Mitomycin C−DNA Adducts Generated by DT-Diaphorase. Revised Mechanism of the Enzymatic Reductive Activation of Mitomycin C[†]

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ABSTRACT: Mitomycin C (MC) was reductively activated by DT-diaphorase [DTD; NAD(P)H:quinone oxidoreductase] from rat liver carcinoma cells in the presence of *Micrococcus lysodeicticus* DNA at pH 5.8 and 7.4. The resulting alkylated MC−DNA complexes were digested to the nucleoside level and the covalent MC−nucleoside adducts were separated, identified, and quantitatively analyzed by HPLC. In analogous experiments, two other flavoreductases, NADH-cytochrome *c* reductase and NADPH-cytochrome *c* reductase, as well as two chemical reductive activating agents $Na_2S_2O_4$ and H_2/PtO_2 were employed as activators for the alkylation of DNA by MC. DTD as well as all the other activators generated the four known major guanine-N^2−MC adducts at both pHs. In addition, at the lower pH, the guanine-N7-linked adducts of 2,7-diaminomitosene were detectable in the adduct patterns. At a given pH all the enzymatic and chemical reducing agents generated very similar adduct patterns which, however, differed dramatically at the acidic as compared to the neutral pH. Overall yield of MC adducts was 3−4-fold greater at pH 7.4 than at 5.8 except in the case of DTD when it was 4-fold *lower*. Without exception, however, cross-link adduct yields were greater at the acidic pH (2−10-fold within the series). The ratio of adducts of bifunctional activation to those of monofunctional activation was 6−20-fold higher at the acidic as compared to the neutral pH. A comprehensive mechanism of the alkylation of DNA by activated MC was derived from the DNA adduct analysis which complements earlier model studies of the activation of MC. The mechanism consists of three competing activation pathways yielding three different DNA-reactive electrophiles **11**, **12**, and **17** which generate three unique sets of DNA adducts as endproducts. The relative amounts of these adducts are diagnostic of the relative rates of the competing pathways *in vitro*, and most likely, *in vivo*. Factors that influence the relative rates of individual pathways were identified.

The antitumor antibiotic mitomycin C (**1**; MC)[1] is used clinically for cancer chemotherapy. The mitomycins act primarily as DNA synthesis inhibitors due to their ability to inflict covalent damage to DNA. In this process, covalent cross-links are formed between the complementary strands of DNA (Iyer & Szybalski, 1963), accompanied by intrastrand cross-links (Bizanek et al., 1992) and monofunctional DNA adducts (Tomasz et al., 1986). These DNA alkylation processes are dependent on activation of MC by reduction of its quinone system by cellular reductases (Iyer & Szybalski, 1964). MC is thus regarded as the prototypical bioreductive alkylating agent (Lin et al., 1976).

The metabolites resulting from the reductive transformations of MC have been identified in cell-free systems (Tomasz & Lipman, 1981; Pan et al., 1984; Siegel et al., 1990; Peterson & Fisher, 1986) and in tumor cells and tissues (Pan, 1990; Chirrey et al., 1995; Cummings et al., 1993). The structure of the DNA cross-link adduct (Tomasz et al., 1987) as well as the other major DNA adducts have been determined (Tomasz et al., 1986; McGuinness et al., 1991; Bizanek et al., 1992). These adducts have also been identified in intact cells (Tomasz et al., 1987; Bizanek et al., 1993; Pan et al., 1993). The structures indicate that MC forms a cross-link in the minor groove between two guanines at their 2-amino groups (bisadducts **2** and **4**). The accompanying monoadducts (**3**, **5a**, and **5b**) are guanine-N^2 adducts as well (Scheme 1).

The complex mechanism of the reductive activation of MC has been widely studied. Such studies focused on the nature of the electron transfer step to the quinone of MC, on the ensuing chemical transformations leading to the ultimate DNA-reactive electrophile and on enzymes involved in the activation of MC in tumor cells. [As examples of chemical mechanism studies, see Tomasz and Lipman (1981), Kohn and Zein (1983), Peterson and Fischer (1986), Hoey et al. (1988), Pan et al. (1984), Danishefsky and Ciufolini (1984), Danishefsky and Egbertson (1986), and Schiltz and Kohn (1993)]. MC is reduced enzymatically under anaerobic conditions by a variety of flavoreductases utilizing NADH or NADPH as electron donors; for example, NADPH-cytochrome *c* reductase (EC 1.6.2.4), xanthine oxidase (EC 1.2.3.2), NADH-cytochrome b_5 reductase (EC 1.6.2.2), and DT-diaphorase [NAD(P)H:quinone-acceptor oxidoreductase; EC 1.6.99.2] (Cummings et al., 1995). A characteristic

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[1] Abbreviations: APase, alkaline phosphatase; MC, mitomycin C; 2,7-DAM, 2,7-diaminomitosene; DCPIP, 2,6-dichlorophenol-indophenol, DTD, DT-diaphorase; ML−DNA, *Micrococcus lysodeicticus* DNA; SVD, snake venom phosphodiesterase; ccr, cytochrome *c* reductase.

