) spectrometer operated at a modulation frequency of 100 kHz and interfaced with a Nicolet Model 1180 Data System (Madison, WI). Enzymatic reactions were conducted anaerobically as mentioned under "Materials and Methods," except that 10% dimethyl sulfoxide was included in the reaction mixture. Immediately after the initiation...
of the reaction, the reaction mixture was transferred to an EPR flat cell which was prepurged with nitrogen. The free radical signal was monitored immediately at room temperature under 10 milliwatts of incident microwave power. The scanning conditions were: 4 × 1 G modulation amplitude, 1.25 × 10⁻⁶ × 10 gain, 20 G/min scan rate, and 0.5-s time constant (the control used 0.25-s time constant). Pitch was used as a standard.

RESULTS

HPLC Analysis of Metabolites

The reduction of MC catalyzed by NADPH-cytochrome P-450 reductase generated several metabolites as determined by HPLC (Fig. 2, a and b). Similar metabolite profiles were obtained from xanthine oxidase-catalyzed reactions (data not shown). Each separated compound was identified by its capacity factor ($k'$). NADPH and NADP (or NADH and NAD) were eluted at $k' = 1.4$ and 3.1, respectively, and unreacted MC was eluted at $k' = 12.7$, exhibiting a high absorbance ratio at 365/313 nm. When reactions were carried out in phosphate buffer with either enzyme, five major and a number of other metabolites resulted (Fig. 2a). Three metabolites ($k' = 4.1, 6.4$, and 7.1) were phosphate-dependent and were not produced in enzyme reactions buffered by 0.1 M Tris-HCl at equivalent pH (Fig. 2b). Other metabolites ($k' = 11.7, 14.4, 16.6, 13.4, $ and 17.7) were produced similarly in either buffer.

In the absence of enzyme or reduced pyridine nucleotide and in the presence of air, the MC was stable and no metabolites or reaction products of MC were detected. MC hydrolyzed in 0.34 M sodium phosphate (pH 3.0) as reported by Tomasz and Lipman (16) revealed five peaks by our HPLC analysis (Fig. 2c). These included MC and reaction products with $k' = 4.1, 6.4, 11.7, $ and 14.4, which had the same retention times as the major enzymatic metabolites. A peak appearing at 7.5 min for all experiments was an unknown impurity in MC.

Identification of Metabolites

UV-visible Absorbance Spectrometry—Six metabolites ($k' = 4.1, 6.4, 7.1, 11.7, 14.4, $ and 16.6) including the three phosphate-dependent metabolites exhibited UV-visible absorbance spectra resembling the 7-aminomitosene chromophore (13, 17). All showed maximum optical absorption near or at 312 and 252 nm (Fig. 3), whereas MC showed maximum absorbance at 367 nm. Two other metabolites ($k' = 13.4$ and 17.7) had different spectra (discussed under “Secondary Metabolites”).

Mass Spectrometry—Metabolites $k' = 11.7, 14.4, $ and 16.6 were acetylated, and the acetylated products were analyzed by electron ionization mass spectrometry. Metabolite $k' = 11.7$ showed m/z 301 (8.0%), 285 (11.3%), 242 (17.9%), 224 (10.9%), Metabolite $k' = 14.4$ showed m/z 404 (1.0%), 361 (3.4%), 343 (2.3%), 301 (61.9%). They were 2,7-diamino-1-hydroxymitosenes of different isomeric forms. The metabolite $k' = 16.6$ was 2,7-diaminomitosene, the new metabolite discovered by Tomasz and Lipman (7) as determined by electron ionization mass spectrometry (m/z 303 (2.2%), 285 (5.3%)) and field desorption mass spectrometry (m/z 347 (MH)+, 330 (MH-NH2)+).

TLC Analysis—The stereoisomers of 2,7-diamino-1-hydroxymitosene ($k' = 11.7$ and 14.4) were verified by TLC systems A and C. Chemically synthesized derivatives were used as standards. The metabolite $k' = 11.7$ was trans-2,7-diamino-1-hydroxymitosene ($R = 0.09$ in A and 0.30 in C), and the metabolite $k' = 14.4$ was the cis-2,7-diamino-1-hydroxymitosene ($R = 0.21$ in A and 0.20 in C). (14).

