Sequence-Selective Alkylation and Cross-Linking Induced by Mitomycin C upon Activation by DT-Diaphorase†

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ABSTRACT: Aerobic reduction of MMC by DTD, an obligate two-electron reductase, or chemical reduction by sodium borohydride results predominantly in monoalkylation of DNA at the guanine N7 position within 5′-GG-3′ and 5′-GTC-3′ sequences. The level of guanine N7 alkylation after DTD reduction increased as the pH was decreased from 7.8 and was optimal at pH 6.6. A similar profile of alkylation was obtained when the major metabolite of DTD-mediated MMC metabolism, 2,7-diaminomitosene, was further reduced by DTD. The sequence preference for DNA interstrand cross-linking (ISC) was also determined using singly end-labeled oligonucleotide duplexes. Reduction of MMC by DTD induced DNA cross-links which were resistant to piperidine cleavage. Exposure of cross-linked DNA to dimethyl sulfate or formic acid and subsequent piperidine cleavage displayed a discontinuity in band pattern which suggested a 5′-CG-3′ preference for DNA ISC. Major groove alkylation is proposed to occur via generation, and subsequent metabolism by DTD, of 2,7-diaminomitosene. Cross-linking of DNA, at 5′-CG-3′ sequences, is proposed to require the formation of either the protonated leucomitomycin C or the leucoaziridinomitosene during DTD-mediated metabolism of MMC.

The generation of reactive intermediates, upon metabolism of MMC,[1] can occur via either one- or two-electron reduction. After either enzymatic or chemical reduction, rearrangement of MMC occurs with the loss of the methoxy substituent as methanol and proton-assisted aziridine ring opening to generate a reactive quinone methide at the C-1 position (Iyer & Szybalsky, 1963; Szybalsky & Iyer, 1964; Peterson & Fisher, 1986). Alkylation of DNA results, and subsequent cross-linking occurs after loss of the carbamate generates a reactive imine at the C-10 position of mitomycin C (Iyer & Szybalsky, 1963; Szybalsky & Iyer, 1964; Tomasz et al., 1987). We have recently implicated DTD (an obligate two-electron reductase) as being involved in the activation of MMC under aerobic conditions (Siegel et al., 1990). In particular, DTD-mediated metabolism of MMC was found to result in DNA alkylation, GSH conjugation, and DNA interstrand cross-linking (Siegel et al., 1992). DNA interstrand cross-linking was found to increase as the pH of the incubation was made more acidic. Identification of the reactive species responsible for such reactions remains the subject of intense investigation.

The most important lesion identified after alkylation of DNA by MMC is thought to involve an initial binding to the NH_2 at the 2 position of guanine and subsequent cross-linking of DNA via alkylation at an adjacent guanine, also at the NH_2 group at the 2 position (Tomasz et al., 1987). MMC-induced DNA ISC has been suggested to prefer 5′-CG sequences although other potential sites have been implicated (Teng et al., 1989). This 5′-CG preference results in the cross-link sitting in the minor groove of DNA (Tomasz et al., 1987). It is important to emphasize that the majority of these studies have used chemical reduction (sodium dithionite or sodium borohydride) under anaerobic conditions (Norman et al., 1990; Teng et al., 1989; Tomasz et al., 1987).

In contrast, enzymatic reduction by the one-electron reductases xanthine oxidase or NADPH–cytochrome *c* reductase resulted in a monofunctional adduct attached to the NH_2 group at the 2 position of guanine (Tomasz et al., 1988; Kumar et al., 1992). This monofunctional adduct accounted for greater than 90% of the bound MMC under these reduction conditions. The picture is complicated further by recent results which show that aerobic chemical reduction (sodium borohydride) of MMC resulted in adducts which were piperidine labile and presumably represent alkylations at the guanine N7 position (Li & Kohn, 1991). In this same study, chemical reduction of MMC under anaerobic conditions generated adducts at either 5′-CG (DNA interstrand cross-links) or 5′-GG (either monofunctional DNA adducts or DNA intrastrand cross-links) sequences. The conclusion from such studies is that the mechanism by which MMC is reduced ultimately determines the level and type of DNA adduct formation.

The purpose of this study was to determine the nucleotide preferences for DNA alkylation and DNA ISC of MMC and its major metabolite, 2,7-diaminomitosene, after enzymatic reduction by DTD. We have compared enzymatic reduction with chemical reduction, particularly under aerobic conditions, in order to clarify the DNA lesions which may be most important to the aerobic cytotoxicity observed with MMC. We propose that 2,7-diaminomitosene is responsible for MMC-induced alkylation within the major groove of DNA and that a protonated leucomitomycin C or the leucoaziridinomitosene formed during DTD mediated metabolism of MMC is responsible for MMC alkylation and DNA ISC within 5′-CG-3′ sequences of DNA.

MATERIALS AND METHODS

MMC was a generous gift from the Bristol Myers Co., Syracuse, NY. 2,7-Diaminomitosene was synthesized as

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[1] Abbreviations: DNA ISC, DNA interstrand cross-linking; 2,7-DAM, 2,7-diaminomitosene; DTD, DT-diaphorase; MMC, mitomycin C.

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described (Siegel et al., 1992). DTD was isolated and purified from uninduced Sprague-Dawley rats according to published procedures (Sharkis & Swenson, 1989). The enzymes T4 polynucleotide kinase, Klenow fragment, *Hin*dIII, and *Bam*HI (Promega), *Eco*RI (BRL), and T4 DNA polymerase (Boehringer-Mannheim) were purchased. [α-^{32}P]dATP and [γ-^{32}P]ATP were purchased from NEN Dupont. Sodium dithionite (J. T. Baker), sodium borohydride and dicoumarol (Aldrich), piperidine (Fisher Scientific), and formic acid and NADH (Sigma) were obtained from the commercial sources indicated.

Labeling and Isolation of the DNA Fragment. A 375-base-pair *Bam*HI/*Eco*RI fragment of pBR322 was either 3'- or 5'-end-labeled at the *Bam*HI site by [α-^{32}P]dATP with Klenow fragment or by T4 PNK in the presence of [γ-^{32}P]ATP, respectively. The labeled fragments were isolated on a 4% nondenaturing polyacrylamide gel (Prakash et al., 1990).