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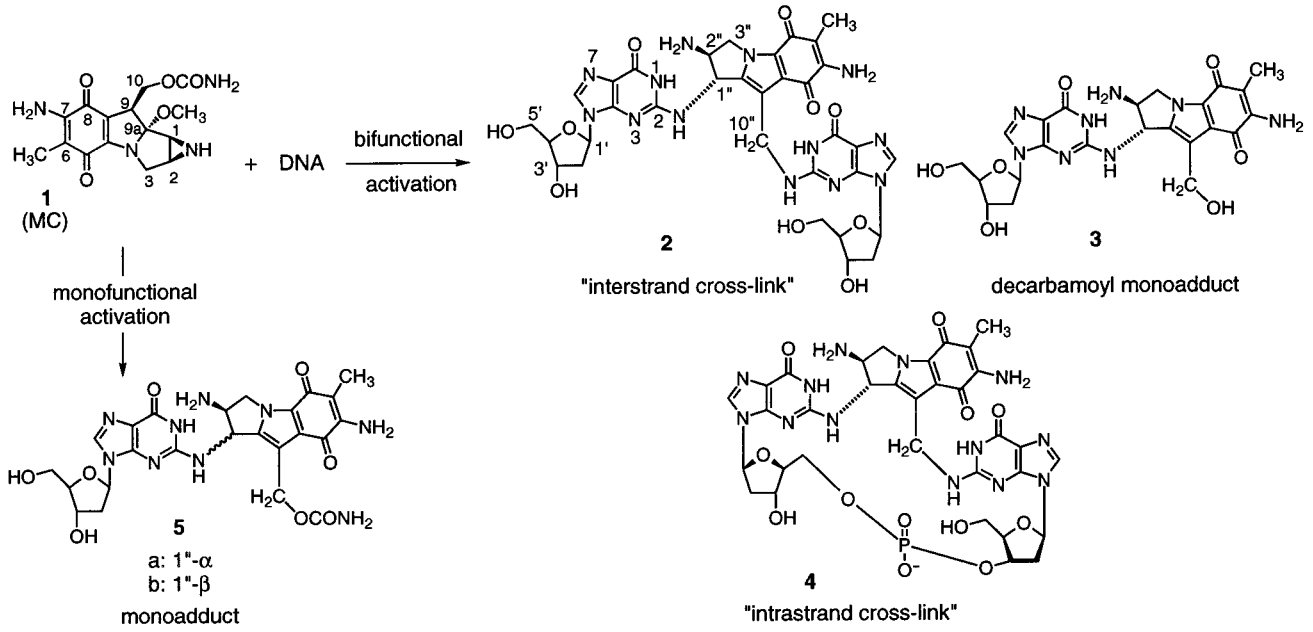
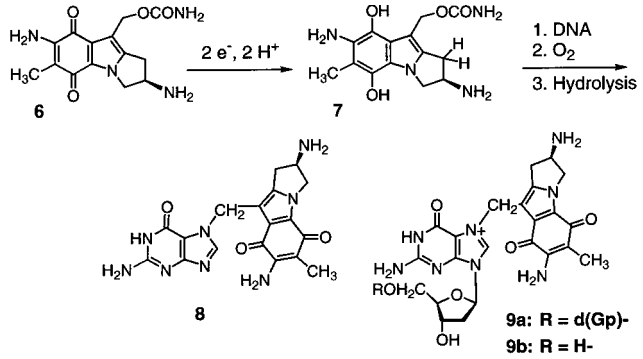
Scheme 1: MC−DNA Adducts (Reductive Activation)



feature *in vitro* of most of the enzymatic activation systems studied is the inhibition of the activation of MC in air, due to the fast reoxidation of the initially formed MC semiquinone by O_2 (Bachur et al., 1979; Kalyanaraman et al., 1980). DT-diaphorase (DTD) presents an exception, however, in that it catalyzes a *two-electron transfer* to quinones (Iyanagi & Yamazaki, 1970). MC activation by DTD is not inhibited in air since the initially formed MC hydroquinone is not susceptible to the fast reoxidation observed with the semiquinone (Siegel et al., 1990).

Activation of MC and other bioreductive anticancer agents is a newly discovered function of DTD. The main role of this enzyme is thought to be the reductive detoxication of xenobiotic quinones (Talalay et al., 1987). However, DTD has been shown recently to play a major role in the metabolic activation of MC and other bioreductive antitumor agents in a number of cancer cell lines as demonstrated by correlation of drug cytotoxicity with cellular DTD level [Fitzsimmons et al., 1996; for a review of additional work, see Workman (1996)]. Furthermore, cells transfected with DTD cDNA resulted in DTD overexpression and increased MC cytotoxicity (Belcourt et al., 1996; Mikami et al., 1996). A unique feature of the reduction of MC by DTD in cell-free systems is that DTD is inactivated by MC at or above physiological pH (Schlager & Powis, 1988; Siegel et al., 1993). This pH-dependent inactivation requires NADH and appears to be due to alkylation of the enzyme by MC as shown in elegant experiments by Siegel and co-workers (Siegel et al., 1993). Consequently, *in vitro* (cell-free) studies of DTD-catalyzed metabolism and DNA cross-linking by MC have been conducted at acidic pH, typically at pH 5.8, at which reductive activation by DTD occurs readily (Siegel et al., 1990, 1992; Prakash et al., 1993; Joseph et al., 1996). Under these conditions, an unexpected heat-labile DNA lesion was detected in addition to the usual, heat-stable cross-linking of DNA. The new lesion was attributed to guanine-N7 alkylation by 2,7-DAM (**6**), formed presumably from reduced MC *in situ* (Prakash et al., 1993). The structure of this lesion was determined as the guanine-N7−2,7-DAM adduct **8**, by the direct isolation of **8** and its derivatives **9a**

Scheme 2: Guanine-N7 Adducts by Reductive Alkylation of DNA by 2,7-DAM (**6**)



and **9b** from 2,7-DAM-treated DNA (Suresh Kumar et al., 1996). It was also isolated from EMT6 murine tumor cells treated with MC (Palom et al., 1997). In view of these results, it seemed possible that DTD activates MC by a different mechanism than the previously studied one-electron reductases, leading to different activated electrophile(s) and consequently, different DNA adducts (Cummings et al., 1995). An alternative possibility was that the difference in adducts is not activating enzyme-specific but rather it is caused by the lower pH employed in the DTD experiments.

Since DTD appears to play a major role in the anticancer activity of MC, an understanding of the mechanism of MC activation and DNA adduct formation effected by this enzyme is desirable. Accordingly, the aim of this work was to elucidate the MC−DNA adducts induced by DTD and derive the mechanism of their formation. In order to establish whether 2,7-DAM and its DNA adduct **8** are unique products of the activation of MC by DTD, the DNA adducts generated by the one-electron donating enzymes NADPH-cytochrome *c* reductase and NADH-cytochrome *c* reductase (EC 1.6.99.3) were also determined for comparison. Furthermore, the adducts formed in representative *chemical* MC-activating systems were identified under the same pH conditions. The results revealed that at a given pH, DT-diaphorase, cytochrome *c* reductases, and the chemical reducing agents all generate a very similar pattern of DNA

adducts (**8**, among them) with MC. The patterns differed dramatically, however, at acidic and neutral pH. From a quantitative analysis a comprehensive mechanism of the alkylation of DNA by activated MC was derived which is fully consistent with the activation mechanism of MC derived from earlier model studies in the absence of DNA (Peterson & Fisher, 1986; Hoey et al., 1988; Schiltz & Kohn, 1993). It consists of three competing activation pathways, only one of which leads to cross-linking of DNA. The three pathways generate three different, diagnostic sets of adducts. The relative rates of these pathways and the relative adduct distributions are determined by the pH rather than the nature of the activating agent. The results allow one to predict certain conditions which maximize the DNA cross-linking pathway of MC activation, desirable for chemotherapeutic activity.

