Bioreductive Activation of Mitomycin C by DT-Diaphorase†

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ABSTRACT: The role of DT-diaphorase (DTD, EC 1.6.99.2) in the bioreductive activation of mitomycin C was examined using purified rat hepatic DTD. The formation of adducts with reduced glutathione (GSH), binding of [^{3}H]mitomycin C to DNA, and mitomycin C-induced DNA interstrand cross-linking were used as indicators of bioactivation. Mitomycin C was metabolized by DTD in a pH-dependent manner with increasing amounts of metabolism observed as the pH was decreased from 7.8 to 5.8. The major metabolite observed during DTD-mediated reduction of mitomycin C was 2,7-diaminomitosene. GSH adduct formation, binding of [^{3}H]mitomycin C and mitomycin C-induced DNA interstrand cross-linking were observed during DTD-mediated metabolism. In agreement with the pH dependence of metabolism, increased bioactivation was observed at lower pH values. Temporal studies and experiments using authentic material showed that 2,7-diaminomitosene could be further metabolized by DTD resulting in the formation of mitosene adducts with GSH. DNA cross-linking during either chemical (sodium borohydride) or enzymatic (DTD) mediated reduction of mitomycin C could be observed at pH 7.4, but it increased as the pH was decreased to 5.8, showing the critical role of pH in the cross-linking process. These data provide unequivocal evidence that the obligate two-electron reductase DTD can bioactivate mitomycin C to reactive species which can form adducts with GSH and DNA and induce DNA cross-linking. The use of mitomycin C may be a viable approach to the therapy of tumors high in DTD activity, particularly when combined with strategies to lower tumor pH.

Mitomycin C is an antitumor quinone which undergoes reductive metabolism to generate reactive electrophilic species which can alkylate cellular nucleophiles (Iyer & Szybalski, 1963, 1964; Kennedy et al., 1980; Powis, 1987). Mitomycin C can be reduced chemically or enzymatically to reactive species capable of either forming monoadducts with DNA or resulting in DNA cross-linking through a second electrophilic site on the molecule (Iyer & Szybalski, 1963, 1964; Moore & Czerniak, 1981; Powis, 1987). The putative electrophilic intermediate generated after reduction of mitocycin C is a quinone methide which arises from elimination of a methoxy group as methanol and aziridine ring opening and leads to alkylation at the C-1 position of mitomycin C. Cross-linking occurs through the C-10 position of the molecule following loss of carbamate and production of an electrophilic imine moiety. The quinone methide derived from mitomycin C is ambivalent and behaves differently dependent on pH. At lower pH values, the quinone methide can be covalently trapped by a proton to form 2,7-diaminomitosene whereas at higher pH values it functions as an electrophile leading to alkylation of biological nucleophiles, such as nucleic acids, or water to form *cis*- and *trans*-1-hydroxyaminomitosenes (Peterson & Fisher, 1986).

The enzymology underlying bioreductive activation of mitomycin C and related quinones has been the subject of extensive research. Cytochrome P450 reductase, xanthine oxidase, cytochrome *b*5 reductase, mitochondrial reductases, and various bacterial and yeast reductases have all been shown to catalyze anaerobic reduction of mitomycin C (Schwartz, 1962; Iyer & Szybalski, 1964; Tomasz & Lipman, 1981; Kennedy et al., 1982; Pan et al., 1984; Peterson & Fisher, 1986; Bligh et al., 1990; Hodnick et al., 1991). The role of DT-diaphorase (DTD) in bioreductive activation of mitomycin C has been controversial (Workman et al., 1989; Pritsos et al., 1988; Marshall et al. 1989b). Dicoumarol, an inhibitor of DTD, has been found to inhibit mitomycin C-induced DNA interstrand cross-linking and cytotoxicity in a variety of tumor cell systems under aerobic conditions (Keyes et al., 1985, 1989; Rockwell et al., 1988a; Marshall et al., 1989a, 1991a; Begleiter et al., 1989; Dulhanty & Whitmore, 1991). L5178Y cell lines which were sensitive to mitomycin C had up to 24-fold higher DTD activity than resistant lines (Begleiter et al., 1989). An association between elevated DTD levels in human colon carcinoma cells or human fibroblasts and aerobic mitomycin C sensitivity has also been observed (Siegel et al., 1990; Marshall et al., 1991b). These data supported a role for DTD in the metabolism and activation of mitomycin C, but the compound did not act as a substrate for purified DTD isolated from either human kidney or rat liver at pH 7.8 (Powis, 1987; Schlager & Powis, 1988). We confirmed these observations but found that metabolism of mitomycin C by either purified rat hepatic or human kidney DTD was pH-dependent (Siegel et al., 1990). At pH 7.8, reduction of mitomycin C by DTD leads to inhibition of the enzyme, whereas at lower pH values, removal of mitomycin C and metabolite formation could be detected (Siegel et al., 1990, 1991). The major metabolite detected during metabolism of mitomycin C by DTD at pH values between 5.8 and 7, under either aerobic or anaerobic conditions, was 2,7-diaminomitosene (Siegel et al., 1990).