Alkaline Phosphatase Digestion—Phosphate-dependent metabolites ($k' = 4.1, 6.4, $ and 7.1) were subjected to alkaline phosphatase digestion. HPLC analysis of the digested mate-

![Fig. 2. HPLC analysis of the metabolites of MC catalyzed by NADPH-cytochrome P-450 reductase. Incubation time was 20 min. Enzyme reactions and HPLC were carried out as described under "Materials and Methods." a, in 0.1 M potassium phosphate buffer at pH 7.4; b, in 0.1 M Tris-HCl at pH 7.4; c, MC hydrolyzed in 0.34 M sodium phosphate at pH 3.0. ———, 0.05 AUFS, 313 nm; ———, 0.2 AUFS, 365 nm; ———, 0.05 AUFS, 280 nm.](http://www.jbc.org/Downloaded from http://www.jbc.org/Downloaded from http://www.jbc.org/Downloaded from http://www.jbc.org/)
The spectra showed that metabolite \( k' = 4.1 \) was converted to trans-2d, and metabolites \( k' = 6.4 \) and the minor peak at \( k' = 7.1 \) both were converted to cis-2d. The two phosphate derivatives isolated from acid-hydrolyzed MC in 0.34 M phosphate reported by Tomasz and Lipman (16) were subjected to the same digestion, and the results were identical. Therefore, metabolite \( k' = 4.1 \) was designated as the trans form and metabolite \( k' = 6.4 \) as the cis form of 2,7-diaminomitosene 1-phosphate. These compounds were unstable when they were subjected to 0.1 N HCl for 1 h, and several degradation products formed instead of the defined conversion shown by phosphatase digestion.

**Secondary Metabolites**

Purified cis-2d and trans-2d are both activated and metabolized further by NADPH-cytochrome P-450 reductase and by xanthine oxidase. HPLC analysis of the enzyme-reduced reaction mixtures resolved one major metabolite each for trans-2d and cis-2d at \( k' = 13.4 \) and 17.7, respectively. These two metabolites co-chromatographed with two of the transient metabolites observed when MC was reduced in the initial reaction \( k' = 13.4 \) and 17.7. The absorbance spectra of the two new products resembled each other but were different from the typical spectra of metabolites containing the 7-aminomitosene chromophore (Fig. 3). Under neutral conditions, the spectra showed maximum absorption at 370 and 248 nm with shoulders around 312 and 280 nm.

trans- and cis-2d-phosphates and 2,7-diaminomitosene were also metabolized further by both enzymes. One major metabolite was resolved by HPLC for each compound after enzymatic reduction. trans- and cis-2d-phosphate yielded metabolites \( k' = 6.20 \) and 10.10, respectively, and 2,7-diaminomitosene yielded a metabolite \( k' = 20.4 \). Absorption spectra of these secondary metabolites were similar to the metabolites of trans-2d and cis-2d \( k' = 13.4 \) and 17.7 \) and were unlike the 7-aminomitosene chromophore. Conclusive identities of the secondary metabolites are unclear, and we are currently working on this project.

**Reduction Kinetics**

The rate of total metabolite production measured by the increase of absorption at 554 nm for the initial 3 min responded linearly to the concentration of both enzymes and MC (data not shown). Under the conditions described, MC saturation was not reached for either enzyme up to 5 mM, and \( K_m \) and \( V_{max} \) were not applicable for the reduction of MC. The activation of MC was apparently first order in MC. Using peak height from HPLC analysis to determine concentration, we plotted the production of the five principal identified metabolites (cis-2d, trans-2d, cis-2d-phosphate, trans-2d-phosphate, and 2,7-diaminomitosene) and the disappearance of MC (Fig. 4). Semilogarithmic plots of MC concentration versus reaction time revealed a biphasic linear relationship, which can be described in terms of an initial rapid phase first order kinetics followed by a slower phase when the substrate (MC) declined to about 0.2 mM (one-fifth of the original 1.0 mM) (Fig. 5).

**Rate of Reduction**—The enzymatic reduction rate of MC and its metabolites by xanthine oxidase was estimated by substrate half-life. Xanthine oxidase at 124 µg/ml of reaction mixture under conditions described under “Materials and Methods” reduced MC, cis-2d, cis-2d-phosphate, and 2,7-diaminomitosene; trans-2d-phosphate; cis-2d-phosphate (all metabolites were monitored at 313 nm, 0.05 AUPS).
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963

-300

E

E

-200

0

-100

0.01

0.05

1

2

3

PHOSPHATE CONC. (M)

PEAK HEIGHTS

METABOLITES at 313 nm (mm)

PEAK HEIGHTS

METABOLITES at 313 nm (mm)