MATERIALS AND METHODS

MC was received from Dr. D. M. Vyas of Bristol-Myers Squibb Co., Wallingford, CT. 2,7-DAM was synthesized from MC as previously described (Tomasz & Lipman, 1981). DT-diaphorase [NAD(P)H:quinone oxidoreductase; EC 1.6.99.2] from rat Walker 256 carcinoma cells was prepared as reported (Boland et al., 1991). NADH-cytochrome *c* reductase (NADH-FMN oxidoreductase; EC 1.6.99.3), NADH, NADPH, and *M. lysodeicticus* DNA (Type XI; 72% GC) were purchased from Sigma, St. Louis, MO. NADPH-cytochrome *c* reductase (EC 1.6.2.4), purified from rabbit heart was received from Dr. Wayne Backes, Louisiana State Medical School, New Orleans, LA. DNAse I (code D), phosphodiesterase I (snake venom diesterase), and *Escherichia coli* alkaline phosphatase (type III-R) were purchased from Worthington, Freehold, NJ.

Enzyme Activities. DTD was assayed by the method of Benson et al. (1980). One unit was defined as that reducing 1.0 μmol of DCPIP/min under the conditions of the assay. NADH-cytochrome *c* reductase and NADPH-cytochrome *c* reductase were assayed by reduction of oxidized cytochrome *c* (Mahler, 1955). One unit reduces 1.0 μmol/min under the conditions of the assay.

Quantitation of DNA, MC−DNA adducts, and other MC derivatives was based on UV spectrophotometry. Extinction coefficients used were the following: MC, 21 800 (367 nm); ML−DNA, 7000 (260 nm; mononucleotide unit); and dT, 6600 (254 nm). For adducts, we used the following extinction coefficients (254 nm): **3**, **5a**, and **5b**, 24 000 and **2**, 30 000.

Molar proportions of individual substances in the HPLC profile were determined by peak area measurements. Peak areas were divided by the appropriate extinction coefficient at the wavelength of the detection; this gave molar proportions.

Yields of adducts were determined by relating their molar proportion to the dT peak of the digest serving as internal standard, then the known dT:dG molar ratio of ML−DNA (0.39) was used for calculating the yield of the adduct based on total dG of ML−DNA (Suresh Kumar et al., 1996).

Reproducibility. The HPLC profiles shown in Figure 1 and 2 were used for calculations of the yields presented in Figure 3. However, each specific type of reaction between drug and DNA was repeated at least three times (some many more times, in the span of two years) and the HPLC profile of the DNA adducts was determined in each such repeated experiment. The profiles were closely reproducible to the eye, without exception. Although not all profiles were evaluated for yields quantitatively, average deviation from the mean value of three experiments never exceeded ± 15% in the cases where this was determined.

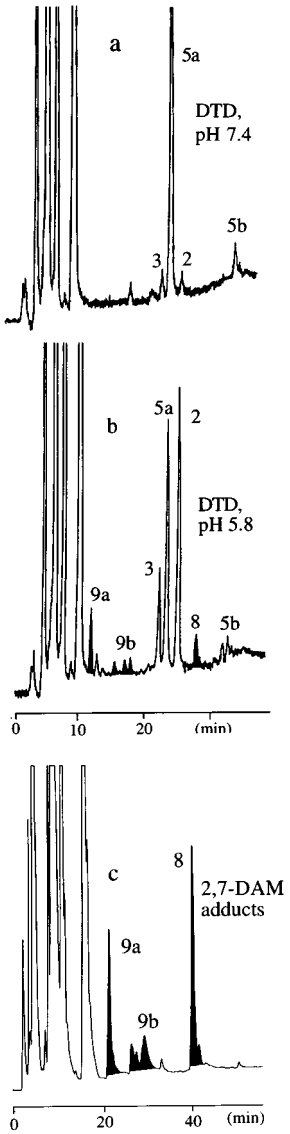


FIGURE 1: HPLC of digests of MC−DNA complexes formed under activation of MC by DT-diaphorase under anaerobic conditions, at two different pHs: (a) pH 7.4 and (b) pH 5.8. HPLC conditions: 6−18% acetonitrile in 0.03 M potassium phosphate, pH 5.4, in 60 min. Flow-rate: 1.0 mL/min. Detection: 254 nm. (c) HPLC of digest of 2,7-DAM−DNA complex formed under activation of 2,7-DAM by DT-diaphorase; reproduced from data from Suresh Kumar et al. (1996). Shaded peaks represent 2,7-DAM adducts.

Standard DNA adducts of MC or 2,7-DAM were prepared by published methods (Tomasz et al., 1986; 1987a; Suresh Kumar et al., 1996).

DNA Alkylation by MC under Activation by Various Reducing Systems: (i) DTD/NADH. ML−DNA (0.5 μmol), MC (0.5 μmol), and NADH (1.0 μmol) MC were dissolved in a 0.01 M potassium phosphate-0.001 M EDTA, pH 5.8 buffer (0.40 mL). DTD (0.044 units in a 10 μL of 0.01 M potassium phosphate, pH 7.0 solution) was added, and the mixture was incubated at 37 °C for 50 min. This procedure was conducted either under aerobic or anaerobic conditions; the latter was achieved by purging the solution continuously

