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# pH-Dependent Inactivation of DT-Diaphorase by Mitomycin C and Porfiromycin

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**SUMMARY**

Mitomycin C and porfiromycin were found to inactivate rat hepatic DT-diaphorase. Inactivation was pH dependent; little inactivation was detected at pH 5.8, but inactivation increased as the pH was raised to 7.8. Inactivation was concentration and time dependent and displayed pseudo-first-order kinetics. Inactivation was NADH dependent, indicating that reductive metabolism was necessary for inhibition. [$^3$H]Mitomycin C was covalently bound to DT-diaphorase during inhibition, and the stoichiometry for inactivation of DT-diaphorase by mitomycin C was approximately 0.8 nmol of mitomycin C bound/nmol of enzyme. A higher molecular mass product (60 kDa) was detected by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and Western blot analysis of DT-diaphorase preincubated with NADH and mitomycin C at pH 7.8, suggesting that mitomycin C is capable of cross-linking DT-diaphorase. The kinetics of inhibition, requirement for NADH for inhibition, covalent binding of [$^3$H] mitomycin C to DT-diaphorase, and approximate 1:1 stoichiometry suggest that this inactivation process may be mechanism based. Inhibition of DT-diaphorase by mitomycin C and porfiromycin is not limited to a cell-free system and could also be observed in HT-29 cells in culture at pH 7.2. Bioactivation of mitomycin C or porfiromycin by DT-diaphorase is favored at lower pH, whereas at higher pH values enzyme alkylation and inactivation of DT-diaphorase occur. These data suggest that the success of attempts to exploit the elevated DT-diaphorase content of certain human tumors for improved chemotherapeutic response using mitomycin C or porfiromycin will depend on intracellular pH.

DTD or NADPH-quinone oxidoreductase (EC 1.6.99.2) is an obligate two-electron reductase that can utilize either NADH or NADPH as an electron donor and that is inhibited by dicumarol (1). The role of DTD in the activation of MMC is controversial. Most of the data indicating the involvement of DTD in the activation of MMC have come from cellular studies using dicumarol as an inhibitor of DTD. Dicumarol has been found to inhibit MMC-induced DNA cross-linking and cytotoxicity in a variety of tumor cell systems under aerobic conditions (2-5). In addition, differences in DTD activities in sensitive and resistant human fibroblast cell lines (6) and Chinese hamster ovary cells (7) have suggested a role for DTD in MMC-induced cytotoxicity. Numerous attempts have been made to demonstrate that DTD can catalyze the metabolism of MMC in cell-free systems. MMC was not metabolized by DTD purified from rat liver[2] or human kidney (9) or by DTD-rich preparations from HT-29 human colon carcinoma cells or rat Walker cells.[3] In fact, a detailed study of the metabolism of MMC by human kidney DTD concluded that MMC was a weak competitive inhibitor, rather than a substrate for the enzyme (9).

Previous work in our laboratory demonstrated that MMC was indeed a substrate for both purified rat hepatic and human HT-29 DTD but that metabolism was pH dependent (5). No metabolism was detected at pH 7.8, but metabolism increased as the pH was decreased to 5.8. Recently, we have shown that pH-dependent metabolism of MMC by DTD does represent a bioactivation process, as measured by covalent DNA binding, DNA cross-linking, and glutathione conjugate formation (11). In this manuscript we report that the metabolism of POR by DTD is also pH dependent and that the mechanism underlying pH-dependent metabolism of MMC and POR is pH-dependent inactivation of DTD.

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[3] M. Walton and P. Workman, unpublished observations, cited in Ref. 10.

**ABBREVIATIONS:** DTD, DT-diaphorase; MMC, mitomycin C; POR, porfiromycin; DCPIP, 2,6-dichlorophenol-indophenol; BSA, bovine serum albumin; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; HPLC, high performance liquid chromatography; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

## Materials and Methods

**Chemicals and reagents.** MMC was a generous gift from the Pharmaceutical Research and Development Division, Bristol-Myers Company (Syracuse, NY). [$^3$H]MMC was provided by Kyowa Hakko Kogyo (Tokyo, Japan). POR was kindly supplied by The Upjohn Company (Kalamazoo, MI). NADH, DCPIP, reactive blue (Cibacron blue) 2-Sepharose CL-6B, BSA, and Tween-20 were obtained from Sigma Chemical Co. (St. Louis, MO). Dicumarol and 5-chloro-2-pyridinol were purchased from Aldrich Chemical Co. (Milwaukee, WI). ScintiVerse II was obtained from Fisher Scientific (Fair Lawn, NJ). Rabbit anti-rat liver DTD polyclonal antibody was kindly provided by Dr. Richard J. Knox, Institute of Cancer Research (Surrey, UK). All reagents were at least of analytical grade.

**Purification of rat hepatic DTD.** DTD was purified from uninduced Sprague-Dawley rats (300 g) using Cibacron blue affinity chromatography as described previously (12). The purified enzyme was resolved as a single band upon SDS-PAGE (12%) and had a specific activity of 880 μmol of DCPIP/min/mg.