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 221          231          241          251          261
5'-GGCGTGCTGC  TAGCGCTATA  TGCGTTGATG  CAATTTCTAT  GCGCACCCGT
3'-CCGCACGACG  ATCGCGATAT  ACGCAACTAC  GTTAAAGATA  CGCGTGGGCA

 271          281          291          301          311
   TCTCGGAGCA  CTGTCCGACC  GCTTTGGCCG  CCGCCCAGTC  CTGCTCGCTT
   AGAGCCTCGT  GACAGGCTGG  CGAAACCGGC  GGCGGGTCAG  GACGAGCGAA

 321          331          341          351          361
   CGCTACTTGG  AGCCACTATC  GACTACGCGA  TCATGGCGAC  CACACCCGTC
   GCGATGAACC  TCGGTGATAG  CTGATGCGCT  AGTACCGCTG  GTGTGGGCAG

 371
   CTGTGGA*TC-3'
   GACACCTAG-5'
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DNA Alkylation by Activated MMC. End-labeled DNA (at 10000 cpm) was reacted with 10 μM MMC in the presence of 100 μM NADH and 0.173 μg of DTD in various pH-adjusted phosphate buffers (pH 5.8, 6.2, 6.6, 7.0, 7.4, and 7.8) at 20 °C for 1 h. For chemical reduction, 100 μM MMC was reacted with DNA in the presence of either 1 mM $Na_2S_2O_4$ or 1 mM $NaBH_4$. Additional aliquots were added at half-hour intervals to the incubation mixture at 20 °C for 1 h. Incubations were stopped by precipitation in ethanol, and modified DNA pellets were then subjected to heat treatment (90 °C for 10 min) in 1 M piperidine, which leads to quantitative conversion of all N7 guanine alkylated sites to strand breaks (Prakash et al., 1990). The samples were then lyophilized and were used for electrophoresis as described below.

The 3' to 5' exonuclease activity of T4 DNA polymerase has been reported to be blocked by alkylations in the minor groove but not the major groove of DNA (Panigrahi & Walker, 1990). The 5'-end-labeled *Bam*HI/*Eco*RI fragments of pBR322 DNA samples were incubated with MMC in the presence of DTD in phosphate buffer (pH = 5.8) for 1 h at 20 °C. Anthramycin (0.2 μM), a known alkylator of the exocyclic amino group of guanine (Graves et al., 1984), was used as a positive control, and alkylation was for 1 h at 37 °C in TE buffer. The reactions were stopped by precipitation in ethanol, and DNA pellets were resuspended in 50 μL of reaction buffer for digestion with *Hin*dIII. This step is included to avoid interference from drug-modified DNA termini (Panigrahi & Walker, 1991). After digestion the samples were then precipitated in ethanol, and the DNA pellets were redissolved in T4 DNA polymerase buffer. Incubations were performed for 1 h at 37 °C in the presence of 2 units of T4 DNA polymerase for 30 min, followed by the addition of another 2 units of polymerase and incubation for a subsequent 30 min. MMC-treated DNA without T4 DNA polymerase and non-drug-treated DNA with T4 DNA polymerase were used as controls. Reactions were terminated by inactivating the enzyme by heating at 65 °C for 10 min, followed by phenol/chloroform extraction and ethanol precipitation.

Prior to being loaded on an 8% denaturing polyacrylamide gel, samples were dissolved in formamide dye. Sequencing lanes corresponding to G, Pu, C, and T were loaded to aid sequence identification. The gel was run on a Bio-Rad Sequigen apparatus at 50 W until xylene cyanol had migrated 26 cm. The gel was then dried and exposed to X-ray film (Kodak 5-XAR) overnight prior to development.

Preparation of Oligonucleotides for Cross-Linking Assay. An oligomer 23 bases in length containing three potential cross-linkable sites, a 5'-GTC-3', a 5'-CG-3', and a 5'-GC-3' sequence, and the complementary strand were synthesized on an Applied Biosystems DNA synthesizer (see legend of Figure 6 for the full sequence). The oligonucleotides were deprotected by heating at 55 °C in concentrated ammonium hydroxide overnight and subsequently purified and isolated on a 20% denaturing acrylamide gel using the UV-shadowing technique. Complementary strands were annealed by combining equal amounts of the two oligomers, heating to 70 °C, and cooling to room temperature overnight. The 3'-ends of the duplex were selectively labeled by Klenow enzyme in the presence of either [α-^{32}P]dATP (bottom strand) or [α-^{32}P]dCTP (top strand). The labeled duplex samples were then precipitated in cold ethanol and resuspended in phosphate buffer (pH = 5.8) prior to drug exposure.

Production, Isolation, and Analysis of Cross-Linked Oligomers. Isolation of cross-linked product relies on the fact that a single strand of DNA runs faster on a denaturing gel than when cross-linked to its complementary strand (Millard et al., 1991). The 3'-end-labeled duplex was reacted with 200 μM MMC in phosphate buffer (pH = 5.8) under the conditions previously described for sequencing reactions. Reactions were terminated by ethanol precipitation, and samples were denatured by heating at 90 °C for 2 min. Samples were then loaded on a 20% denaturing polyacrylamide gel and run at 3000 V until the xylene cyanol dye had migrated 12 cm. The gel was visualized by exposure to X-ray film, and the bands corresponding to unmodified, monoalkylated, and cross-linked oligomers were excised from the gel and recovered into TE buffer.

Each sample was split into two prior to analysis of the excised DNA fragments. Samples were subjected to 90 °C heat treatment in 1 M piperidine in order to cleave alkylations at the guanine N7 position. The remaining samples, and in particular the DNA ISC products, were treated with dimethyl sulfate or formic acid and then subsequently subjected to piperidine treatment. This procedure allowed us to identify the location of the cross-link since only those lesions between the cross-link and the labeled end, upon piperidine cleavage, will run faster than the unmodified fragment. Formic acid and DMS exposure was controlled to provide less than one hit per fragment. All the samples were then lyophilized and dissolved in formamide dye and were run on a 20% denaturing polyacrylamide gel.