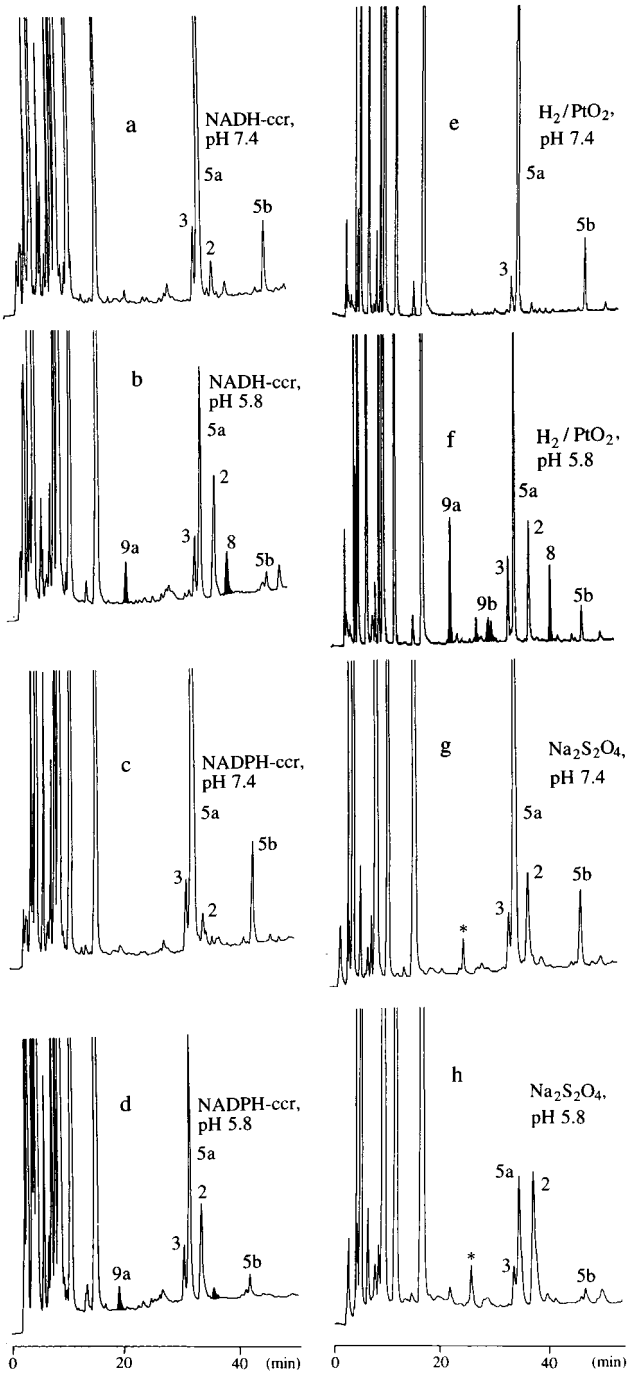


FIGURE 2: HPLC of digests of MC−DNA complexes formed under activation of MC by various reducing agents at two different pHs. (a) NADH-cytochrome *c* reductase/NADH, pH 7.4. (b) Same, pH 5.8. (c) NADPH-cytochrome *c* reductase/NADPH, pH 7.4. (d) Same, pH 5.8. (e) H_2/PtO_2, pH 7.4. (f) Same, pH 5.8. (g) $Na_2S_2O_4$, pH 7.4. (h) Same, pH 5.8. Shaded peaks represent 2,7-DAM adducts. In panels g and h, the peaks marked with an asterisk are C-10″ bisulfite derivatives of **5a** (McGuinness et al., 1991). HPLC conditions: 3−18% acetonitrile in 0.03 M KH_2PO_4, pH 5.5, in 90 min. Flow-rate: 1 mL/min. Detection: 254 nm.

with argon. The same reaction was carried out in a pH 7.4 buffer also. The reaction was stopped by applying the mixture on a Sephadex G-100 column (2.5 × 56 cm; 0.02 M NH_4HCO_3 as eluant). The MC−DNA complex was isolated from the void volume fraction by lyophilization.

(ii) NADPH-Cytochrome c Reductase/NADPH. ML−DNA (0.5 μmol mononucleotide), MC (0.5 μmol) and NADPH (1 μmol) were mixed in a 0.01 M potassium phosphate−0.001 M EDTA, pH 5.8 buffer (0.40 mL) and

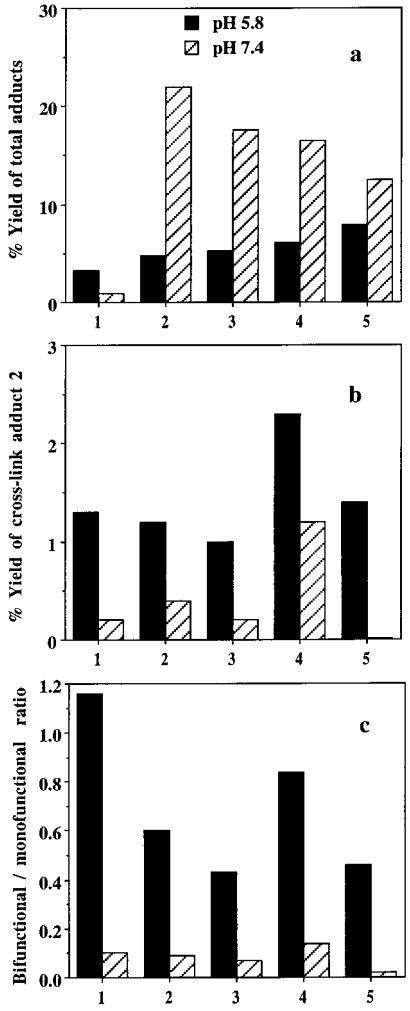


FIGURE 3: Yields of MC-deoxyguanosine adducts formed in ML−DNA using various activating agents (1−5) for activation of MC at pH 5.8 and pH 7.4. 1, DTD/NADH; 2, NADH-cytochrome *c* reductase/NADH; 3, NADPH-cytochrome *c* reductase/NADPH; 4, $Na_2S_2O_4$; 5, H_2/PtO_2. (a) Percent yield of total MC adducts, defined at percent DNA-guanine converted to adducts, **2**, **3**, **5a**, and **5b**. (b) Percent yield of the cross-link adduct **2**, defined as percent DNA-guanine converted to adduct **2**. (c) Bifunctional:monofunctional (adduct yield) ratio is defined as the ratio of percent yield of (**2** + **3**) to percent yield of (**5a** + **5b**).

deaerated by bubbling argon through the solution. NADPH-cytochrome *c* reductase (0.5 unit; 1 μL) was then added from a stock solution in 0.1 M potassium phosphate, pH 7.4. Incubation followed for 50 min at 37 °C under argon bubbling. The same procedure was also carried out in a pH 7.4 buffer. The MC−DNA complex was isolated as above.

(iii) NADH-Cytochrome c Reductase/NADH: The analogous procedure as in point ii was employed except that NADPH was replaced by NADH.