**HPLC analysis of MMC and POR metabolism.** HPLC analysis of the metabolism of MMC and POR by DTD was performed as described (13). Reactions were analyzed by HPLC using a Supelco $C_{18}$ reverse phase column (5 μm), at a flow rate of 1 ml/min and a detection wavelength of 314 nm. The reactions were stopped with an equal volume of ice-cold methanol containing 5-chloro-2-pyridinol (internal standard; final concentration, 10 ng/ml), and aliquots (100 μl) were immediately analyzed by HPLC. HPLC-mass spectrometry was performed using a Hewlett-Packard 5988A mass spectrometer equipped with a thermospray source and interfaced with a negative ion filament. A Supelco $C_{18}$ reverse phase column (5 μm) was used with a solvent program of 20% solution B for 2 min, 20–80% solution B over 20 min, and 80% solution B for 6 min (solution A, 20 mM ammonium acetate, pH 6.5; solution B, 20 mM ammonium acetate/methanol, 1:1, v/v). The flow rate was 1 ml/min. The thermospray source temperature was maintained at 276°, and the interface control temperature was adjusted to maintain 95% vaporization of the mobile phase (118–112°). The fragment analysis for $N^2$-methyl-2,7-diaminomitosene was as follows: $m/z$ 377, $(M + CH_3COO)^-$; $m/z$ 317, $(M - H)^-$; $m/z$ 274, $(M - CONH_2)^-$.

**Inactivation of DTD by MMC and POR.** The effects of MMC and POR on the activity of DTD were measured using the following procedure. DTD (2.0 μg/ml) was incubated with MMC (0–4.0 μM) and 100 μM NADH in 0.05 M potassium phosphate buffer containing Tween-20 (2 mg/ml), pH 5.8–7.8. At various times, 50-μl aliquots were removed and diluted with 200 μl of ice-cold 0.05 M potassium phosphate, pH 7.4, containing 0.25 M sucrose (1:1, v/v). Fifty microliters of this mixture were added to 50 mM potassium phosphate buffer, pH 7.4, containing 0.7 mg/ml BSA and 200 μM NADH. DTD activity was measured spectrophotometrically at 600 nm via the reduction of 40 μM DCPIP, in 1-ml reactions, at 27°. The final dilution of NADH and MMC from the inactivation reaction was 100-fold. Partition ratios were generated using methods described above except that 3.2 μg/ml DTD was used with 0.125–4.0 μM MMC or POR and the reactions were allowed to proceed for 2 hr before assaying for DTD activity.

**Stoichiometry of MMC binding to DTD.** In method A, DTD (10 or 20 μg) was incubated with 5.0 μM [$^3$H]MMC (22 mCi/mmol) and 100 μM NADH in 50 mM potassium phosphate buffer, pH 7.8, containing 125 mM sucrose (final volume, 2 ml), at 27° for 2 hr. After 2 hr, the enzyme was assayed for activity (DCPIP) and exhibited >99% inactivation. The reaction was transferred to dialysis tubing (molecular weight cut-off, 12,000–14,000) and dialyzed extensively (72 hr) against deionized water. The contents of the dialysis tube were freeze-dried and resuspended in 200 μl of water. Scintillation fluid (6 ml) was then added and the samples were counted for radioactivity. In method B, DTD (200 μg), isolated from *Escherichia coli* (JM109 strain) as described by Forrest *et al.* (14), was incubated with 500 μM NADH and 62.5 or 125 μM [$^3$H]MMC in 50 mM Tris·HCl, pH 7.8 (final volume, 1 ml), at 27° for 2 hr. After 2 hr, the enzyme was assayed for activity (DCPIP) and exhibited >95% inactivation. The enzyme was then heated to 60° for 1 hr to remove FAD. Protein was separated from FAD by centrifugation at 10,000 × *g* for 10 min. The supernatant was analyzed for FAD-MMC adducts by diode-array HPLC (250–500 nm) and scintillation counting. The protein was washed twice with 1 ml of 70% methanol/water and then 1 ml of 70% methanol/water and ethyl ether (1:1, v/v). The protein was air-dried and resuspended overnight in 200 μl of 1 N NaOH at 37°. Aliquots were removed for scintillation counting and protein determination (15).