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That questions still remain with respect to the potential role of DTD in bioreductive activation of mitomycin C is due, in part, to an overreliance on the use of enzyme inhibitors in cellular systems and the lack of studies in cell-free systems where both metabolism and bioactivation can be examined. The purpose of this work was to examine in a cell-free system using purified DTD whether metabolism of mitomycin C by DTD represented an activation step. We have used the formation of glutathione conjugates of mitomycin C, binding of mitomycin C to DNA, and mitomycin C-induced cross-linking of DNA as indicators of bioactivation. Our data confirm that DTD can metabolize mitomycin C in a pH-dependent manner but also show that metabolism by DTD results in activation of mitomycin C to reactive species that can alkylate both glutathione and DNA and lead to DNA cross-linking. These data may also be of some significance for elucidation of pathways of activation of mitomycin C and the identity of reactive species formed after reduction.

EXPERIMENTAL PROCEDURES

Materials. Mitomycin C was a generous gift from the Pharmaceutical Research and Development Division, Bristol Myers Co., Syracuse, NY. [6C-$^3H_3$]Mitomycin C ([^{3}H]MC) was synthesized as described previously (Arai & Kasai, 1991; Kasai et al., 1991). NADH, dichlorophenolindophenol (DCPIP), reactive blue 2-Sepharose CL-6B (Cibacron blue), calf thymus DNA, and protease K were obtained from Sigma Chemical Co., St. Louis, MO. Dicoumarol was purchased from Aldrich Chemical Co., Milwaukee, WI. [^{3}H]glutathione was obtained from NEN Research Products, Boston, MA. pBR322 DNA was obtained from Boehringer Mannheim, Indianapolis, IN. All other reagents used were of analytical grade.

Purification of Rat Hepatic DT-Diaphorase. DT-Diaphorase was purified from uninduced Sprague-Dawley rats (Sasco, NE; 250–300 g) using Cibacron blue affinity chromatography as described (Sharkis & Swenson, 1989). The purified protein resolved as a single band using SDS–PAGE at a molecular mass of 32 kDa. The purified enzyme had a specific activity of 520 μmol/(min·mg of protein) with DCPIP as the electron acceptor. All reductase activity was inhibited by 1 μM dicoumarol.

DNA Binding. Binding to calf thymus DNA was determined using [^{3}H]MC. Briefly, reactions were terminated by the addition of cold ethanol containing 0.5 M sodium chloride, and the samples were cooled to −70 °C for 30 min. The DNA was pelleted by centrifugation at 10000*g* for 5 min, washed with ether to remove unbound MC, and dried under nitrogen. The DNA was resuspended in 25 mM Tris-HCl buffer, pH 6.8, containing protease K (1 mg/mL) for 45 min at 37 °C. The DNA was then purified by column chromatography (Nucleobond X500; Machery-Nagel, Duren, Germany), as described by the manufacturer. DNA recovery was greater than 80% as determined by A_{260}. Residual binding under these conditions probably represents covalent binding, although the possibility of tight noncovalent binding cannot be excluded. Scintillation fluid (15 mL, Scintiverse II) was added to 200 μg of DNA for scintillation counting.

HPLC Analysis. Analytical HPLC analyses were performed on a gradient system consisting of two Waters 501 pumps and a controller equipped with a Waters 991 photodiode array detection system. HPLC analysis of 2,7-diaminomitosene and the glutathione conjugate of MC was performed as described previously (Siegel et al., 1990). Radioelution profiles were determined by column eluate collection at 1-min intervals, addition of 10 mL of Scintiverse II (Fisher Scientific) scintillation fluid, and subsequent scintillation counting. Preparative HPLC analysis was performed essentially as described (Pan et al., 1984) but using a Lichrosorb RP-18, 25 × 1 cm column.

Preparation of 2,7-Diaminomitosene. 2,7-Diaminomitosene was prepared from mitomycin C either enzymatically using DTD at pH 5.8 or via sodium borohydride mediated reduction of mitomycin C. We have previously shown that 2,7-diaminomitosene was the major metabolite formed during DTD-mediated metabolism of mitomycin C by DTD at acidic pH (Siegel et al., 1990). The material formed has identical chromatographic, NMR, and mass spectral behavior to 2,7-diaminomitosene prepared by catalytic hydrogenation (Tomasz & Lipman, 1981). Enzymatic preparation was performed as follows; 110 μL of purified DTD (3.5 mg/mL) was added to 5 mg (15 μmol) of mitomycin C and 21 mg (30 μmol) of NADH in 10 mL of 0.1 M potassium phosphate buffer (pH 5.8) under aerobic conditions. The reaction mixture was stirred at 22 °C for 4–6 h. The mixture was added to a Prep-Sep-C18 extraction column (Fisher Scientific) which was then washed with 90–100 mL of water. 2,7-Diaminomitosene and any remaining mitomycin C were eluted with 10 mL of methanol, and 2,7-diaminomitosene was collected after preparative HPLC analysis. Methanol was evaporated under reduced pressure from the pooled eluates containing 2,7-diaminomitosene and the resultant aqueous solution was added to a second extraction column. Salts were removed by washing with 50 mL of water, and 2,7-diaminomitosene was eluted with 5 mL of methanol. The methanol was evaporated under reduced pressure to give 1.8 mg (39% yield) of a dark purple solid. Purity was checked by analytical scale HPLC with diode array detection (HPLC/DAD).