(iv) $Na_2S_2O_4$. ML−DNA (0.5 μmol), MC (2 μmol), and $Na_2S_2O_4$ (3 μmol; from a freshly prepared anaerobic 0.12 M solution in water) were incubated aerobically in a 0.01 M potassium phosphate−0.001 M EDTA, pH 5.8 buffer (0.4 mL) for 1 h at 37 °C. The same procedure was also carried out in a pH 7.4 buffer. The MC−DNA complex was isolated as above.

(v) H_2/PtO_2. ML−DNA (0.5 μmol) and MC (1.0 μmol) were dissolved in a 0.01 M potassium phosphate−0.001 M EDTA, pH 5.8 buffer (0.5 mL), to which approximately 100 μg of PtO_2 was then added. The mixture was deaerated by

bubbling argon for 5 min followed by hydrogenation by bubbling H_2 gas for 5 min. The PtO_2 was removed by centrifugation. The same procedure was repeated in a pH 7.4 buffer. The MC−DNA complex was isolated as above.

(vi) Control (No Reducing Agent). ML−DNA and MC were incubated under the same conditions described under point i, except that NADH and DT-diaphorase or just DT-diaphorase were omitted. Isolation procedures were also identical.

Enzymatic Digestion of MC−DNA Complexes to Nucleosides and MC−Nucleoside Adducts. DNAse I (345 units/μmol DNA), SVD (21 units/μmol DNA), and APase (5 units/μmol DNA) were used by a previously described protocol (Tomasz et al., 1986). The digest was directly fractionated by HPLC.

HPLC. A C-18 reverse-phase column (Rainin; Microsorb Analytical; 0.45 × 25 cm) was used, attached to a Beckman gradient HPLC apparatus equipped with integrator and variable wavelength detector. Various gradients of 0.03 M KH_2PO_4 (pH 5.5) and acetonitrile were used as the eluant as specified in the figures.

Rate of MC Consumption by DTD- and NADH-Cytochrome c Reductase-Catalyzed Reactions. MC consumption was measured by HPLC. Reaction conditions were MC (1.25 mM), NADH (2.5 mM), and DTD (0.625 units/mL) in 0.1 M potassium phosphate, pH 5.8 or 7.4 buffers, at 37 °C under aerobic conditions. Reaction conditions using NADH-cytochrome *c* reductase were the same as with DTD, except that incubation was conducted under anaerobic conditions. Aliquots were directly injected into HPLC at appropriate incubation time intervals. A control incubation of MC + NADH was analyzed similarly. The incubation mixture included 2′-deoxythymidine (1.25 mM) as a quantitative marker for measuring MC consumption, as previously described (Sharma & Tomasz, 1994).

RESULTS

MC−DNA adducts formed under activation by DT-Diaphorase: Comparison at pH 7.4 and 5.8. The MC−DNA alkylation complex was digested to nucleosides and nucleoside−MC adducts. HPLC of the digest (Figure 1, panels a and b) shows that the adduct profiles are radically different at the two pHs. At pH 7.4, adduct **5a** is dominant, while at pH 5.8, the cross-link adduct **2** is the major component. In addition, at pH 5.8, but not at pH 7.4, adducts **8**, **9a**, and **9b** are also detectable. Adducts **2**, **3**, **5a**, and **5b**[2] are minor groove G-N[2] alkylation products (Tomasz et al., 1986, 1987; Scheme 1), while **8**, **9a**, and **9b** are 2,7-DAM adducts (Scheme 2). The identity of adducts **8**, **9a**, and **9b** is illustrated by Figure 1c, which shows formation of the same adducts from DNA directly exposed to activated 2,7-DAM [reproduced from Suresh Kumar et al. (1996)]. HPLC of control reactions in the absence of DT-diaphorase (not shown) indicate that a small amount of adduct **5a** is formed at pH 5.8 due to activation of MC by acidic pH (Tomasz et al., 1987b). There was no significant difference between adduct profiles from aerobic and anaerobic reactions conducted at the same pH (data not shown). Overall, less MC−DNA adduct is formed at pH 7.4 than at pH 5.8 (Figure 3a).

[2] The intrastrand cross-link **4** is known to hydrolyze to **2** under the digestion conditions used herein; therefore, it is not detected separately (Bizanek et al., 1992).

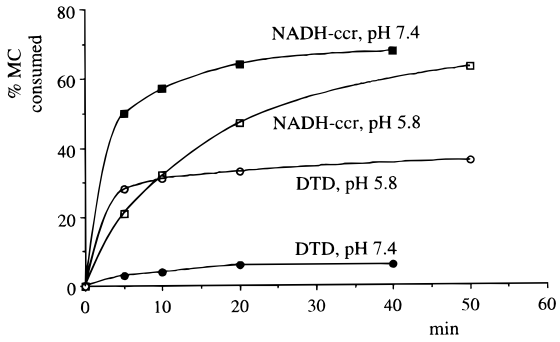


FIGURE 4: Rates of reduction of MC catalyzed by DTD and NADH-cytochrome *c* reductase at pH 5.8 and 7.4.

MC−DNA Adducts Formed under Activation by NADH-Cytochrome c Reductase and NADPH-Cytochrome c Reductase at pH 5.8 and 7.4. Qualitatively, these two enzymes induced the same DNA adduct profiles as DT-diaphorase, above (Figure 2, panels a−d). Thus, at pH 7.4, monoadduct **5a** is the major product of DNA alkylation, while at pH 5.8, the cross-link adduct **2** is formed in more substantial amounts, along with the decarbamoyl adduct **3b** and the G-N7 adducts of 2,7-DAM (**8** and **9**). In contrast to the DTD system, however, the total MC−DNA adduct yield is greater at pH 7.4 than at pH 5.8 using NADPH-cytochrome *c* reductase NADH-cytochrome *c* reductase (4.6 and 3.3 times, respectively; see Figure 3a).