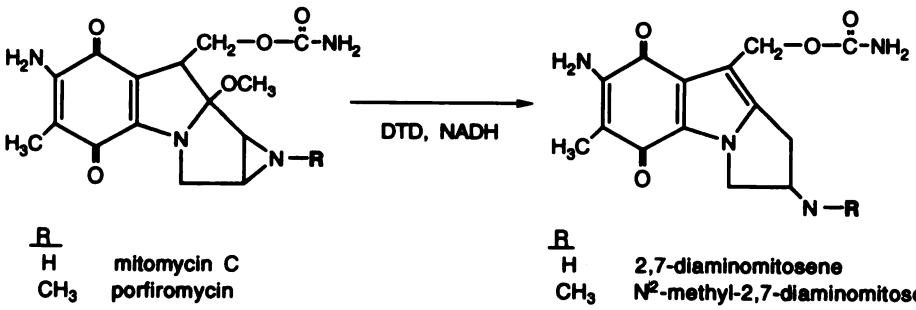
**SDS-PAGE of DTD.** SDS-PAGE (16) was used to analyze DTD after incubation with MMC and NADH. Reactions were performed using the following conditions: 1.12 μg of DTD, 50 μM NADH, and 20 μM MMC in 0.05 M sodium phosphate buffer, pH 5.8–7.8 (final volume, 50 μl), at 25°. After 60 min, the reactions were terminated by snap-freezing in liquid nitrogen, followed by freeze-drying. Each sample was resuspended in 5 μl of water and then 20 μl of SDS sample buffer and processed as described previously (16), except that the samples were not boiled before being loaded onto the gel (12% Minigel; GIBCO, Grand Island, NY). The gels were stained for protein using Coomassie blue. Western blot analysis was performed using an anti-rat liver DTD rabbit polyclonal antibody. DTD (10 μg) was incubated with 100 μM MMC, 500 μM NADH, in 0.05 M sodium phosphate buffer, pH 7.8 (final volume, 10 ml), for 2 hr at 25°. Less than 4% of the initial DTD activity remained after 2 hr. The sample was dialyzed against 20 liters of deionized water over 24 hr, freezed-dried, and then resuspended in 50 μl of water followed by 250 μl of SDS sample buffer. Samples (0.05–0.2 μg) were analyzed by SDS-PAGE as described above, followed by transfer to a nitrocellulose membrane (25 V × 14 hr) in 25 mM Tris, 192 mM glycine, with 20% methanol. Exposure to primary antibody (1/2500) was for 1 hr, followed by exposure to secondary antibody (goat anti-rabbit IgG alkaline phosphatase conjugate, 1/5000) for 30 min and then transfer to 50 mM Tris·HCl, pH 9.5, containing nitro blue tetrazolium (0.17 mg/ml) and 5-bromo-4-chloro-3-indolyl-1-phosphate (0.85 mg/ml) for 10 min.

**Inhibition of HT-29 DTD by MMC and POR.** HT-29 human colon carcinoma cells were grown in culture as described previously (5). Cells ($1 \times 10^4$) were seeded onto 10-mm culture plates and allowed to grow for 4 days. The medium was removed and the cells were washed with 10 ml of Hanks' balanced saline. Ten milliliters of 20 mM HEPES-buffered Hanks' balanced saline, pH 7.2, were added to each plate. After a 30-min preincubation in buffer, MMC or POR (3.1–50 μM) was added to each plate for 2 hr. Drug-containing medium was removed and the cells were washed extensively with Hanks' balanced saline (4 × 10 ml), to remove unincorporated MMC or POR. The cells were then scraped into 2 ml of 25 mM Tris·HCl, pH 7.4, containing 250 mM sucrose (1:1, v/v), and the cell suspension was sonicated on ice for 30 sec. The cell sonicates were maintained on ice until assayed for DTD using the reduction of DCPIP, as described previously (5).

## Results

The metabolism of MMC and POR by DTD (Scheme I) was examined at pH 5.8 and 7.8 using HPLC (Fig. 1). No metabolism was detected at pH 7.8, but metabolism was observed as the pH was lowered to 5.8. The metabolism of both compounds by DTD at pH 5.8 was NADH dependent and could be inhibited by dicumarol. Nonenzymatic degradation of either MMC or POR was not observed at pH 5.8. The major metabolite formed after the reduction of MMC by DTD at pH 5.8 has been previously characterized as 2,7-diaminomitosene (5). The major metabolite formed after the reduction of POR by DTD at pH 5.8 was isolated and characterized by HPLC-mass spectrometry as $N^2$-methyl-2,7-diaminomitosene (see Materials and Methods).

Experiments to examine the effects of MMC and POR on the specific activity of DTD were performed. DTD was prein-




**Scheme I.** Structures of MMC and POR and their major metabolites 2,7-diaminomitosene and $N^2$-methyl-2,7-diaminomitosene.

cubated with MMC or POR and NADH in a potassium phosphate/Tween-20 buffer, pH 5.8–7.8, and at various times an aliquot of the reaction mixture was removed, diluted (100-fold), and then assayed for DTD specific activity. Tween-20 was added to stabilize DTD, and in control samples (without MMC or POR) loss of DTD specific activity was <5% over 2 hr at pH 5.8 or 7.8. Preincubation with either MMC or POR (2 $\mu$M) and NADH had no effect on DTD specific activity when the reactions were performed at pH 5.8. When the pH was increased towards 7.8, however, a time-dependent decrease in DTD specific activity was observed (Fig. 2). Enzymatic activity could not be restored after Sephadex G-10 gel filtration (data not shown), demonstrating irreversible inhibition. Time-dependent inactivation of DTD by MMC or POR at pH 7.8 was not observed in the absence of NADH. At pH 7.8, DTD was inactivated in a time- and concentration-dependent process by both MMC and POR (Fig. 3), and inactivation was characterized by pseudo-first-order kinetics.