Preparation of the Glutathione Conjugate Formed during Metabolism of Mitomycin C by DTD. This was performed by sodium borohydride (10 mM) mediated reduction of mitomycin C (0.1 mM) under aerobic conditions in potassium phosphate buffer, (100 mM), pH 5.8 (100 mL), containing GSH (1 mM). The mixture was stirred overnight and then applied to a Prep-Sep C18 extraction column, washed with 50 mL of water, and then eluted with 5–10 mL of methanol. The conjugate was collected after preparative HPLC, methanol was evaporated under reduced pressure, and the aqueous solution was applied to a new extraction column and washed with 50 mL of water. The conjugate was eluted with 5 mL of methanol, and the methanol was evaporated under reduced pressure to give 1.7 mg (21% yield) of a dark purple solid. Purity was checked by analytical scale HPLC/DAD. The proton nuclear magnetic resonance spectrum (^{1}H-NMR) was recorded on a Varian VXR-300S spectrometer: ^{1}H-NMR (D_2O) δ 1.51 (s, C(6)CH_3), 1.82–1.92 (m, Glu(β)CH_2), 2.25 (t, J = 7 Hz, Glu(γ)CH_2), 2.57 (dd, J = 9, 17 Hz, C(1)H_β), 2.78–2.88 (m, Cys(β)CH_2), 3.16 (dd, J = 7, 17 Hz, C(1)H_α), 3.49 (s, C(10)CH_2), 3.47–3.58 (m, Glu(α)CH, expected triplet partially obscured by singlets at 3.49 and 3.56 ppm), 3.56 (s, Gly(α)CH_2), 3.94–4.02 (m, C(2)H), 4.08 (dd, J = 4, 14 Hz, C(3)H_β), 4.26 (dd, J = 7, 14 Hz, C(3)H_α), 4.35–4.42 (m, Cys(α)CH).

pBR322 DNA Cross-Linking. Analysis of cross-linking of pBR322 DNA was carried out as described by Hartley et al. (1991b). Briefly, reactions were terminated by the addition of 50 μL of 600 mM sodium acetate buffer containing 1 mM EDTA and tRNA (300 μg/mL). The DNA was precipitated by the addition of 300 μL of cold 95% ethanol and storage at −70 °C for 1 h. DNA was pelleted by centrifugation at 10000*g*

Table I: pH-Dependent Binding of [^{3}H]Mitomycin C to Calf Thymus DNA Catalyzed by Rat Hepatic DT-Diaphorase (DTD)[a]

	pmol of [^{3}H]mitomycin C/μg of DNA
1. complete system, pH 7.8	5.7[b] (3.6, 7.8)
2. complete system, pH 5.8	64.7 (60.1, 69.3)
3. complete system + dicoumarol (10 μM), pH 5.8	5.3 (6.9, 3.6)
4. complete system (-DTD), pH 5.8	2.3 (4.1, 0.4)
5. 2,7-DAM, pH 5.8	52.7 (60.6, 44.8)
6. 2,7-DAM + dicoumarol (10 μM), pH 5.8	9.9[c]

[a] Complete system: 50 μM [^{3}H]mitomycin C, 200 μM NADH, 400 μg of calf thymus DNA, and 3 μg of DTD in 0.1 M potassium phosphate buffer, pH 5.8 and 7.8 for 2 h. [b] Mean of two separate experiments; the values are given in parentheses. [c] Single determination. 2,7-DAM was prepared (conditions 5 and 6) by incubating 50 μM [^{3}H]mitomycin C, 200 μM NADH, and 3 μg of DTD for 30 min at pH 5.8, after which 400 μg of calf thymus DNA was added and the reaction was allowed to proceed for 2 h. In the 30-min incubation period prior to the addition of DNA, removal of MC was >90%, as measured by HPLC, with 82% recovery of the MC removed as 2,7-DAM.

for 15 min. The DNA was washed with 300 μL of 70% ethanol and then centrifuged using a Speed-Vac (Savant) until dry. The DNA was resuspended in 2 μL of water followed by 18 μL of strand separation buffer (30% DMSO in 10 mM Tris-HCl buffer, pH 7.4, plus 1 mM EDTA, 0.04% bromophenol blue, and 0.04% xylene cyanol). Samples were heated to 90 °C for 3 min and then rapidly cooled in ice-cold water prior to horizontal agarose gel (1%) electrophoresis. Nondenatured control samples were dissolved in 18 μL of buffer (10 mM Tris-HCl buffer, pH 7.4, containing 1 mM EDTA, 0.04% bromophenol blue, and 0.04% xylene cyanol), and loaded directly without heating. Autoradiography was performed on Kodak X-omat AR film using intensifying screens at -70 °C for 4–6 h. Quantitation of the percent of cross-linked DNA was performed using a laser densitometer (LKB, Stockholm).