MC−DNA Adducts Formed under Chemical Activation of MC: Comparison at pH 5.8 and 7.4. Catalytic hydrogenation (Figure 2, panels e and f) as an activating agent resulted in similar adduct distributions to those observed under enzymatic activation: at pH 7.4 mostly the monoadduct **5a** and its isomer **5b** were formed, while at pH 5.8, the other adducts also appeared in the HPLC profile. The $Na_2S_2O_4$-activated reaction at pH 7.4 (Figure 2g) produced mostly monoadduct **5a**, while at pH 5.8 (Figure 1h), the total adduct yield decreased drastically (2.7-fold; Figure 3a), but the yield of the cross-link (**2**) alone *increased* as compared to that at pH 7.4 (2.3% vs 1.2%; Figure 3b). No 2,7-DAM adducts (**8**, **9a**, and **9b**) were apparent, however, under the $Na_2S_2O_4$ activation at either pH.[3]

Rates of Reduction of MC by DTD and NADH-Cytochrome c Reductase at pH 7.4 and 5.8. This is illustrated in Figure 4. The pH dependence of reduction of MC shows a sharp contrast between the two enzymes. Reduction of MC by NADH-cytochrome *c* reductase at pH 7.4 is faster than at pH 5.8; reduction of MC by DTD at pH 7.4 on the other hand is very slow compared to that at pH 5.8, in agreement with the known pH dependence of MC reduction by this enzyme (Siegel et al., 1990).

DISCUSSION

DNA Adducts of DT-Diaphorase-Activated MC. A conspicuous finding is that overall, much less DNA adduct is formed at pH 7.4 than at pH 5.8, in sharp contrast to all the other activating agents (Figure 3a). This is qualitatively consistent with the unique inactivation of DTD by reduced MC with increasing pH, resulting in low efficiency of MC reduction at pH 7.4 (Siegel et al., 1992, 1993; see also Figure 4). At pH 5.8, however, the MC adduct yield is comparable

[3] 2,7-DAM reacts with HSO_3 ions, formed in the reduction mixture (Schiltz & Kohn, 1993) rather than it alkylates DNA (Li & Kohn, 1992).

to that obtained with other activating agents (Figure 3a). Detection of individual adducts by HPLC shows conclusively that the previously observed cross-linking of DNA and oligonucleotides by DTD-activated MC (Siegel et al., 1992; Prakash et al., 1993) is due to the minor groove cross-link adduct, **2**. The minor groove monoadducts, **3**, **5a**, and **5b** are detected in DTD-activated MC−DNA complexes for the first time. It is also important that the major groove 2,7-DAM adduct **8** and its derivatives **9a** and **9b** are shown to be formed from activated MC, side-by-side with the MC adducts **2**, **3**, and **5a** (Figure 1b). Formation of **8** was shown before directly from reaction of DNA with DTD-activated 2,7-DAM but not with MC (Suresh Kumar et al., 1996). Prakash and coworkers, however, found that both drugs induced the same piperidine-labile lesions in DNA restriction fragments, indicative of guanine-N7 alkylation by 2,7-DAM in both cases (Prakash et al., 1993). It is now confirmed that the MC-induced heat-labile sites of DNA are due to formation of **8**, an adduct of the reduction product of MC, i.e., 2,7-DAM (**6**), and guanine-N7 of DNA, as originally proposed by Prakash and co-workers (Schemes 1 and 2). Significantly, we found recently that **8** is formed as a major adduct also in MC-treated tumor cells (Palom et al., 1997), indicating that this is a substantial pathway *in vivo*, although enzymes other than DTD could equally be responsible for the activation (see below).

A main conclusion from these findings is that DTD is capable of generating all previously known MC−DNA adducts formed *in vitro* and *in vivo* and thus remains a potential candidate for being an *in vivo* activator of MC, although its low activity of MC reduction and low DNA adduct yields at the physiological pH 7.4 seen in the cell-free system are somewhat difficult to reconcile with this notion. Reduction of MC by DTD, however, may be more favorable in intracellular settings than in *in vitro* systems.

DNA Adducts from One-Electron vs Two-Electron Reduction of MC. We find here that the obligatory two-electron-donating reductase DTD (Ernster, 1967) qualitatively generates the same adducts, in similar distribution, as the one-electron-donating enzymes NADPH-cytochrome *c* reductase and NADH-cytochrome *c* reductase under anaerobic conditions, at both pHs (Figures 1 and 2). A quantitative difference, namely the anomalously low adduct yield in the case of DTD at pH 7.4 (Figure 3a) is readily explained by the limited reduction of MC by DTD at pH 7.4, due to inactivation, as discussed above. The other enzymes reduce MC somewhat faster at pH 7.4 than at 5.8 (Figure 4; Pan et al., 1984). The identical nature of the products of one-electron- and two-electron-reduced MC with DNA might have been expected since under anaerobic conditions the initial one-electron semiquinone radical reduction product of MC rapidly disproportionates to quinone and hydroquinone (Hoey et al., 1988; Machtalere et al., 1988). Thus, the DNA adduct profiles observed in the present study support the conclusion that there is a *common intermediate after one- and two-electron reduction* and that the intermediate is the hydroquinone. Note, however, that under aerobic conditions, the one-electron reductant-generated MC semiquinone radical is reoxidized to MC by O_2 faster than it is disproportionated, resulting in redox cycling but no activation (Pan et al., 1984, 1986; Dusre et al., 1990; Iyanagi & Yamazaki, 1970). The greater cytotoxicity of MC to hypoxic cells has been generally attributed to this mechanism of inhibition of its activation by O_2. DTD-mediated activation of MC (two-electron reduction) is insensitive to O_2, however (Siegel et al., 1990).

The chemical reducing agents H_2/PtO_2 and $Na_2S_2O_4$ produced DNA adduct patterns which were very similar to those produced by the enzymes (Figure 2), except that $Na_2S_2O_4$ yielded no 2,7-DAM adducts **8** and **9**.[3] One may conclude that both the enzymatic and the chemical reducing agents play the same, single role in the activation of MC: reduction of the quinone.