RESULTS

Figure 1 shows an autoradiogram which represents the pattern of piperidine-labile DNA alkylation produced by $Na_2S_2O_4$ or DTD-activated MMC, under aerobic conditions, within the *Bam*HI/*Eco*RI 375-bp fragment of DNA. Lability of alkylated DNA to piperidine is indicative of alkylation at the guanine N7 position within the major groove of DNA. Enzymatic reduction of MMC alkylates DNA in a sequence-selective fashion with 5'-guanine in 5'-GTC-3' and 3'-guanine

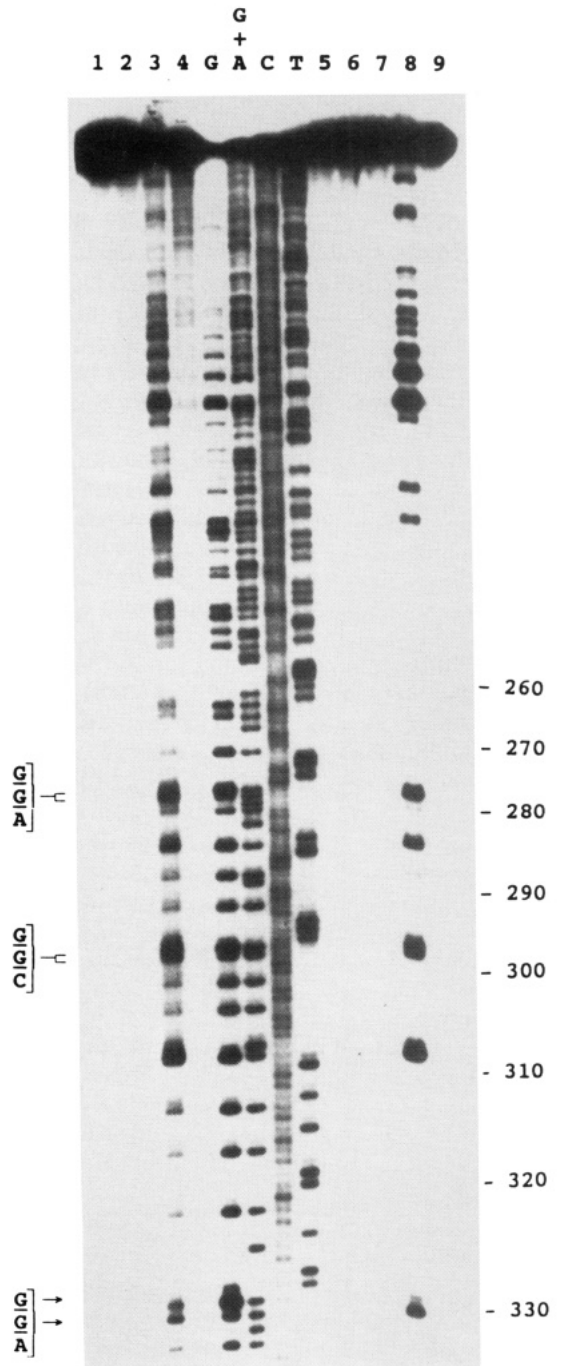

FIGURE 1: Autoradiogram of an 8% denaturing polyacrylamide gel showing piperidine-induced DNA strand cleavage of 3′-end-labeled pBR322 DNA alkylated with MMC after reduction. The reactions were carried out in 10 mM phosphate/1 mM EDTA buffer (pH 5.8) for 1 h at 37 °C: lane 1, control; lane 2, 25 μM MMC; lane 3, 25 μM MMC and 2 mM $Na_2S_2O_4$; lane 4, 2 mM $Na_2S_2O_4$; lane 5, 0.173 μg DTD; lane 6, 2.5 μM MMC; lane 7, 2.5 μM MMC and 100 μM NADH; lane 8, 2.5 μM MMC, 100 μM NADH, and 0.173 μg of DTD, lane 9, 2.5 μM MMC, 100 μM NADH, 0.173 μg of DTD, and 20 μM dicoumarol. Lane G is the guanine-specific reaction, lane G+A or Pu represents the purine-specific reaction, lane C is the cytosine-specific reaction, and lane T is the thymine-specific reaction.

in 5′-GGN-3′ sequences when N = T, A, or G but not when N = C (lane 8). In 5′-GGC-3′ sequences both guanines are moderately alkylated (Figures 1 and 5). Consistent with previous metabolic studies was the fact that enzymatic reduction, and hence alkylation, required the presence of NADH and DTD, with no DNA alkylation being observed with MMC alone or MMC plus NADH (compare lanes 6–8, Figure 1). Dicoumarol, a known inhibitor of DTD (Ernster, 1967), was able to prevent DTD-mediated MMC alkylation. Chemical reduction of MMC by sodium dithionite at pH 5.8, under aerobic conditions, also produced a similar pattern of guanine N7 alkylation as that found with DTD.

Previous metabolic studies have shown that the level of MMC-induced DNA interstrand cross-linking was found to be greater at more acidic pH values (Siegel et al., 1992). Figure 2 shows the pH dependence of piperidine-labile DNA alkylation by MMC either after activation by DTD (panel A, lanes 1–12) or upon chemical reduction by sodium dithionite (panel B, lanes 1–12). All drug treatments were performed under aerobic conditions. The optimum level of DTD-mediated MMC alkylations was found to occur at pH 6.6 (lane 6). A high level of alkylation was observed at pH 5.8 (lane 2) with little alkylation being observed at pHs above 7.0 (lanes 10 and 12). In contrast, when MMC was activated by $Na_2S_2O_4$, the level of alkylation was maximum at pH 5.8 and negligible at pH 6.6 or higher (Figure 2, panel B, lanes 1–12).

The pH dependence of alkylation by MMC upon chemical reduction by sodium borohydride was also determined (Figure 3). The levels of DNA alkylation remain nearly constant at pHs between 5.8 and 7.4 (lanes 2, 4, 6, 8, and 10), but at pH 7.8 very little alkylation was observed (lane 12). It is interesting to note that with both sodium dithionite and sodium borohydride a 10-fold higher MMC concentration was required to produce similar levels of alkylation than that observed for DTD-mediated MMC alkylation.