RESULTS

We first examined whether DTD-mediated metabolism resulted in binding of [^{3}H]mitomycin C to calf thymus DNA. After [^{3}H]mitomycin C was incubated with DTD, NADH, and calf thymus DNA at pH 5.8, DNA was precipitated and subjected to protease K digestion, reprecipitation and column chromatography (see Experimental Procedures). After enzymatic reduction by DTD, radioactivity could be detected bound to DNA at pH 5.8, and this could be inhibited by dicoumarol or by exclusion of DTD (Table I). Interestingly, that mitomycin C equivalents were bound to DNA after DTD-mediated metabolism could be detected by visual comparison of DNA between sample and control incubates—the DNA contained in metabolically competent reactions was colored purple relative to control incubates. When DNA was added to the reaction after greater than 90% of the mitomycin C had been metabolized (as detected by HPLC), essentially equivalent binding to DNA still occurred and this could be inhibited by dicoumarol (Table I, conditions 5 and 6). Since the major metabolite formed during DTD-mediated metabolism of mitomycin C is 2,7-diaminomitosene (Siegel et al., 1990), these data suggested that 2,7-diaminomitosene may be activated by DTD to a reactive electrophilic species capable of alkylating DNA. Whether the same adduct was formed when DNA was present from the start of the reaction or was added at later times could not be elucidated from these experiments.

Chemical precedent exists for reductive activation of 2,7-diaminomitosene. Chemical reduction leads to loss of carbamate and alkylation of guanine or sulfur nucleophiles such as thiocarbonate at the C-10 position (Iyengar et al., 1989; Bean & Kohn, 1983). Using reduced glutathione (GSH) as a nucleophile, we examined the potential formation of glutathione conjugates during DTD-mediated metabolism of mitomycin C. When mitomycin C was incubated with DTD, NADH, and GSH, an additional and more polar product could be detected during HPLC analysis (Figure 1), which had the characteristic chromophore of a mitosene when examined using HPLC/DAD. This product was confirmed as a GSH-conjugate by the use of [^{3}H]glutathione and collection of the eluate corresponding to this peak and subsequent scintillation counting (Figure 1). The formation of this product was dependent on the presence of both enzyme and cofactor and could be inhibited by dicoumarol or by prior reaction of glutathione with excess *N*-ethylmaleimide, a thiol-blocking agent (not shown). The time course of formation of this adduct was examined in more detail relative to the formation of 2,7-diaminomitosene. During metabolism of mitomycin C by DTD at pH 5.8, 2,7-diaminomitosene predominanted at early time points whereas at later times, the amount of 2,7-diaminomitosene decreased and the concentration of conjugate increased (Figure 1). These data suggested that the species responsible for conjugation could be derived from 2,7-diaminomitosene. To confirm this, experiments were performed using authentic 2,7-diaminomitosene, prepared enzymatically and purified using HPLC, which was incubated with DTD and NADH in the presence of GSH. A product with the same retention time as that of the GSH conjugate generated from mitomycin C was formed from 2,7-diaminomitosene, and these products cochromatographed and had identical spectra when examined using HPLC/DAD (not shown).

The putative GSH conjugate could also be formed after chemical reduction of mitomycin C with sodium borohydride in the presence of glutathione at pH 5.8. Authentic 2,7-diaminomitosene (prepared enzymatically as described), when reduced chemically by sodium borohydride in the presence of GSH at pH 5.8, also led to the formation of the glutathione adduct. When the conjugate was concentrated and subjected to FAB-MS, a protonated molecular ion was observed at m/z 551 which corresponded to the C-10 monoglutathione mitosene adduct which can be formed from 2,7-diaminomitosene after reduction. The proton NMR spectrum of the adduct (experimental procedures) was consistent with the proposed structure of a C-10 glutathione adduct.

DNA interstrand cross-linking was examined using pBR322 DNA and an agarose gel electrophoresis method to separate single-stranded and double-stranded DNA. Our data show that DTD-mediated metabolism of mitomycin C resulted in increased DNA cross-linking as the pH was lowered from pH 7.8 to pH 5.8 (Figures 2 and 3). Cross-linking was dependent on mitomycin C, enzyme, and NADH and could be inhibited by dicoumarol (Figure 2). Increased cross-linking at lower pH values is consistent with previous studies of DNA cross-linking induced by mitomycin C and diaziquone analogs after chemical reduction (Dzielendziak et al., 1990; Teng et al., 1989; Lee et al., 1992). Since DTD is inhibited by mitomycin C at pH 7.8 (Siegel et al., 1991), we utilized sodium borohydride as a reductant in order to examine the pH dependence of DNA cross-linking after reduction. Reduction of mitomycin C by sodium borohydride at both pH values was verified using spectrophotometry and by HPLC. Using a 100:1 ratio of sodium borohydride:mitomycin C, greater than 90%