ARSENATE CONC. (M)

FIG. 6. Effect of buffer concentration on MC reduction and metabolite formation catalyzed by xanthine oxidase. Reactions were carried out as described under "Materials and Methods" except the concentration of buffers varied from 0.01 to 0.3 M. Incubation time was 20 min. ▲, (365 nm, 0.2 AUFS), MC; △, trans-2d; ■, cis-2d; □, trans-2d-phosphate; ○, cis-2d-phosphate (all metabolites were monitored at 313 nm, 0.05 AUFS). a, in phosphate buffer (pH 7.8); b, in arsenate buffer (pH 7.8). (trans-2d-phosphate is plotted at one-half of its actual amount.)

diaminomitosene at half-lives of 4, 20, 27, and 13 min, respectively. The phosphorylated metabolite was least reactive.

Phosphate and Arsenate Effect—The production of 2d-phosphates was phosphate concentration-dependent since the amount of cis- and trans-2d-phosphate increased almost linearly with increasing concentration of the reaction pH 7.8 buffer from 0.01 to 0.3 M (Fig. 6a). However, the concentration of phosphate (at constant pH) had little effect on the amount of cis- and trans-2d produced. As a result, the disappearance rate of MC in higher phosphate concentrations was faster. This concentration effect was further investigated with arsene, a stronger nucleophile, as a buffer at pH 7.8. With arsene, 1-arsenomitosene, which is the equivalent of 1-phosphomitosene, would undergo arseneolysis to cis- and trans-2d spontaneously. As expected, accumulation of arsenomitosene was not seen, and the amount of cis-2d and trans-2d increased with increasing concentrations of arsene (Fig. 6b).

The generation of 2,7-diaminomitosene was not affected by the concentration of either phosphate or arsene. The dependency of the concentration of MC and phosphate suggested second order kinetics for the formation of 2d-phosphate.

pH Effect—Enzymatic reduction of MC in 0.1 M phosphate buffer from pH 6.5 to 8.0 produced a series of different metabolite profiles. The distribution of each metabolite and unreacted MC was plotted by their peak heights to show the differences at each pH (Fig. 7). At pH 6.5, 2,7-diaminomitosene was essentially the only metabolite. Only trace amounts of cis-2d and trans-2d, cis-2d-phosphate, and trans-2d-phosphate were observed. Increasing the pH of the reaction to 7.8 and above resulted in a sharp decrease of the amount of 2,7-diaminomitosene formed and an increase of the other four metabolites. The disappearance rate of MC at higher pH was faster, indicating a greater reactivity at a higher pH. When

FIG. 7. pH effect on the production of metabolites catalyzed by xanthine oxidase. The reactions were carried out as described under "Materials and Methods" except the pH of the buffer varied. Incubation time was 20 min. ●, (365 nm, 0.2 AUFS), MC; ■, trans-2d; △, cis-2d; ○, trans-2d-phosphate; □, cis-2d-phosphate (all metabolites were monitored at 313 nm, 0.05 AUFS).

FIG. 8. EPR spectrum of MC radical anion. Reduction of MC was catalyzed by xanthine oxidase. Details of EPR detection are described under "Materials and Methods". a, pitch; b, MC radical anion; c, control in absence of enzyme. Spectra of b and c were the sum of 10 scans.
cis and trans isomers were produced, the ratio of cis and trans isomers of 2d and 2d-phosphate was between 1 and 2 or 3. Incubation time (Fig. 4), phosphate or arsenate concentration (Fig. 6, a and b), or pH (Fig. 7) had little effect on the ratio of the isomers generated, although these conditions had other effects on the reduction of MC. MC is hydrolyzed nonenzymatically to cis- and trans-2d and cis- and trans-2d-phosphate in phosphate buffer when the pH is below 5.0. MC is also converted nonenzymatically to unknown compound(s) at pH higher than 9.0 (data not shown).

**Free Radical Formation**

The free radical of MC was detected upon activation by xanthine oxidase in a reaction mixture containing 10% dimethyl sulfoxide. The signal was somewhat weak and necessitated 10 sweeps at 2 min/sweep accumulated by computer to observe the EPR spectrum with a g-value of 2.0046 (Fig. 8). Fine structure was not detected in this aqueous reaction mixture. In the absence of enzyme or NADH, no free radical signal was detected. Since NADPH-cytochrome P-450 reductase lost most of its activity in 10% dimethyl sulfoxide, NADPH-cytochrome P-450 reductase was not effective in these experiments.