The major metabolite of DTD-mediated metabolism of MMC has been found to be 2,7-diaminomitosene (2,7-DAM), and we have found that this species, upon reduction by DTD, is capable of binding to DNA (Siegel et al., 1992). Figure 4 shows the piperidine-labile alkylation pattern produced by 2,7-DAM upon reduction under aerobic conditions by DTD. This structure is only capable of monofunctional alkylation of DNA via nucleophilic attack at the C-10 position (Iyengar et al., 1990). The pH dependence of, and sequence preference for, DNA alkylation by this analog was found to be similar to that observed with MMC. Alkylation was found to occur within 5′-GG-3′ and 5′-GTC-3′ sequences, and a greater degree of alkylation was observed at pH 5.8 than that observed at pH 7.8. Alkylation by 2,7-DAM required the presence of DTD and NADH (lane 8), thus implicating an enzymatic mechanism.

In order to determine the degree of alkylation and nucleotide preferences for alkylation within the minor groove, we took advantage of the fact that the exonuclease activity of T4 DNA polymerase can be blocked by adducts within the minor groove but not by adducts within the major groove of DNA (Panigrahi & Walker, 1990). In these experiments, we failed to detect any blockage of exonuclease activity of T4 DNA polymerase upon alkylation of 5′-end-labeled DNA by 100 μM MMC (Figure 5, compare lanes 2, 5, 6, and 7). Lane 5 shows that under the incubation conditions of the nuclease treatment, which includes an enzyme inactivation step (heating the samples at 65 °C), a considerable amount of depurination occurs specifically at the sites of guanine N7 alkylation (compare with lane 7). In contrast, the known minor groove alkylating agent, anthramycin (which alkylates the exocyclic amino group at the 2 position of guanine within 5′-AGPu-3′ sequences), was found to block the progression of the exonuclease activity of T4 DNA polymerase (lane 3). In agreement with previous work (Graves et al., 1984), piperidine treatment of anthramycin-modified DNA does not produce any strand cleavage (lane 4), which suggests that this antibiotic does not alkylate DNA in the N7 position of guanine. For

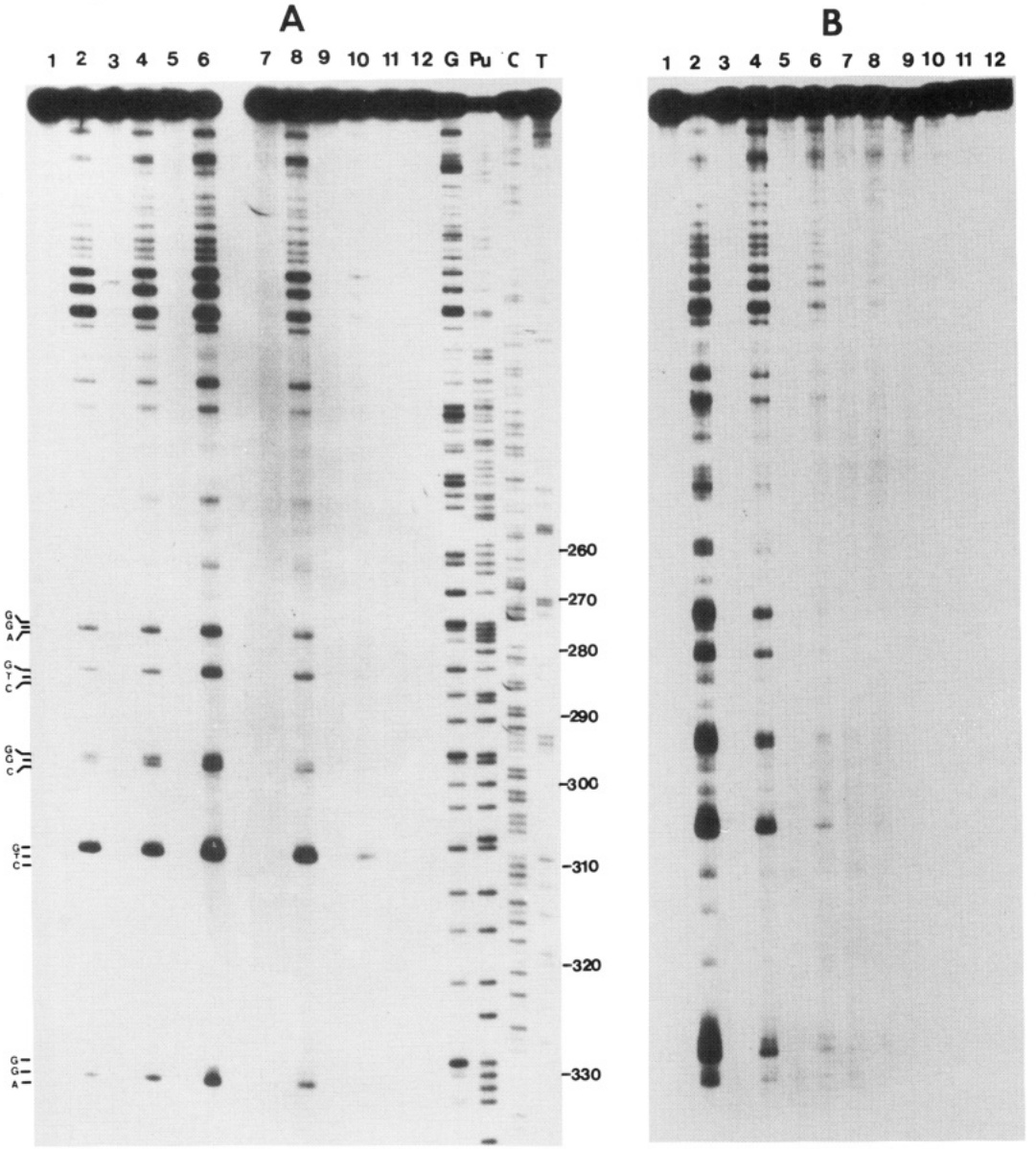

FIGURE 2: Autoradiograms of pH-dependent alkylation of 3′-end-labeled DNA by MMC activated by DTD (panel A, includes sequencing lanes) or by $Na_2S_2O_4$ (panel B). The enzyme-activated drug samples contained 10 μM MMC, 100 μM NADH, and 0.173 μg of DTD, while the chemically reduced samples contained 100 μM MMC and 1 mM $Na_2S_2O_4$ in the first 30 min of incubation which was raised to 2 mM in the second 30 min. All reactions were carried out in 10 mM phosphate/1 mM EDTA buffer at 20 °C for 1 h. Even-numbered lanes are DNA samples with drug, and odd-numbered lanes are the corresponding control DNA samples without drug. The pHs of the reaction mixtures are 5.8 for lanes 1 and 2, 6.2 for lanes 3 and 4, 6.6 for lanes 5 and 6, 7.0 for lanes 7 and 8, 7.4 for lanes 9 and 10, and 7.8 for lanes 11 and 12. Sequencing lanes, as described in Figure 1, are as shown.