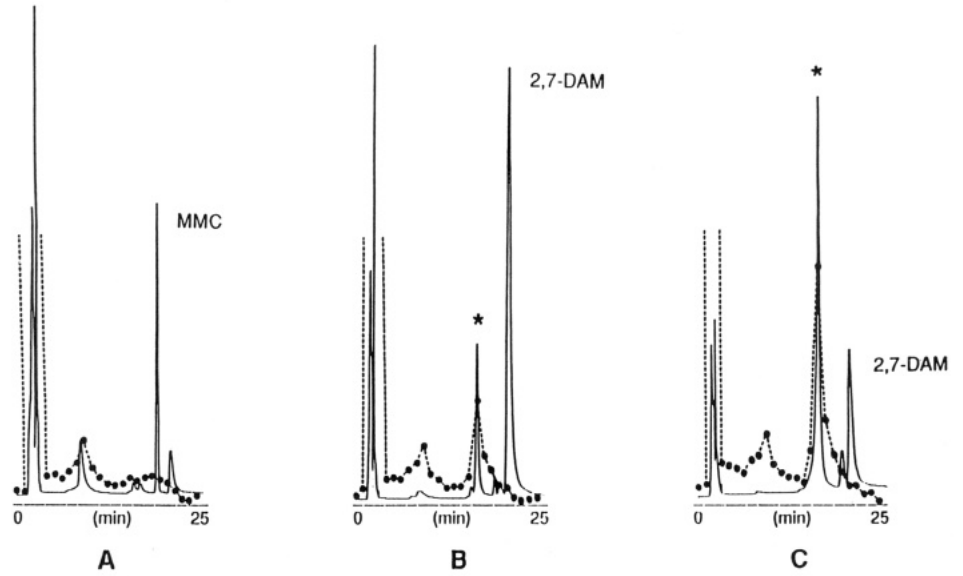


FIGURE 1: Mitomycin C–glutathione conjugate formation catalyzed by rat hepatic DT-diaphorase. HPLC was used to analyze the time course of formation of a mitomycin C–GSH conjugate [(A), 2 min; (B), 30 min; (C) 120 min]. Reaction conditions: 100 μM mitomycin C, 200 μM NADH, 1 mM [^{3}H]GSH and 5.2 μg of rat hepatic DT-diaphorase in 0.1 M potassium phosphate buffer (pH 5.8). Reactions were performed in a total volume of 0.5 mL at 25 °C. HPLC conditions were as described in Experimental Procedures. (Symbols: solid line, detection at 314 nm; dashed line, ^{3}H elution; *, mitomycin C–GSH conjugate).

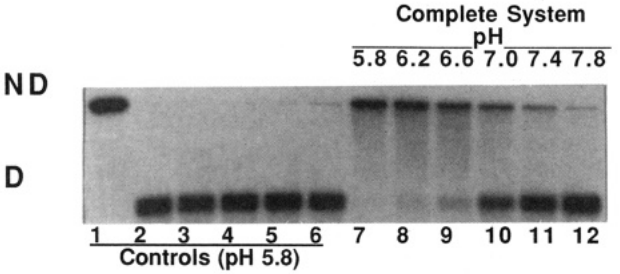


FIGURE 2: pH-dependent mitomycin C-induced cross-linking of pBR322 DNA catalyzed by rat hepatic DT-diaphorase. Cross-linking of pBR322 DNA was used as a measure of the ability of rat hepatic DT-diaphorase to bioactivate mitomycin C to a DNA cross-linking species. Complete system: 10 μM mitomycin C, 0.173 μg of rat hepatic DT-diaphorase, 100 μM NADH, and 10 ng of [^{32}P]pBR322 DNA. Reactions were performed in 10 mM potassium phosphate containing 1 mM EDTA, pH 5.8–7.8 at 25 °C for 1 h. Cross-linked DNA migrated as nondenatured DNA (ND) and non-cross-linked DNA migrated as denatured DNA (D). Lane 1, nondenatured control; lane 2, denatured control; lane 3, NADH and DT-diaphorase; lane 4, mitomycin C only; lane 5, mitomycin C and NADH; lane 6, complete system plus 20 μM dicoumarol; lanes 7–12, complete system, pH 5.8–7.8.

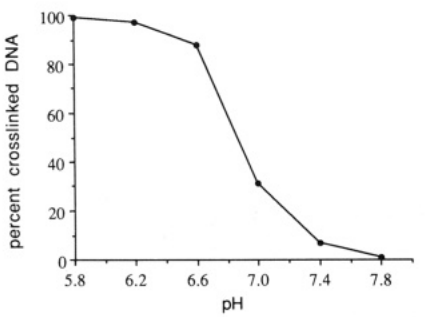


FIGURE 3: Effect of pH on mitomycin C-induced DNA cross-linking catalyzed by DT-diaphorase. Percent of cross-linked DNA (from Figure 2) as determined by laser densitometry.