**DISCUSSION**

We have found that the detergent-solubilized NADPH-cytochrome P-450 reductase activated MC without the requirement of either cytochrome P-450 or phospholipid. As for xanthine oxidase, xanthine served as an electron donor for the reduction of MC just as well as NADH. Due to substrate inhibition by xanthine at high concentrations (18), NADH was chosen as the electron donor throughout the course of our studies. Both enzymes required anaerobic conditions for the reduction of MC. If air were present, NADPH or NADH was oxidized due to the cyclic electron transfer by MC, augmenting O2 consumption (19).

The HPLC method developed in our laboratory for the analysis of MC metabolites has several advantages over other methods (8). For our study of the biochemical activation of MC, this method enabled us to determine the kinetics of the production of several metabolites and to detect transient metabolites that may be significant for the elucidation of the mechanism of MC action. The k1 values we report varied somewhat from the previous observation (8) as the result of using a different column. Co-chromatography with standards was always used for confirmation in our experiments.

Five major metabolites identified as cis- and trans-2d, 2,7-diaminomitosene, and cis- and trans-2d-phosphate were produced during the course of reduction of MC by both enzymes in the presence of phosphate buffer. Our findings are comparable to Tomasz and Lipman's (7) report of rat liver microsomes with some differences. They reported a 1 to 1 ratio of cis and trans isomers, whereas our system detected a 1 to 2 or 3 ratio of the cis and trans isomers. In addition, we detected a number of additional unidentified metabolic products through the high resolution of our HPLC method.

The activation of MC by either NADPH-cytochrome P-450 reductase or xanthine oxidase obeyed first order kinetics, since the saturation of MC was not reached within a reasonable concentration (5 mM), and total metabolite production (sum of all metabolites measured at 554 nm) was proportional to both enzyme concentration and substrate (MC) concentration. Substrate disappearance from the reaction was not linear. A semilogarithmic plot of substrate disappearance showed a biphasic linear relationship which was probably due to the accumulation of primary metabolites (Fig. 5). After the reaction proceeded for 30 min, the primary metabolites accumulated to sufficient concentrations such that they competed with MC for the enzyme.

The relative distribution of the five major identified metabolites is directly dependent on reaction conditions. This is seen in the effects of the nucleophilic reactants, phosphate and arsenate, on metabolite profile and is also seen when proton concentration is varied (pH effect). From these data, we propose that for a reaction mechanism only the initial step of MC activation is enzyme-catalyzed and the primary metabolites produced are dependent on reaction conditions. The formation of cis- and trans-2d followed pseudo-first order kinetics with MC and H2O, whereas the formation of cis- and trans-2d-phosphate followed second order kinetics (first order with MC and first order with phosphate). We believe that the kinetics of 2,7-diaminomitosene formation also obeyed a pseudo-first order kinetics where hydrogen is abstracted from H2O (Scheme 1). Proton participation directly in the formation of 2,7-diaminomitosene through second order kinetics is unlikely because hydrolysis at pH 3.0 could not generate any 2,7-diaminomitosene as shown by Tomasz and Lipman (16) and our data (Fig. 2c). It is obvious that the overall kinetics for MC metabolism is complicated by the reactivity of its reaction products. These observations may be extrapolated to the cellular condition where MC is activated enzymatically, and the subsequent reactions and product formation depend on the intracellular milieu at the reaction site.

Other workers have proposed that the critical step in the enzymatic activation of MC is a two-electron reduction of MC to the hydroquinone form (3, 6, 7). We believe, however, that one-electron reduction activates MC and leads directly to the metabolites observed. We base our belief on four lines of evidence. The first is that NADPH-cytochrome P-450 reductase and xanthine oxidase favor one-electron transfer to suitable receptors (20, 21). MC thus receives one electron to form MC radical anion which then undergoes subsequent conversions to metabolites. Although the free radical signal is very weak due to the short half-life and high reactivity of the MC radical anion in aqueous environment, EPR evidence confirms this concept. Second, we believe that one-electron reduction is sufficient to generate an activated form from a...
chemical viewpoint. Upon one-electron reduction of MC, the radical anion (II) is formed (Scheme 1), and the quinone moiety becomes an aromatic ring. Subsequently, loss of methanol between C-9 and C-9a is favored by the resonance stabilization energy released upon formation of the aromatic indole moiety (III). Nucleophilic attack at C-1 is now facilitated in this indole intermediate as the aziridine ring cleaves. The key step for initiating this sequence of transformations that leads to metabolite formation is the aromatization of the quinone ring, and one-electron reduction serves equally well at effecting this aromatization as two-electron reduction. Our third line of evidence that enzymatic transfer of one electron to MC is sufficient to generate a reactive intermediate comes from our electrochemical studies. One-electrochemical reduction gave a group of products that resembled the profile of metabolites reported here for enzymatic reduction (27). Two-electron electrochemical reduction, however, resulted in two products of the 10-decarbamoylmitosene type that were different from all the mitosenes produced by enzymatic reduction (27). Fourth, the enzymatic kinetics for reduction and product formation is first order and linear. If free radical disproportionation were a factor to produce the hydroquinone form prior to reaction, then the kinetics would not be linear.