comparison, the strand cleavage pattern obtained upon piperidine treatment of DNA alkylated by MMC reduced by DTD is shown (lane 8).

The observation that DTD-mediated MMC alkylations were predominantly found to occur in the major groove of DNA prompted us to investigate the nucleotide preferences for DNA ISC. Using a multi-target-site duplex oligonucleotide (which contained 5′-GC-3′, 5′-CG-3′, and 5′-GNC-3′ target sequences), we detected the formation of MMC-induced DNA ISC upon enzymatic reduction. Figure 6 shows a denaturing 20% acrylamide gel which shows the separation of cross-linked DNA from single-stranded DNA. Two distinct bands with mobility consistent with cross-linked DNA were observed when either the top strand of the duplex or the bottom strand of the duplex was labeled and analyzed separately.

We subsequently isolated each band and subjected them to a variety of different chemical procedures in order to determine the site of DNA cross-linking. Figure 7 (panel A, top-strand-labeled duplex; panel B, bottom-strand-labeled duplex) shows a sequence analysis of both the cross-link bands and the monoalkylated bands. Lanes 5–7 in panels A and B correspond to the cross-link band 1 while lanes 8–10 in the two panels correspond to cross-link band 2. Sequence analysis of both the cross-linked products gave identical results. Each cross-link was resistant to piperidine cleavage, suggesting that the cross-link did not involve linkage via two guanine N7 positions. DMS treatment of the cross-linked products followed by piperidine treatment resulted in a discontinuity after a 5′-CG-3′ sequence. This can be determined by comparing panel A and panel B where the discontinuity is between the cleaved purine and the full-length single-stranded product. In the case of formic acid exposure a discontinuity is observed one purine before the 5′-CG-3′ sequence. Again, this was found whether the DNA was end-labeled on the top or bottom strand (compare lanes 7 and 10, panel A, and lanes 7 and 9, panel B). These data indicate that the major site of DNA cross-

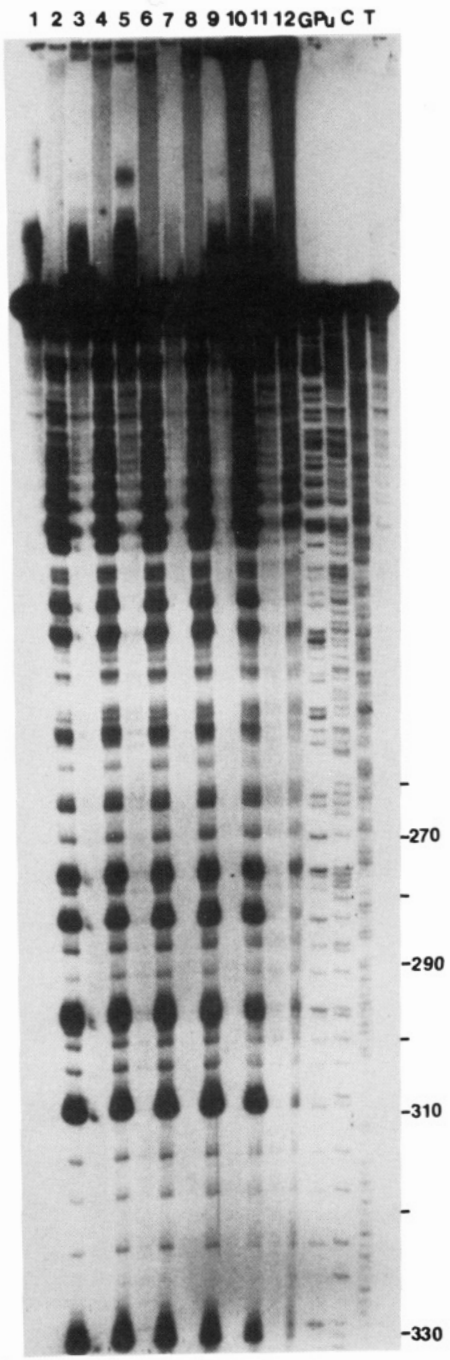

FIGURE 3: Autoradiogram of pH-dependent alkylation of 3′-end-labeled DNA by $NaBH_4$-reduced MMC. All reactions were carried out in 10 mM phosphate/1 mM EDTA buffer at 20 °C for 1 h. Even-numbered lanes are DNA samples with drug, and odd-numbered lanes are the corresponding control DNA samples without drug. Each sample contained 100 μM MMC and 1 mM $NaBH_4$ in the first 30 min of incubation and was further raised to 2 mM in the second 30 min. The pHs of the reaction mixtures are as indicated in Figure 2. Sequencing lanes, as described in Figure 1, are as shown.