of mitomycin C was removed at pH 5.8 and almost 100% at pH 7.8 after 1 h. DNA cross-linking was observed after sodium borohydride mediated reduction of mitomycin C and greater cross-linking occurred at pH 5.8 than at pH 7.8 (Figure 4). This shows that even when reduction of mitomycin C occurs

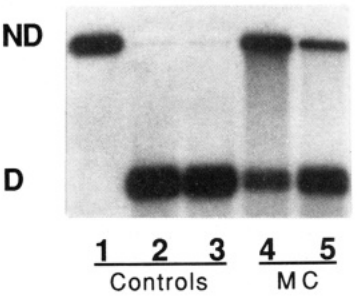


FIGURE 4: Mitomycin C-induced cross-linking of pBR322 DNA catalyzed by sodium borohydride. Cross-linking of pBR322 DNA was used as a measure of the ability of sodium borohydride (10 mM) to bioactivate mitomycin C (10 μM) at pH 5.8 and 7.8. Reactions were performed in 10 mM potassium phosphate containing 1 mM EDTA at pH 5.8 and 7.8 at 25 °C for 1 h. Lane 1, nondenatured control, pH 5.8; lane 2, denatured control, pH 5.8; lane 3, sodium borohydride only, pH 5.8; lane 4, mitomycin C and sodium borohydride, pH 5.8; lane 5, mitomycin C and sodium borohydride, pH 7.8.

at pH 7.8, cross-linking is not as pronounced as at lower pH values (Figure 4).

DISCUSSION

There is considerable evidence implicating DT-diaphorase in the bioreductive activation of mitomycin C to DNA cross-linking and cytotoxic species, which has been obtained mainly by the use of inhibitors of DTD such as dicoumarol in cellular systems. Dicoumarol was found to inhibit mitomycin C-induced DNA cross-linking and/or cytotoxicity in a variety of cell types under aerobic conditions. Associations between aerobic mitomycin C sensitivity and elevated DTD levels have also been observed in human colon carcinoma cells and human fibroblasts (Marshall et al., 1991b; Siegel et al., 1990). Indirect evidence relating to the role of individual reductases in metabolism and bioactivation can be obtained from such studies. In order to confirm the role of individual enzymes in bioactivation, however, experiments need to be performed using purified enzymes in cell-free systems. In previous work, we have demonstrated that mitomycin C can be metabolized by purified rat and human DTD in a pH-dependent manner

(Siegel et al., 1990). We have extended this work to examine whether DTD-mediated metabolism results in bioreductive activation of mitomycin C using purified DTD. Utilizing binding to DNA, the formation of glutathione conjugates and DNA interstrand cross-linking as indicators of bioactivation of mitomycin C, we have shown that metabolism by DTD represents a true activation step. These data suggest that DTD is likely to be an important determinant of the cytotoxicity of mitomycin C in tumor cell systems. As would be expected from the dependence of metabolism of mitomycin C by DTD on pH, activation to reactive species was also pH-dependent with greater activation occurring at lower pH values.