Results of the stoichiometric analysis of [$^3$H]MMC binding to DTD are presented in Table 1. The dialysis procedure (method A) showed an average of 0.77 nmol of MMC bound/nmol of DTD. Heat separation of FAD from the protein (method B) gave a similar result, with an average of 0.85 nmol of MMC bound/nmol of DTD in the protein fraction. HPLC analysis of the FAD fraction showed no alteration in retention time or peak area for FAD and no evidence of additional peaks that might correspond to a MMC-FAD adduct. The approximate 1:1 stoichiometry of binding, the pseudo-first-order kinetics, and the requirement for NADH for effective inhibition suggest a mechanism-based inhibition of DTD by MMC at pH 7.8. $K_i$ and $k_{inact}$ values for the inactivation of DTD by MMC and POR at pH 7.8 were obtained by using Kitz and Wilson (17) plots (Table 2). Partition ratios for the inactivation of DTD by MMC and POR (Table 2) were measured in similar experiments (Fig. 4) at pH 7.8 in which the drug to enzyme ratio was varied (18).

SDS-PAGE analysis of DTD preincubated with MMC and NADH at pH 7.8 revealed the presence of a higher molecular mass product (60 kDa) indicative of a DTD cross-link (Fig. 5A). The molecular mass of monomeric DTD has been estimated to be 32 kDa (19). DTD cross-linking was not observed when the complete reaction was performed at pH 5.8. DTD cross-linking could be detected at pH 7.0, however, and cross-linking increased as the pH was increased to 7.8 (Fig. 5B). Cross-linking of DTD at pH 7.8 was NADH dependent and could be inhibited by dicumarol (Fig. 5A). Addition of excess BSA (25 $\mu$g/lane) had no effect on DTD cross-linking (data not shown). Cross-linking of DTD by MMC at pH 7.8 was also detected by Western blot analysis using a rabbit anti-DTD polyclonal antibody (Fig. 6), where both the monomer and cross-linked dimer demonstrated immunoreactivity.

The ability of MMC or POR to inactivate DTD in a cellular system was examined using DTD-rich HT-29 human colon carcinoma cells (Fig. 7). After a 2-hr treatment with either MMC or POR at pH 7.2, DTD activity was examined in cell sonicates. If a sufficient concentration of drug was used, effective inhibition of cellular DTD could be observed even at pH 7.2. Both MMC and POR were able to inactivate HT-29 DTD and inactivation was concentration dependent.

## Discussion

The role of DTD in the bioreductive activation of MMC has been questioned, mainly due to the lack of data demonstrating metabolism using the purified enzyme (8–10). We have demonstrated, however, that MMC can be metabolized by purified rat and human DTD in a pH-dependent manner (5). MMC removal or metabolite formation could not be detected at pH 7.8, but both removal and metabolite formation increased as the pH was decreased to 5.8. The major metabolite was isolated and characterized as 2,7-diaminomitosene. Additional experiments demonstrated that pH-dependent metabolism of MMC by DTD represented an activation process, as measured by covalent DNA binding, DNA cross-linking, and glutathione conjugation (11).

The influence of pH on MMC-induced cytotoxicity and metabolism has been investigated in many systems. pH has been shown to influence DNA cross-linking and cytotoxicity induced by MMC in EMT6 tumor cells (20). DNA cross-linking and cytotoxicity increased as the pH of the cell was decreased. Experiments using HeLa cells also demonstrated that cellular acidity increased MMC-induced cytotoxicity (21). The role of pH in the activation of MMC has been studied in a number of chemical systems (22–24), and lower pH values favor increased DNA cross-linking after chemical reduction (11, 25). This presumably occurs because of facilitated aziridine ring opening at lower pH values (26). In enzymatic systems, pH has been shown to influence the metabolism of MMC by purified cytochrome P-450 reductase (13), cytochrome $b_5$ reductase (27), xanthine oxidase (13), and Old Yellow enzyme (28).

In experiments using Old Yellow enzyme [brewers' yeast NADPH (acceptor) oxidoreductase, EC 1.6.99.1], the authors observed that the enzyme isolated by gel filtration after the reduction of MMC at basic pH had changed color from yellow to brown. This suggested that MMC was covalently bound to the enzyme, although the enzyme still retained catalytic activity (28). When similar experiments were performed using DTD in the present study, no activity could be detected in the purified enzyme after preincubation with MMC and NADH at pH 7.8