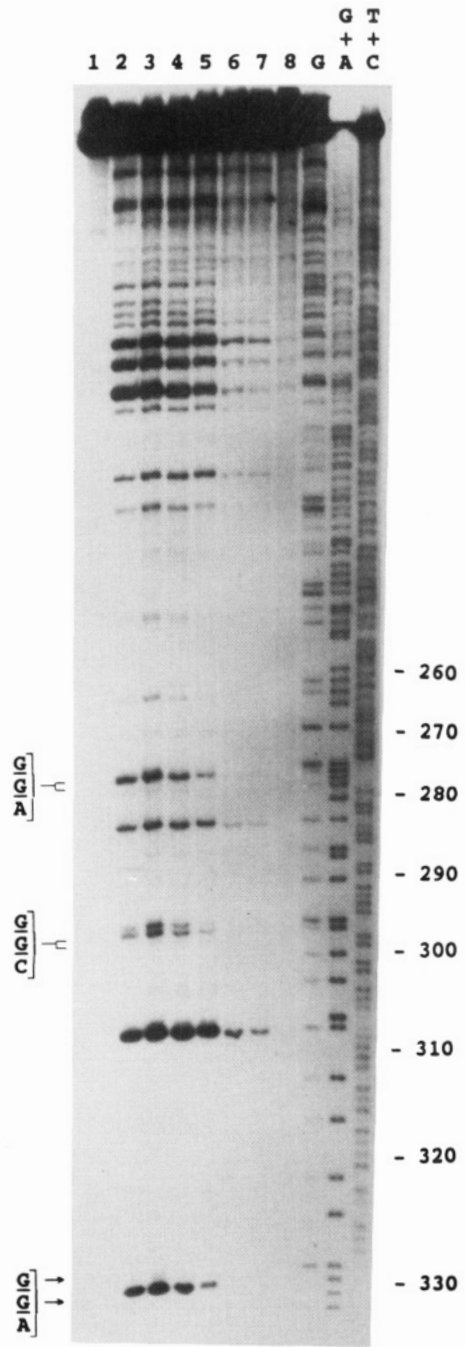

FIGURE 4: Autoradiogram of pH-dependent alkylation of 3′-end-labeled DNA by DTD-activated DAM. The reaction mixtures contained 10 μM DAM, 100 μM NADH, and 0.173 μg of DTD. Lanes 2–7 are DAM-treated DNA samples at pH 5.8 (lane 2), 6.2 (lane 3), 6.6 (lane 4), 7.0 (lane 5), 7.4 (lane 6), and 7.8 (lane 7). Lane 1 is the control DNA at pH 5.8. Lane 8 contains the DNA sample at pH 5.8 with drug alone. Sequencing lanes, as described in Figure 1, are as shown.

linking is at 5′-CG-3′ sequences and that DMS can alkylate the base involved in the DNA cross-link at the guanine N7 position. Further, depurination by formic acid at the cross-linked site is inhibited. The appearance of bands between the DNA cross-links and single-stranded DNA represents cleaved product where the end-labeled DNA is full length with a short fragment of the complementary strand remaining via linkage by the DNA cross-link.

The mobility of duplex oligonucleotide DNA containing monofunctional alkylations can also be separated from either nonmodified single-stranded DNA or DNA cross-links during denaturing electrophoresis. When such monofunctionally alkylated oligonucleotides were subjected to piperidine treatment, a similar sequence selectivity was observed as found in our 375-bp pBR322 DNA fragment (compare Figure 1 with Figure 7). In brief, the major sites of alkylation involved the guanines in 5′-GTC-3′ and 5′-GG-3′ sequences (Figure 7, lanes 4A and 4B). In these experiments a significant amount of monofunctionally alkylated DNA was found to be resistant to piperidine. Piperidine treatment alone leads to bands which comigrate with unmodified fragments, indicating that some of the cross-links reverse to regenerate unmodified fragments.

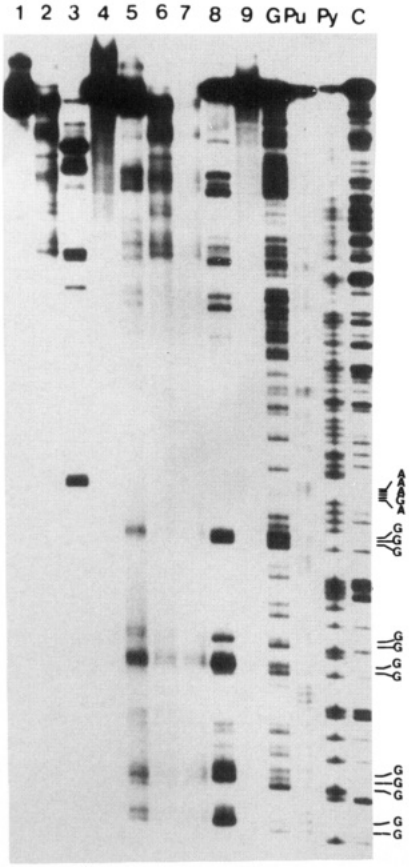

FIGURE 5: Autoradiogram of T4 DNA polymerase assay. MMC treatment of 5′-end-labeled DNA was carried out in 10 mM phosphate/1 mM EDTA buffer at pH 5.8 in the presence of 100 μM NADH and 0.173 μg of DTD. The exonuclease reaction was carried out with the addition of 2 units/30 min of the T4 DNA polymerase at 37 °C for 1 h. Lane 4 is DNA alkylated with anthramycin (0.2 μM), and lanes 8 and 9 are DNA alkylated with MMC (5 μM) either with DTD (lane 8) or without (lane 9) and subjected to piperidine treatment. The following are 5′-end-labeled DNA in the nuclease buffer containing non-drug-treated control DNA (lane 1), non-drug-treated control DNA and T4 DNA polymerase (lane 2), 0.2 μM anthramycin and T4 DNA polymerase (lane 3), 5 μM MMC (lane 5), 5 μM MMC and T4 DNA polymerase (lane 6), and 100 μM MMC and T4 DNA polymerase (lane 7). Sequencing lanes, as described in Figure 1, are shown.

This last result is in agreement with a previously published report (Borowy-Borowski et al., 1990).

DISCUSSION

In this study we have determined that metabolism of MMC by the two-electron reductase DTD under aerobic conditions results in the generation of reactive species which can alkylate and cross-link DNA. In contrast to the majority of previous studies, which either utilized one-electron reductases or performed reduction under anaerobic conditions and observed minor groove alkylations, we found that the major site of alkylation was within the major groove of DNA and was susceptible to piperidine cleavage. This strongly suggests the formation of guanine N7 alkylations (Prakash et al., 1990). As the pH of the drug incubations was made more acidic, a greater degree of guanine N7 alkylation was observed, and this did not alter the sequence preferences for alkylation. Alkylation of guanines was preferred at 5′-GTC-3′ and 5′-GG-3′ sequences.