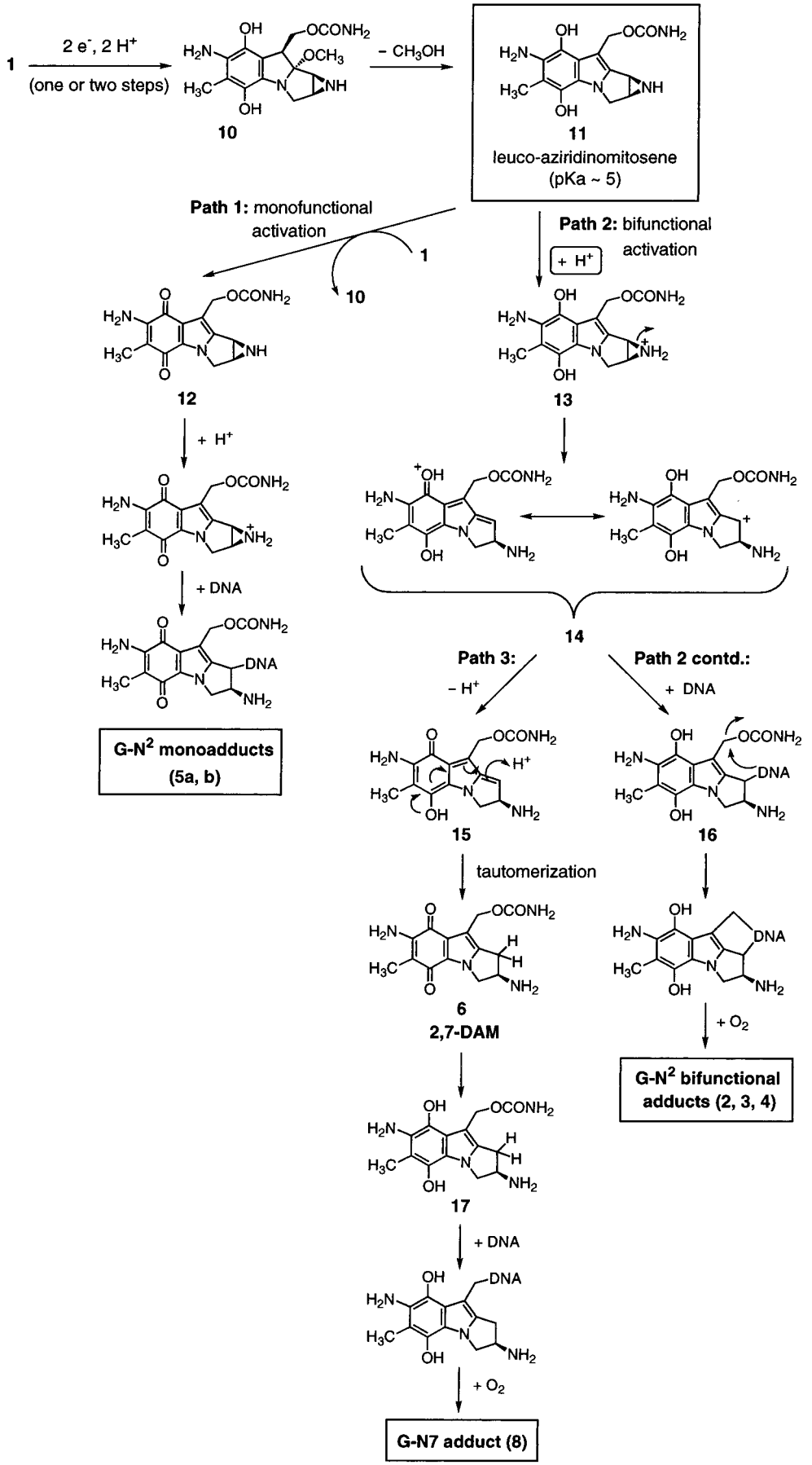
Switch from Monofunctional to Bifunctional DNA Alkylation by MC at the Lower pH. A common dramatic difference is observed between the distribution of the adducts formed at pH 5.8 and 7.4, independently of the activating system used. At pH 7.4, the predominant adduct is **5a**, indicating monofunctional activation at C-1 of MC. In contrast, at pH 5.8, the pair of adducts **2** and **3**, characteristic of bifunctional activation (Tomasz et al., 1988) is predominant; the monoadduct **5a** is suppressed (Figures 1, 2, and 3c). Consistent with this result, a trend of increased DNA cross-linking at acidic pH by reduced MC has been reported previously (Teng et al., 1989; Siegel et al., 1992). Similarly, the amount of bifunctional adduct **3** relative to monoadduct **5** increased when activation was conducted at acidic pH (Yu & Pan, 1993).

Another major difference between adducts at pH 5.8 and 7.4 is the appearance of the 2,7-DAM adduct **8** and its derivatives **9a** and **9b** selectively in the lower pH adduct patterns. The acidic pH selectivity of formation of this guanine-N7 adduct was shown first indirectly by a piperidine-cleavage assay (Prakash et al., 1993).

In summary, the switch from monofunctional to bifunctional alkylation products of MC at lower pH is now shown to be a general phenomenon, regardless of what reducing agent is used. This indicates that the pH alone determines further conversions of the reduced MC (leucomitomycin C **11**) to various different ultimate alkylating species. Importantly, a pH effect *in vivo* was observed in MC-treated tumor cells: when the cells were grown in pH 5.7 culture medium, they contained more cross-links in their DNA and were more sensitive to MC than those under physiological growth conditions (Kennedy et al., 1985). This suggests that the intracellular chemical path of alkylation of DNA by MC is the same as that in the cell-free systems. Alternatively, increased activity of an activating enzyme at low pH such as that of DT-diaphorase would explain this result just as well. Further work is needed to distinguish between these two alternatives.

Mechanism of Monofunctional and Bifunctional Alkylation of DNA by MC. Since DNA interstrand cross-links are generally considered to be more cytotoxic, knowledge of the factors that promote bifunctional vs monofunctional alkylation of DNA by MC is important. Studies of MC activation conducted previously in model systems which did not include DNA have laid the groundwork for a mechanism which displays considerable complexity. An important recent model study is reductive alkylation by MC employing aniline as nucleophile instead of DNA, at various pHs (Schiltz & Kohn, 1993). In the present work, we analyzed the DNA alkylation products themselves. We found that the observed DNA adducts and their pH-dependent distribution are analogous to the aniline model products and their pH dependence. By applying the results of the model studies

Scheme 3: Mechanism of Monofunctional and Bifunctional Alkylation of DNA by MC



with those of the direct DNA study presented herein, the mechanism of mono- and bifunctional alkylation of DNA by reduced MC can be described as follows (Scheme 3).