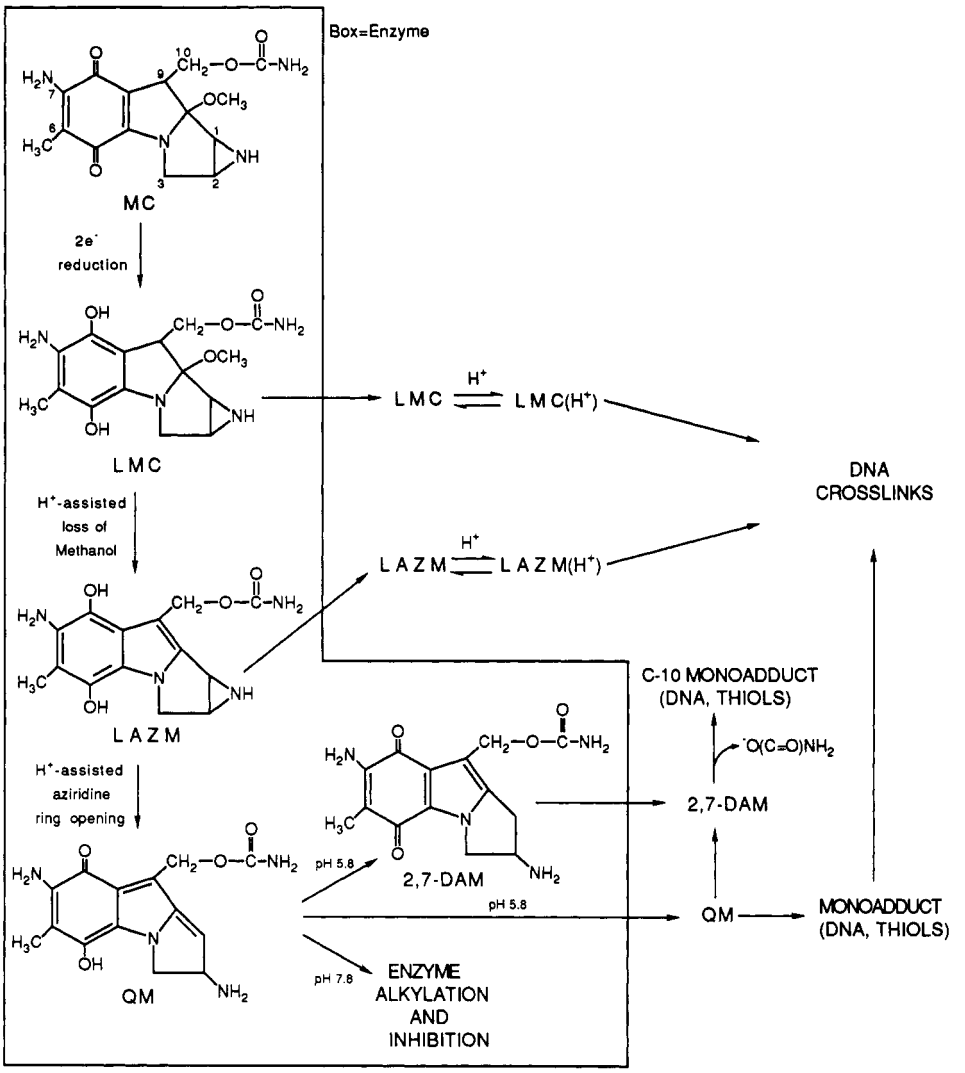
Although the major focus of this study was to address the question of whether DTD catalyzed metabolism of MC resulted in activation, these data also provided some indications as to the identity of reactive species. The major metabolite that was detected during metabolism of mitomycin C by DTD was 2,7-diaminomitosene and a detailed examination of the stoichiometry of this reaction and its pH dependence showed that it did not arise via autocatalytic mechanisms (Siegel et al., 1990) as previously described for other enzymatic systems (Peterson & Fisher, 1986). On the basis of temporal studies and profiles of product formation, it appeared that 2,7-diaminomitosene could be metabolized by DTD to generate reactive species which would bind to GSH. This suggestion was confirmed by isolation of the same glutathione conjugate during metabolism of either MC or 2,7-diaminomitosene by DTD. The same product was also formed during chemical reduction of mitomycin C or 2,7-diaminomitosene by sodium borohydride at pH 5.8. Using chemical reducing systems, it has recently been shown that 2,7-diaminomitosene can be activated to an alkylating species via loss of carbamate and generation of a reactive imine at the C-10 position resulting in alkylation of DNA (Iyengar et al., 1989). In agreement with this proposal ^{1}H-NMR data were consistent with the proposed structure of a C-10 glutathione mitosene adduct. These data show that reductive activation of 2,7-diaminomitosene by DTD can occur and that adducts after DTD-mediated metabolism of MC are likely to occur, at least in part, at the C-10 position via the intermediacy of 2,7-diaminomitosene. Consistent with the ability of mitomycin C to form a glutathione adduct after reductive activation, glutathione depletion has recently been shown to enhance mitomycin C cytotoxicity in HT-29 human colon carcinoma cell lines (Perry & Rasberry, 1991).

DNA cross-linking induced by mitomycin C is thought to be an important parameter in the mechanism of action of this compound (Iyer & Szybalski, 1964; Powis, 1987). Similar to DNA and glutathione adduct formation, we observed increased DNA cross-linking during DTD-mediated metabolism as the pH was decreased from 7.8 to 5.8. It is important to point out that cross-linking was still observed at pH values up to 7.4 and thus, cross-linking would be expected to occur, at least to some degree, under physiological conditions. DNA cross-linking after chemical reduction of mitomycin (Teng et al., 1989) and diaziquone (Dzielendziak et al., 1990; Hartley et al., 1991a; Lee et al., 1992) analogs has previously been shown to depend on pH with lower pH favoring increased cross-linking. Since mitomycin C inhibits DTD at pH 7.8, we utilized sodium borohydride as a reductant to explore the pH dependency of DNA cross-linking. Little DNA cross-linking occurred when mitomycin C was reduced by sodium borohydride at pH 7.8 but cross-linking increased as the pH was lowered. These data, in agreement with previous studies in EMT6 cells, where increased cross-linking was observed as the pH was decreased (Kennedy et al., 1985), show the importance of pH in mitomycin C-induced DNA cross-linking. This presumably reflects proton-assisted aziridine ring opening which facilitates the generation of reactive electrophilic intermediates. Previous work has shown that mitomycin C exhibits greater toxicity to EMT6 cells (Kennedy et al., 1985) and to HeLa cells (Kohnoe et al., 1991) at acidic pH values, and we have observed similar effects in human colon HT-29 cells (unpublished).