We have previously indicated that the major product of metabolism of MMC by DTD is 2,7-DAM and that this structure is capable of binding to DNA upon reduction by

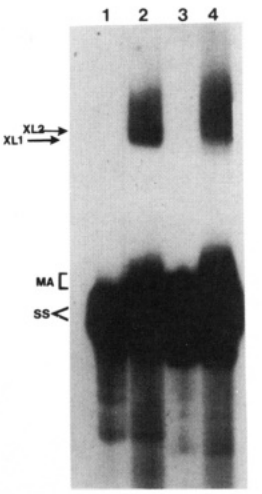

FIGURE 6: Autoradiogram of a 20% denaturing polyacrylamide gel used in the separation of the MMC-induced cross-linked duplex from the parent oligonucleotides. Target sites for DNA interstrand cross-linking are shown in bold. The top and bottom strands of the 23-mer duplex were selectively labeled at the 3′-end by Klenow enzyme in

5′- CTA CAT **CGT** **GTC** ATG **C**AC AGG AT
T GTA **GC**A **CAG** TAC **G**TG TCC TAG A-5′

the presence of either [α-^{32}P]dCTP (top strand) or [α-^{32}P]dATP (bottom strand). Lanes 1 and 2 are top-strand-labeled and lanes 3 and 4 are bottom-strand-labeled oligonucleotides. The duplexes were incubated with 200 μM MMC in the presence of 100 μM NADH and 0.173 μg of DTD in 10 mM phosphate/1 mM EDTA buffer (pH 5.8) for 1 h at 20 °C. Lanes 1 and 3 are controls, and lanes 2 and 4 contain drug-treated samples. The bands corresponding to the cross-linked products and the monoalkylated products are indicated in the figure.

DTD (Siegel et al., 1992). Remers and colleagues have also shown that 2,7-DAM, when reduced by sodium dithionite, can alkylate DNA and 2′-deoxyguanosine (Iyengar et al., 1990). In the current study 2,7-DAM was found to alkylate DNA with the nucleotide preferences for alkylation being identical to those obtained with DTD-mediated MMC-induced alkylations. The ability of 2,7-DAM to alkylate DNA required enzymatic reduction by DTD. Thus, our data would suggest that MMC, upon metabolism by DTD, generates 2,7-DAM and an additional cycle of metabolism generates the hydroquinone form of 2,7-DAM (Figure 8). Loss of the carbamate moiety at the C-10 position is then facilitated, yielding a reactive iminium derivative which can alkylate DNA at the guanine N7 position within the sequences described above. This work is in contrast to previous studies which implicated the site of binding as being the exocyclic amino group of 2′-deoxyguanosine utilizing ^{13}C NMR (Iyengar et al., 1990). Whether the differences relate to the nucleophile being used (DNA versus 2′-deoxyguanosine) or whether this reflects differences in reactive intermediates formed via the different methods of reduction (sodium dithionite versus DTD) is unknown.

DTD, sodium borohydride, and sodium dithionite reduce MMC with generation of 2,7-DAM. With all three methods 2,7-DAM formation is greater at lower pH values and is not observed at pH 7.8 (data not shown). This finding, along with the similar alkylation patterns seen for MMC and 2,7-DAM, suggests that MMC-induced alkylation at the guanine N7 position is the result of 2,7-DAM formation. Differences in the DNA alkylation pH profiles for the three reduction methods can also be explained. Enzymatic reduction of MMC at higher pH values results in mechanism-based inactivation of DTD (Siegel et al., 1992). Enzyme inhibition may be the

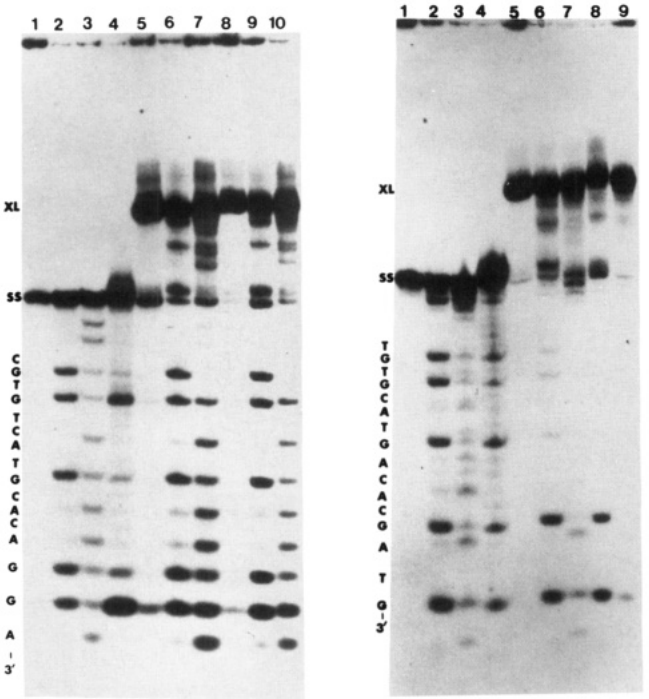

FIGURE 7: Sequencing analysis of products obtained from the cross-linking gel in Figure 6: panel A (left), top strand; panel B (right), bottom strand. All samples were subjected to piperidine treatment. Incubations are as follows: lanes 1A and 1B, control; lanes 2A and 2B, guanine lane; lanes 3A and 3B, purine lane; lanes 4A and 4B, monoalkylated top strand; lanes 5A and 5B, control cross-linked product with higher mobility (XL1); lanes 6A and 6B, DMS-treated XL1; lanes 7A and 7B, formic acid-treated XL1; lane 8A, control cross-linked product with lower mobility (XL2); lanes 9A and 8B, DMS-treated XL2; lanes 10A and 9B, formic acid-treated XL2.