Leucoaziridinomitosene **11**, a well-established intermediate in the reductive activation process (Egbertson & Danishefsky, 1987) is the branch point at the competing *monofunctional* and *bifunctional activation* pathways (path 1 and path 2, respectively). On path 1, it transfers one or two electrons to MC, driven by a large redox potential difference between mitosenes and mitosanes (Peterson & Fisher, 1986), to give the aziridinomitosene **12** (Schiltz & Kohn, 1993), constituting autocatalytic activation of MC. Kohn's group showed that **12** is a reactive C-1 monofunctional alkylating agent which monoalkylates DNA (Li et al., 1996). Thus, pathway 1 accounts for the formation of MC monoadducts **5a** and **5b**.

On the competing pathway 2 the leucoaziridinomitosene **11** opens its aziridine via **13** by acid catalysis (Hoey et al., 1988)[4] to give the C-1 carbonium ion (**14**). Evidence for S_N1 reactivity of **11** (and thus proof for existence of **14**) has been obtained using model nucleophiles (Schiltz & Kohn, 1993) and also using DNA (Gargiulo et al., 1995). The extensive charge delocalization in **14** by the "quinone methide" resonance form of the dihydroxyindole system allows **14** to have sufficient lifetime to react with the nucleophile DNA (path 2) or, in the deprotonated state (**15**), to tautomerize (enol → keto) to 2,7-DAM (path 3). This competition between paths 2 and 3 was powerfully demonstrated by Schiltz and Kohn (1993), using aniline as model nucleophile. It is clear from their quantitative product analysis that path 3, i.e., the electrophilic protonation of **15** to give 2,7-DAM is a substantial competitor of the bifunctional, nucleophilic path 2. In fact, the latter functions only in the presence of strong nucleophiles, e.g., aniline, or in the present case, of DNA. In the absence of such nucleophiles, the reactive species **14** is mostly drawn into the electrophilic path 3, to give 2,7-DAM. It has been well-established that at acidic pH 2,7-DAM is the major product of reduction of MC (Tomasz & Lipman, 1981; Pan et al., 1984; Peterson & Fisher, 1986; Hoey et al., 1988). Qualitative evidence for formation of 2,7-DAM (path 3) in the present work is provided by detection of 2,7-DAM−DNA adducts **8**, **9a**, and **9b**.

This comprehensive mechanism provides a precise explanation for the predominant formation of monofunctional DNA adducts (**5a** and **5b**) at pH 7.4 or above. At such higher pH, the monofunctional path 1 predominates since its competitor, the bifunctional path 2, being acid-catalyzed, is relatively slow, as shown by many studies (Schiltz & Kohn, 1993; Egbertson & Danishefsky, 1987; Tomasz & Lipman, 1981; Pan et al., 1983; Peterson & Fisher, 1986; Hoey et al., 1988; Teng et al., 1989). At the lower pH 5.8, however, products of the bifunctional activation pathway 2 predominate (adducts **2** and **3**), because the irreversible aziridine-opening step of the bifunctional pathway 2 is acid catalyzed while path 1 does not involve acid catalysis. This mechanism accounts for previous observations that DNA cross-linking by reduced MC *increases* with decreasing pH in the pH 5−8 region (Teng et al., 1989; Siegel et al., 1992; Prakash et al., 1993). It also explains why total MC−DNA adduct yield is generally lower at pH 5.8 than at pH 7.4 (Figure 3a): Acid catalysis promotes not only path 2 but also its branch, path 3, which leads to the "waste product", 2,7-DAM.

Conclusion. Reduced MC **10** is converted spontaneously to leucoaziridinomitosene **11**, which is then partitioned between paths 1 and 2. Path 1 constitutes monofunctional activation of MC and is selectively promoted by *high concentration of unreduced MC* (**1**) due to bimolecular kinetics with respect to MC. *In vitro* activation at neutral pH usually proceeds mainly by path 1. The DNA-alkylating species of path 1 is 7-aminoaziridine **12**. Path 2, i.e., bifunctional activation of MC, is selectively promoted by *decreasing pH* since its irreversible first step is protonation-catalyzed opening of the aziridine ring. The DNA-alkylating species of this path is the protonated leucoaziridinomitosene **13**. Path 2 is branched off also to path 3, *via* quinone methide **15** leading to 2,7-DAM, and a third alkylating species **17**. Low pH decreases the rate of path 3 relative to path 2 (Schiltz & Kohn, 1993). All-in-all, bifunctional DNA alkylation is favored over monofunctional alkylation by (i) fast-reducing conditions which remove MC,[5] (ii) acidic pH, or (iii) very low overall MC concentration (Palom et al., 1997).

Mechanism in Vivo. As shown here, *in vitro* reductase-catalyzed DNA cross-linking is quite inefficient relative to monofunctional alkylation at the physiological pH 7.4 (Figure 3c). DNA cross-linking, the hallmark of MC's activity, does occur *in vivo*, however. The mechanism above explains this apparent paradox as follows. At the low concentration of MC in MC-treated cells or tissues, the monofunctional activation pathway 1 is expected to be relatively inactive, especially when MC is removed rapidly by reduction, as under hypoxic conditions or by high reductase activity. These conditions allow pathway 2 to proceed instead, leading to DNA cross-linking. It follows from the mechanism, however, that unavoidably, a portion of the reduced MC is protonated *via* path 3, to give 2,7-DAM, a relatively nontoxic MC metabolite. This is reminiscent of the reductive metabolism of doxorubicin in which protonation generates the nontoxic 7-deoxyaglycone rather than a DNA-alkylating species (Cummings et al., 1992). Determination of DNA cross-linking (Fracasso & Sartorelli; 1986; Begleiter et al., 1992; Siegel et al., 1990) and DNA adducts (Bizanek et al., 1993; Palom et al., 1987) in MC-treated tumor cells verify this scenario.

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[4] The pK_a of **11** may be estimated as approximately 5, by assuming that it is the same as that measured for **10** (Hoey et al., 1988).

[5] For example, excess $Na_2S_2O_4$ under anaerobic conditions (Tomasz et al., 1988). If a high yield of cross-linked DNA is desired *in vitro* the procedure of incremental addition of the total $Na_2S_2O_4$ is to be chosen (Tomasz et al., 1987a; Borowy-Borowski et al., 1990). This reaction is likely to proceed in two steps: first monoalkylation *via* path 1, followed by reductive activation of the DNA monoadduct (Schiltz & Kohn, 1993).

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