Nucleophilic attack on the free radical intermediate (III) by either water or phosphate generates the 1-hydroxy or 1-phospho radical anion. The radical anion remaining may then transfer its electron to molecular oxygen or another MC molecule, or another scavenger to regenerate the quinone. The production of 2,7-diaminomitosene occurs where hydrogen is abstracted from a suitable source (i.e. protein, lipids, water). This route must be favored at a pH slightly lower than 7.0 since 2,7-diaminomitosene was virtually the only product of the reduction of MC at pH 6.5 and 6.8. At higher pH, there is increasing formation of 2d-phosphates since at pH 8.0, phosphate in the more nucleophilic, dibasic form predominates over the monobasic form.

After the initial reductive activation of MC, the resulting products and adducts are capable of at least secondary enzymatic activation. Reduction of the primary metabolites (cis- and trans-2d, 2,7-diaminomitosene, and cis- and trans-2d-phosphate) generated a group of products with maximum absorption at 370 and 248 nm, and shoulders at 312 and 280 nm. These spectra are different from both 7-hydroxymitosene (13) or the primary metabolites (7-aminomitosenes). These data provide definite evidence that reductive center(s) other than C-1 or C-7 of the indoloquinone molecule can be activated. The unknown secondary products shared similar absorbance spectra, suggesting the possibility of sharing similar structural changes. We have not observed any primary metabolite with a structural change that involved a second reactive center, although two transient metabolites \( k' = 13.4 \) and 17.7 were seen during the initial reduction of MC that had characteristics indicating other reactive centers. We believe these two transient products were generated from the primary metabolites instead of directly from MC. Clarification of the identity of the secondary products should confirm the occurrence of C-10 carbamate displacement as proposed by other workers (3, 6). We feel that secondary reduction of the primary alkylated product provides a second reactive center for MC which is probably a main route for bifunctional binding.

The cytotoxicity of MC can be envisioned occurring in several stages by several mechanisms (Scheme 2). In the cell, MC undergoes primary molecular activation under anaerobic conditions via flavoenzyme single electron transfer to the quinone moiety. From this activation, the MC radical produces cis and trans adducts, cis- and trans-mitosenes, and 2,7-diaminomitosene. The cis and trans adducts are monofunctional adducts presumably of DNA (3-5) or other nucleophilic molecules (7). All of these primary metabolic products can undergo secondary flavoenzyme activation through a second anaerobic free radical, semiquinone step. From the secondary activation, a second series of unidentified metabolites is produced. Whereas the free mitosene substrates yield new alkylated products, the monofunctional adducts may undergo secondary condensation reactions to yield bifunctional adducts which cross-link DNA as historically described (4). Additional reactions do occur from the evidence of other unidentified metabolites but these have not been examined in detail, yet.

In addition to the sequence of anaerobic reactions that we have described, a second series of reactions may be possible which parallel the anaerobic activation pathway. These are aerobic flavoenzyme-catalyzed free radical transfer steps. Free radical transfer may be possible at each reaction level; MC, primary metabolites (cis and trans adducts, etc.), and the secondary metabolites. As long as the quinone nucleus remains available, single electron transfers may occur. If oxygen is available as the free radical scavenger, the electron transfer may continue to proceed through oxygen activation. Thus, each quinone-containing metabolic product of MC may be a site for the continued production of active oxygen products such as superoxide and hydroxyl radical. Active oxygen products generated by MC were shown in vitro by a number of laboratories (22-24), and pulmonary toxicity in the clinical use of MC (25, 26) strongly suggests the induction of active oxygen species in vivo by MC. These observations and possibilities demonstrate the cytotoxic versatility nature has built into the MC molecule.

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