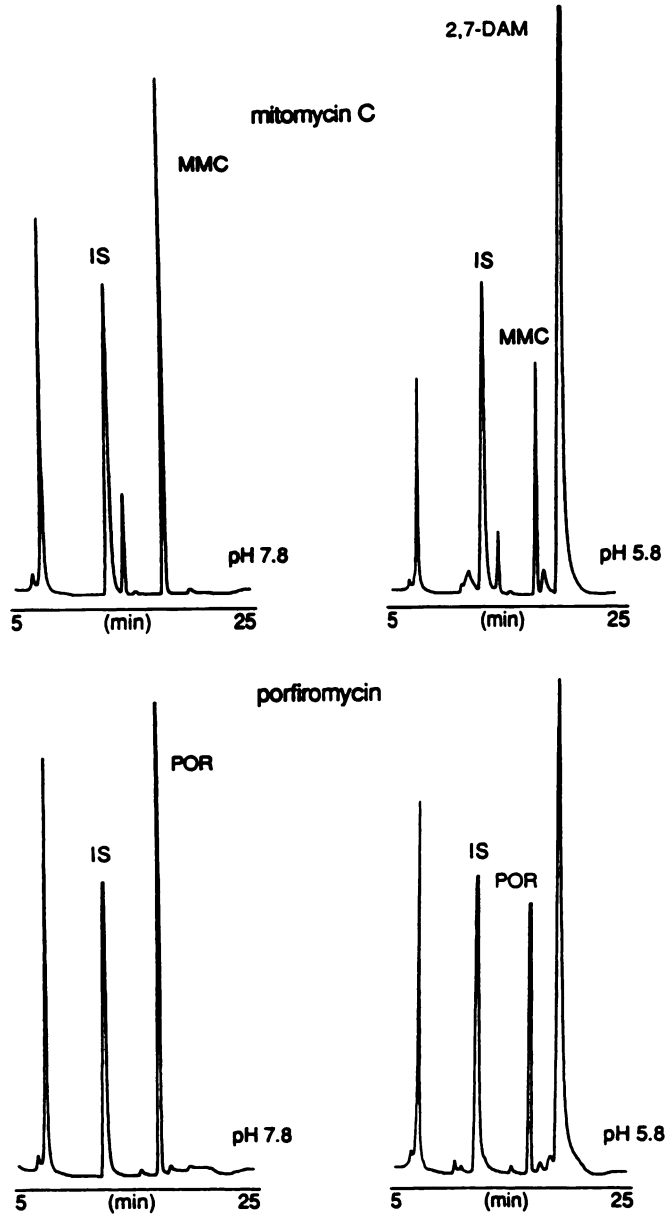



**Fig. 1.** HPLC analysis of the metabolism of MMC and POR by DTD. Reaction conditions: 100 $\mu$M MMC or POR, 200 $\mu$M NADH, and 5.2 $\mu$g/ml DTD in 0.1 M potassium phosphate buffer, pH 5.8 or 7.8. Reactions were performed in a total volume of 0.5 ml at 25°. After 30 min (MMC) or 45 min (POR), 100-$\mu$l aliquots were removed and analyzed by HPLC. Reactions were stopped by the addition of 0.5 ml of ice-cold methanol containing an internal standard. A, MMC, pH 5.8 and 7.8; B, POR, pH 5.8 and 7.8. *IS*, internal standard; *2,7-DAM*, 2,7-diaminomitosene; *I*, $N^2$-methyl-2,7-diaminomitosene.

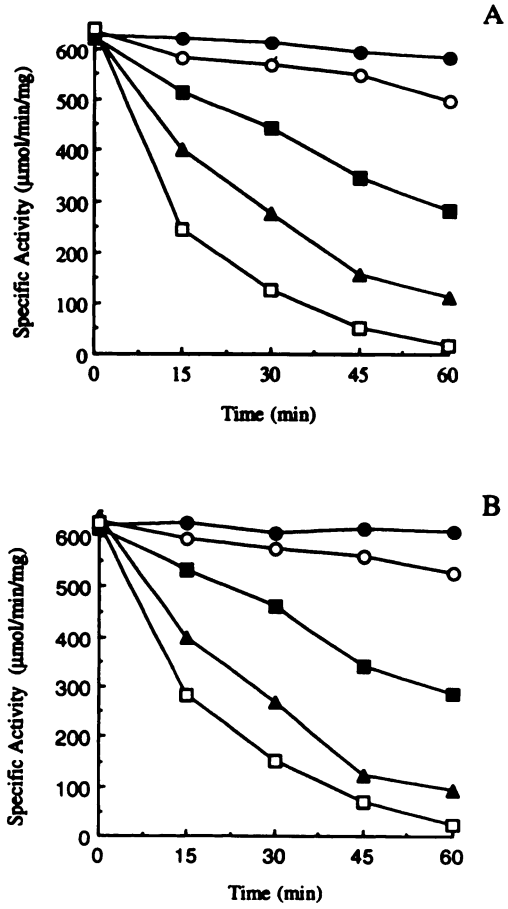



**Fig. 2.** Effect of pH on the inactivation of DTD by MMC and POR. The ability of MMC and POR (2 $\mu$M) to inactivate DTD was examined at pH values from 5.8 to 7.8. Reaction conditions are described in Materials and Methods. A, MMC; B, POR. ●, pH 5.8; ○, pH 6.5; ■, pH 7.0; ▲, pH 7.4; □, pH 7.8.

for 2 hr, and activity could not be restored by gel filtration. This suggests that the inhibition observed is a true inactivation process and not simply a decrease in catalytic rate. Enzyme inactivation was pH dependent, because reactions performed under identical conditions at pH 5.8 contained active DTD. POR, an analog of MMC, inactivated DTD in a similar pH-dependent manner. Inactivation of DTD by MMC or POR at pH 7.8 was both time and concentration dependent. Plots of log percent activity remaining versus time were linear, indicating pseudo-first-order kinetics. NADH was also required for enzyme inactivation, suggesting that a reactive species generated during reductive metabolism of either MMC or POR was responsible for enzyme inactivation. MMC has also been shown to inhibit the flavoprotein glutathione reductase, although it was proposed that inhibition was the result of hydrogen bond formation between FAD and unreduced MMC (29). The requirement for NADH for effective inhibition and the recovery of MMC equivalents bound to DTD during the inhibition process show that inhibition of DTD by MMC and POR occurs by a different mechanism.