The complexity of the bioreduction of mitomycin C (Scheme I) makes the assignment of cross-linking species difficult. The major metabolite generated during DTD-mediated metabolism of mitomycin C is 2,7-diaminomitosene, and increasing amounts are formed as the pH is lowered. At higher pH values such as 7.8, mitomycin C inhibits DT-diaphorase in a manner resembling suicide inactivation (Siegel et al., 1991). This probably reflects the ambivalent behavior of the quinone methide of mitomycin C (Peterson & Fisher, 1986), which at pH 7.8 can function as an electrophile leading to enzyme alkylation and inhibition. At lower pH values, the quinone methide is covalently trapped by a proton leading to 2,7-diaminomitosene formation and maintenance of enzyme integrity. Alternatively, at pH 5.8, leucomitomycin C or its protonated form may be released from the enzyme prior to quinone methide formation. In our studies, increasing amounts of DNA cross-linking were observed during DTD-mediated metabolism of mitomycin C as the pH was lowered from 7.8 to 5.8. Although 2,7-diaminomitosene can give rise to monofunctional adducts, it is not a DNA cross-linking species (Iyengar et al., 1989). At pH 5.8, it is possible that the quinone methide could be released from the enzyme into solution where the majority of it would partition, at this pH, to form 2,7-diaminomitosene (Peterson & Fisher, 1986). It is also possible that the remaining quinone methide could alkylate nucleophiles such as DNA and lead to cross-linking. This mechanism might appear inconsistent with increased cross-linking at low pH, since low pH values would be expected to favor protonation of the quinone methide to form the non-cross-linking 2,7-diaminomitosene. Lower pH, however, may also favor formation of the quinone methide from the leucomitomycin because of the proton-assisted loss of the methoxy group and aziridine ring opening. A suggestion which is more consistent with our data is that the precursor to DNA cross-linking could be the protonated leucomitomycin C (Hoey et al., 1988) or the leucoaziridinomitosene (Cera et al., 1989). The pK_a of the aziridine ring in leucomitomycin C has been reported to be 5.1 (Hoey et al., 1988), which would allow significant protonation at pH 5.8.

Since DTD is an obligate two-electron reductase (Ernster, 1967), our results, in agreement with those of Hoey et al. (1988), imply that activation occurs via the intermediacy of the hydroquinone of mitomycin C. This does not preclude subsequent radical involvement after initial enzymatic reduction, but we failed to observe any oxygen uptake during DTD-mediated metabolism of mitomycin C under aerobic conditions (Siegel et al., 1990). In addition, we did not observe any differences in metabolism of mitomycin C under aerobic or hypoxic conditions (Siegel et al., 1990) suggesting that bioactivation of mitomycin C should be similar in each case. In support of this, we observed little difference in glutathione conjugate formation during DTD-mediated metabolism of mitomycin C under aerobic or hypoxic conditions in this study. Paradoxically, although dicoumarol inhibits mitomycin C-induced toxicity in cellular systems under aerobic conditions, it potentiates under hypoxic conditions (Keyes et al., 1985,

Scheme I: Potential Routes of DTD-Catalyzed Activation of Mitomycin C[a]



[a] Abbreviations: MC, mitomycin C; LMC, leucomitomycin C; LAZM, leucoaziridinomitosene; QM, quinone methide; 2,7-DAM, 2,7-diaminomitosene.

1989; Rockwell et al., 1988b). If the effects of dicoumarol reflect only the involvement of DTD in cells, then these data seem contradictory. Assuming that cytotoxic events are induced with greater efficiency in the order of semiquinone > hydroquinone > oxygen radicals, these data can be rationalized by considering the involvement of other enzyme systems (Sartorelli, 1986, 1988; Dulhanty & Whitmore, 1991). Thus, under aerobic conditions metabolism occurs via both one- and two-electron reductases. The toxicity of the semiquinone is limited, however, by redox cycling and oxygen radical generation which is *relatively* nontoxic. Most bioreductive activation therefore occurs as a result of hydroquinone generation via DTD. Dicoumarol therefore inhibits aerobic toxicity. Under hypoxic conditions, the concentration of semiquinone is not limited by redox cycling, and this leads to enhanced toxicity. Dicoumarol blocks any DTD-mediated reduction allowing enhanced one-electron reduction and therefore increased toxicity. Indeed, there is some mechanistic evidence to support the purported increased toxicity of the semiquinone relative to the hydroquinone. Departure of the carbamate from the C-10 position of a monoadducted mitosene, which allows cross-linking to occur, has been suggested to be more efficient from the semiquinone rather than from the hydroquinone oxidation state (Egbertson & Danishefsky, 1987; Cera et al., 1989). Recent work employing nitroxide-scavenging agents has also implied that the toxicity of mitomycin C in hypoxic cells is due to semiquinone generation (Krishna et al., 1991).

In summary, these data show that DTD-mediated metabolism of mitomycin C represents an activation step as shown by the occurrence of DNA binding, GSH conjugate formation, and DNA cross-linking. Metabolism and bioactivation of mitomycin C is pH-dependent and increases as the pH is decreased from 7.8 to 5.8. This provides the first unequivocal evidence of the importance of DTD in the bioreductive activation of mitomycin C in vitro. Our data also suggest that attempts to modulate pH in combination with mitomycin C treatment could be a viable approach for the therapy of tumors high in DTD activity such as certain human colon (Traver et al., 1992; Mekhail-Ishak et al., 1989), and non-small-cell lung cancers (Malkinson et al., 1991).

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