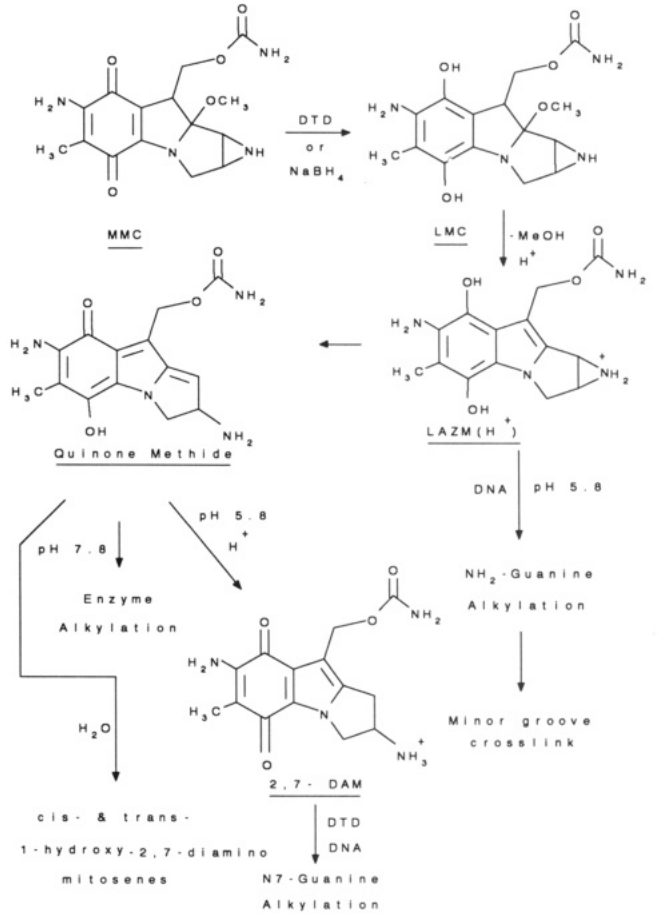

FIGURE 8: Schematic diagram of MMC reaction pathways.

direct result of generation of the ambivalent quinone methide which acts as an electrophile at pH 7.0–7.8 and alkylates the enzyme (Siegel et al., 1992). Thus the generation of 2,7-DAM and the subsequent alkylation of DNA in this pH range are prevented. pH-dependent alkylation is also observed with sodium borohydride and sodium dithionite since more of the intermediate quinone methide is converted to 2,7-DAM at lower pH values. Surprisingly, DNA alkylation by 2,7-DAM is also pH-dependent. This may simply reflect proton-assisted loss of carbamate at lower pH values, a process that may also play a role in the MMC-induced alkylations.

MMC-induced DNA ISC has been suggested to prefer 5′-CG sequences although other potential sites have been implicated (Teng et al., 1989). The formation of this DNA ISC is thought to involve an initial binding to the NH_2 at the 2 position of guanine and subsequent cross-linking of DNA via alkylation at an adjacent guanine, also at the NH_2 group at the 2 position (Tomasz et al., 1987). As this work progressed, the possibility that DTD-mediated MMC-induced DNA ISC may be located within the major groove and not the minor groove of DNA became plausible. Results obtained with duplex oligonucleotides suggest that the nucleotide preference for DNA ISC formation occurs within the 5′-CG-3′ sequence. This is in accordance with numerous published reports which have reduced MMC both enzymatically or chemically under anaerobic conditions (Tomasz et al., 1988). Our cross-link products are resistant to piperidine cleavage, thus supporting the thought that this represents cross-linking between two exocyclic amino groups at the 2 position of guanine on opposite strands of DNA within the 5′-CG-3′ sequence and thus in the minor groove of DNA. Studies are currently underway to test this interpretation.

The one caveat in this argument is our failure to detect MMC-induced minor groove alkylation by T4 exonuclease activity. It is possible, although unlikely, that the T4 exonuclease is blocked by anthramycin adducts at the exocyclic amino group but not by mitomycin C adducts at the same position. The fact, however, that 100 μM MMC failed to block the progression of T4 DNA polymerase suggests that the level of initial alkylation of DNA within the minor groove is not as great as that observed in the major groove.

The two cross-linked products observed in Figure 6 give rise to identical sequence analysis in Figure 7. This indicates that they arise from different conformations of cross-linking occurring in the 5′-CG-3′ sequence. It is possible that the two conformers are due to the orientation of the cross-link, one running from top strand G-C1-C10-G bottom strand while the other running from bottom strand G-C1-C10-G top strand. This is consistent with alkylation by the C-1 position of mitomycin C within a 5′-CG-3′ sequence where both DNA strands are potential targets for the initial alkylation event. Computer modeling analysis would support such a view (Bizanek et al., 1992).

The complexity of the bioreduction of mitomycin C makes the assignment of the cross-linking species difficult. In previous work we have shown that increasing amounts of DNA ISC were observed during DTD-mediated metabolism of MMC as the pH was lowered from 7.8 to 5.8 (Siegel et al., 1992). Although 2,7-DAM can give rise to monofunctional alkylations, it is not a DNA ISC agent (Iyengar et al., 1990). Tomasz and her colleagues have previously suggested that the quinone methide may alkylate nucleophiles such as DNA and lead to DNA ISC. This mechanism might appear inconsistent with increased DNA ISC at lower pHs since this

would favor protonation of the quinone methide to generate the monofunctional 2,7-DAM. Further, the quinone methide is thought to be more nucleophilic at acidic pH values and would thus be incapable of alkylating DNA (Petersen & Fisher, 1986). A suggestion which is more consistent with our data is that the precursor to DNA cross-linking is either the protonated leucomitomycin C or the leucoaziridinomitosene (Siegel et al., 1992).

In conclusion, the results presented within this paper show that enzymatic reduction of MMC under aerobic conditions by the two-electron reductase DTD results in monofunctional alkylation in the major groove of DNA. Major groove alkylation occurs predominantly within 5′-GG-3′ and 5′-GTC-3′ sequences at guanine N7 positions, whereas DNA interstrand cross-linking occurs within 5′-CG-3′ sequences and may involve the exocyclic NH_2 groups of guanine. These data, when combined with previous metabolic studies (Siegel et al., 1992), suggest that major groove alkylation occurs via generation, and subsequent metabolism by DTD, of 2,7-diaminomitosene. Cross-linking of DNA is proposed to require the formation of either the protonated leucomitomycin C or the leucoaziridinomitosene during DTD-mediated metabolism of MMC.

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