When DTD was preincubated with MMC and NADH, the formation of a higher molecular mass product (60 kDa), indicative of a DTD cross-link, was observed. DTD cross-linking by MMC was pH dependent, with cross-linking decreasing as the pH of the reaction was lowered from 7.8 to 5.8. DTD cross-linking was unaffected by the addition of excess BSA, indicating that MMC alkylation was DTD specific. Cross-linking of DTD by MMC was also examined in greater resolution using 10% SDS-PAGE (20 mm) with silver staining (data not shown). No products with a molecular mass greater than that of the 60-kDa cross-linked dimer were detected, indicating that oligomer formation did not occur. Cross-linking was also examined by Western blot analysis. Both the monomer and the cross-linked dimer demonstrated immunoreactivity, suggesting that cross-linking induces little conformational change in the enzyme. Whether simple monofunctional binding of MMC to DTD is

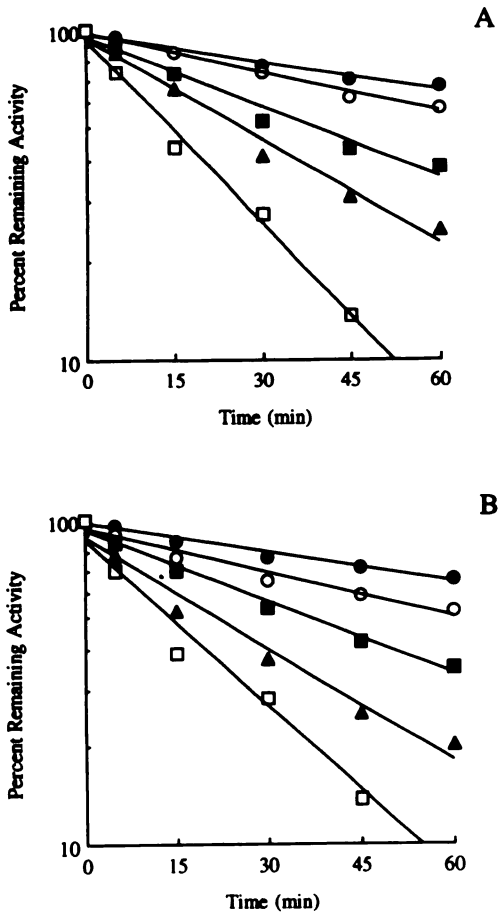



Fig. 3. Semilogarithmic plot showing time- and concentration-dependent inactivation of DTD by MMC and POR at pH 7.8. Reaction conditions are described in Materials and Methods. The *lines* were generated by linear regression analysis and are plotted as best fits. No inactivation of DTD was observed at time 0 with either MMC or POR. A, MMC; B, POR. ●, 0.25 μM; ○, 0.5 μM; ■, 1.0 μM; ▲, 2.0 μM; □, 4.0 μM.

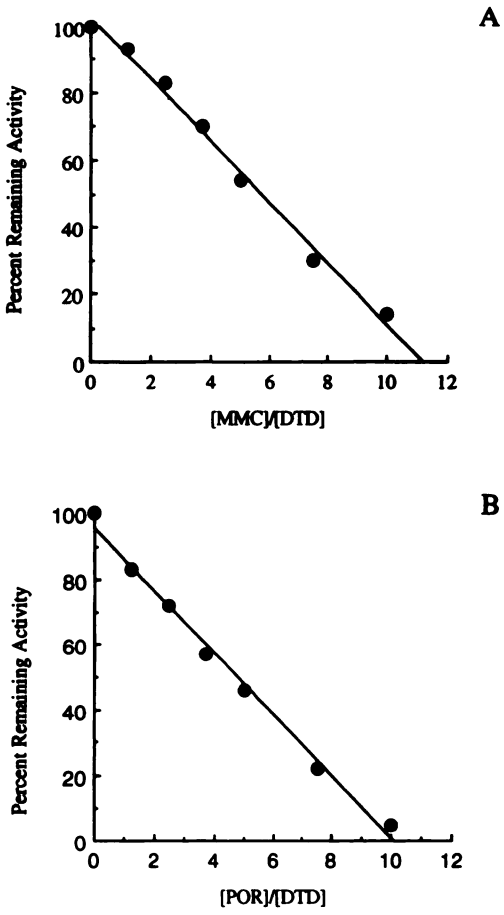



Fig. 4. Partition ratios for the inactivation of DTD by MMC and POR. Percent remaining activity is plotted versus the MMC/enzyme ratio. Reaction conditions are described in Materials and Methods. A, MMC; B, POR.

TABLE 1

**Stoichiometry of mitomycin C binding to DT-diaphorase**

| Method[a] | Mitomycin C[b] | DT-diaphorase[b] | Binding ratio[c] |
|---|---|---|---|
| | *nmol* | | *nmol/nmol* |
| A | 10.0 | 0.625 | 0.66 |
| A | 10.0 | 0.313 | 0.88 |
| B | 62.5 | 6.25 | 0.69 |
| B | 125 | 6.25 | 1.00 |

[a] Methods A and B are described in Materials and Methods.
[b] Total number of nanomoles of mitomycin C and DT-diaphorase present in reaction.
[c] Number of nanomoles of mitomycin C bound per nmol of DT-diaphorase.

TABLE 2

**Kinetic constants for the inactivation of DT-diaphorase by mitomycin C or porfiromycin[a]**

| | $K_{inact}$ | $K_I$ | Partition ratio |
|---|---|---|---|
| | $min^{-1}$ | μM | |
| Mitomycin C | 0.017 | 1.28 | 11 |
| Porfiromycin | 0.025 | 2.11 | 10 |

[a] Reaction conditions are described in Materials and Methods.

sufficient to inactivate the enzyme or whether bifunctional alkylation by MMC to form the cross-linked product is required has not been established. However, because >99% inactivation occurs in conjunction with an approximate 1:1 stoichiometry for inactivation and because complete cross-linking of the enzyme has not been observed, it is likely that monoalkylation is sufficient to inactivate the enzyme.

Mechanism-based inhibition occurs via production of a reactive intermediate that inactivates the enzyme before its release from the active site. Because the active site of DTD has not been characterized, the question of whether alkylation at the active site occurs during metabolism of MMC and POR must await further studies. Our data based on the pseudo-first-order kinetics of inhibition, the requirement for NADH for inactivation, covalent binding of [$^3$H]MMC to DTD, the approximate 1:1 stoichiometry for inactivation by MMC (30), and the cross-linking of the enzyme suggest that MMC and POR may be pH-dependent mechanism-based inhibitors of DTD. Additional studies are in progress to confirm this and to identify the target(s) for the covalent binding of MMC to DTD.

The ability of pH to influence the reactivity of MMC may reflect the ambivalent nature of the quinone methide formed after the reduction of MMC (28, 31). At acidic pH, the quinone methide can be trapped by a proton to form 2,7-diaminomitosene, whereas at higher pH values the quinone methide can function as an electrophile capable of alkylating DTD. Thus,

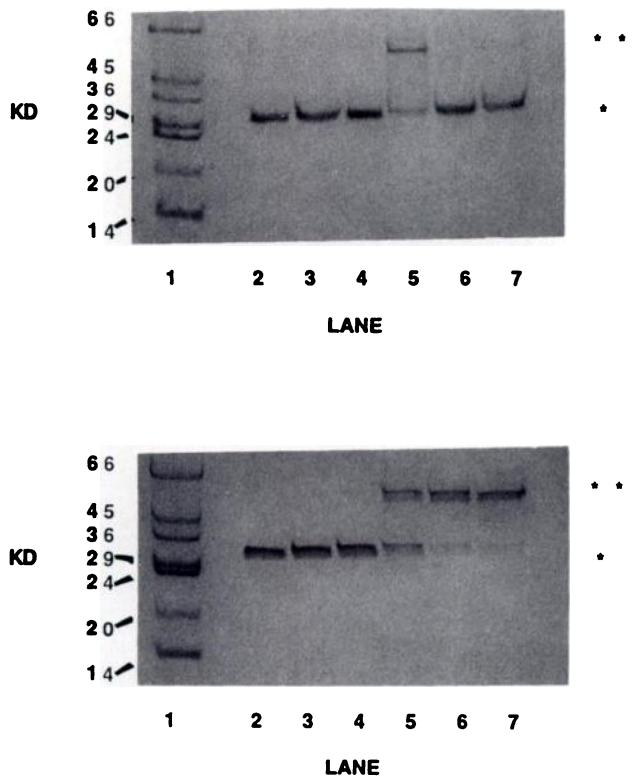



**Fig. 5.** SDS-PAGE of rat hepatic DTD incubated with MMC. SDS-PAGE was used to examine DTD after incubation with MMC. Reaction conditions are described in Materials and Methods. A, Molecular weight markers (*lane 1*), NADH and DTD, pH 5.8 (*lane 2*), NADH and DTD, pH 7.8 (*lane 3*), NADH, DTD, and MMC, pH 5.8 (*lane 4*), NADH, DTD, and MMC, pH 7.8 (*lane 5*), DTD and MMC, pH 7.8 (*lane 6*), and NADH, DTD, MMC, and dicumarol (20 μM), pH 7.8 (*lane 7*); B, molecular weight markers (*lane 1*), complete system (NADH, DTD, and MMC), pH 5.8 (*lane 2*), complete system, pH 6.2 (*lane 3*), complete system, pH 6.6 (*lane 4*), complete system, pH 7.0 (*lane 5*), complete system, pH 7.4 (*lane 6*), and complete system, pH 7.8 (*lane 7*). *, monomer; **, dimer.

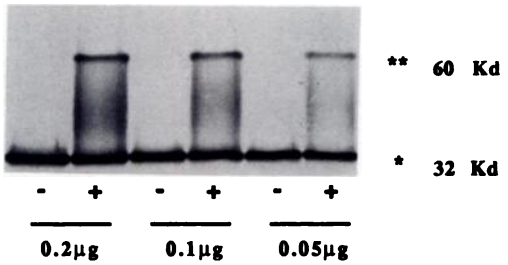



**Fig. 6.** Western blot analysis of rat hepatic DTD incubated with MMC. Western blot analysis was used to examine DTD incubated in the presence (+) or absence (−) of MMC at pH 7.8, as described in Materials and Methods. Analysis was performed using a rabbit polyclonal antibody.

at lower pH values metabolism of MMC by DTD can be observed, resulting in DNA cross-linking, whereas at higher pH values inhibition of DTD occurs. pH could also influence the metabolism of MMC by DTD at the level of the hydroquinone generated after the two-electron reduction. Because it has been suggested that the protonated hydroquinone is the immediate precursor to DNA cross-linking (23), this may explain increased DNA cross-linking at acidic pH (11). At basic pH, the hydroquinone could undergo transformation to the quinone methide in or near the active site of DTD, leading to enzyme

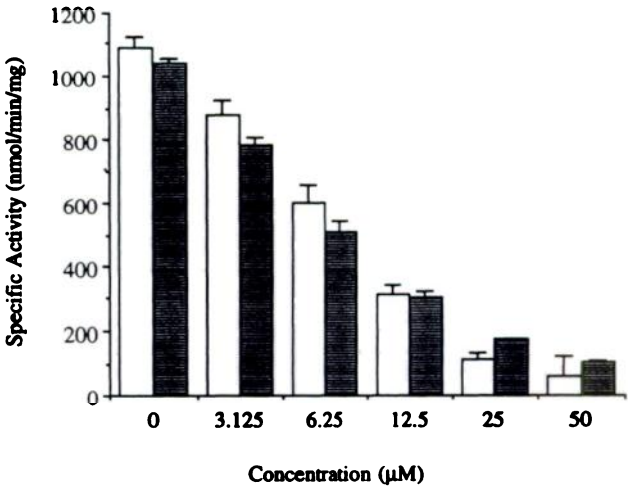



**Fig. 7.** Inactivation of HT-29 DTD by MMC or POR. DTD specific activity was measured in HT-29 human colon carcinoma cells after a 2-hr exposure to either MMC or POR (3.1–50 μM). Drug treatment and analysis were performed as described in Materials and Methods. □, MMC; ■, POR.

inactivation. pH may also influence the reactivity of certain amino acid residues such as the imidazole ring in histidine ($pK_a$ 6.5); at basic pH this group would be un-ionized and more likely to undergo nucleophilic attack. Another possibility involves the FAD cofactor, which could be alkylated to form a C(4a) adduct (32). However, when FAD was isolated from DTD and examined by diode-array HPLC, no evidence for the formation of a MMC-FAD adduct was observed. Preliminary data using a series of mitomycin analogs have shown that pH-dependent inactivation of DTD occurs only in compounds that contain a 7-$NH_2$ substituent, such as MMC and POR (33). It is possible that hydrogen bonding between the 7-$NH_2$ group and DTD may be important to align and hold the reactive species in the correct position for enzyme alkylation leading to enzyme inactivation (34).

In previous work using purified DTD in a cell-free system, we showed that DNA interstrand cross-linking induced by MMC could occur at pH values up to pH 7.4 but at pH 7.8 metabolite formation and DNA cross-linking could not be detected (11). In this manuscript, we have shown that this behavior reflects the pH-dependent inhibition of DTD by MMC. Inactivation of DTD by MMC or POR, however, is not limited to cell-free systems. HT-29 human colon carcinoma cells treated with either MMC or POR at pH 7.2 exhibited a concentration-dependent decrease in DTD specific activity, although whether a mechanism of inhibition of DTD occurs that is similar to that observed in a cell-free system is unknown. Whether MMC is bioactivated by DTD in a particular cell type or whether MMC inactivates DTD depends on intracellular pH, the concentrations of both MMC and DTD, and other enzyme systems that may be responsible for MMC metabolism. Because the bioactivation of MMC can be demonstrated at pH 7.4 and increased cytotoxicity of MMC in DTD-rich cell lines has been demonstrated (5–7), the use of MMC for the therapy of tumors high in DTD activity seems a viable approach. Because of the pH-dependent inactivation of DTD by MMC and POR, however, the use of strategies to lower intratumoral pH in combination with MMC or POR may produce greater effectiveness. A recent study has shown that lowering of intra-

cellular pH in DTD-rich cells, but not in DTD-deficient cells, enhances MMC cytotoxicity (35). Other compounds such as the aziridinylbenzoquinone 2,5-dimethyl-3,6-diaziridinyl-1,4-benzoquinone (36, 37) or the indoloquinone 3-hydroxy-5-aziridinyl-1-methyl-2-(1H-indole-4,7-dione)-propenol (38), which can be efficiently bioactivated by DTD, may also be useful candidates for the therapy of tumors high in DTD, such as non-small cell lung cancers (39).

In summary, the metabolism of both MMC and POR by DTD at basic pH results in the inactivation of the enzyme. Our data suggest that the inactivation process may be mechanism based and that reactive species generated during the metabolism of either MMC or POR are responsible for alkylation and inactivation of DTD.

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