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DNA Alkylation by Enzyme-Activated Mitomycin C

SU-SHU PAN, THOMAS IRACKI, and NICHOLAS R. BACHUR

The University of Maryland Cancer Center, The New Bressler Research Laboratories, Baltimore, Maryland 21201

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SUMMARY

After anaerobic reductive activation by either NADPH cytochrome P-450 reductase (EC 1.6.2.4) or xanthine oxidase (EC 1.2.3.2), mitomycin C readily alkylated DNA. When the mitomycin C-alkylated DNA is digested by DNase, snake venom phosphodiasterase, and alkaline phosphatase, only partial release of the monofunctionally linked mitomycin C nucleotide adduct occurs. Cross-linked adducts are not released into dinucleotides but resist nuclease digestion and remain in oligonucleotides and insoluble precipitates. Kinetic analyses show that the nuclease-resistant fraction which is indicative of DNA cross-linking by mitomycin C takes place quite readily. This nuclease-resistant fraction is particularly significant when the amount of total bound mitomycin C is less than 15 μmol/mmol of DNA. The cross-linked mitomycin C product accounts for more than half of the total alkylation under all pH conditions tested. Our data suggest that particular DNA sites are available for DNA cross-linking by mitomycin C, and these sites are probably the preferred and immediate alkylating targets. Furthermore, DNA cross-links by mitomycin C are not the secondary product of monofunctional adducts. Activity of both flavoenzymes is pH dependent, hence, mitomycin C activation and the rate of DNA alkylation are pH dependent. At elevated mitomycin C alkylation of DNA, the highest amount of cross-linking occurs at neutral pH. High pressure liquid chromatographic separation of the nuclease-digested DNA detected one major and two less prominent mitomycin C adducts. These were verified to be mononucleotide mitosene types by UV spectra showing maximum absorbance at 312 and 250 nm. The major adduct was purified and identified as O^6-(2′-deoxyguanosyl)-2,7-diaminomitosene by NMR, indicating that the O^6 position of guanine is a preferred site in DNA for at least monofunctional linkage formation.

MC (Fig. 1), a potent antitumor antibiotic, is a bioreductive alkylating agent. Reductively activated MC binds covalently by single linkage to DNA, RNA, and protein (1), cross-links complementary strands of DNA (2–5), and cross-links DNA with protein (6). The covalent binding of MC to DNA is believed to be the principle action to account for the cytotoxicity of this drug. Earlier studies of bacterial DNA alkylation by MC have concluded that monofunctional linkage is the predominant product whereas DNA interstrand cross-links by MC are relatively rare (1, 3). Recent studies by Dorr *et al.* (4), conducted in mammalian tumor cell cultures, indicated that MC caused rapid DNA interstrand cross-links in a dose-dependent fashion. Good correlations between DNA cross-links and cytotoxicity have also been reported (4, 5). Although the exact nature of the interstrand cross-links caused by MC remains unknown, the guanine residue was found to be crucial for both monofunctional and bifunctional binding of MC to DNA (7, 8). The chemical structure of monofunctionally linked MC adducts was recently reported by Hashimoto *et al.* (9, 10) from MC-DNA alkylation through catalytic hydrogenation. Three monofunctional adducts showed alkylation by the C-1 center of MC occurring at the O^6 and N^2 positions of guanine and at the N^6 position of adenine. Furthermore, Tomasz *et al.* (11) showed that binding of MC to a dinucleotide, 2′-deoxyguanylyl-(3′→5′)-2′-deoxycytidine, was also at the O^6 position of guanine by the C-1 center in a *trans* configuration.

Using flavoenzyme-activated MC, we found that reaction conditions such as pH and nucleophile concentrations had a decisive effect on the generation of specific metabolites (12). We believe that the nature of DNA alkylation by MC is also effected by bioreductive conditions. Therefore, we conducted a study of DNA alkylation by enzymatically activated MC. We describe the *in vitro* preparation MC-DNA by enzyme-activated MC, the kinetics of the adduct formation, the effects of reaction conditions on adduct formation, and the isolation and structural analysis of MC adducts. A preliminary account of this study was presented (13).

Materials and Methods

MC was kindly supplied by the Drug Development Branch, Division of Cancer Treatment, National Cancer Institute and by Dr. W. Bradner, Bristol Laboratories (Syracuse, NY). It was further purified by repeated extraction with methanol until the molar absorptivity reached 21,800 at 367 nm, and only one blue spot showed on thin layer chromatographs. Bovine milk xanthine oxidase (EC 1.2.3.2) and rat

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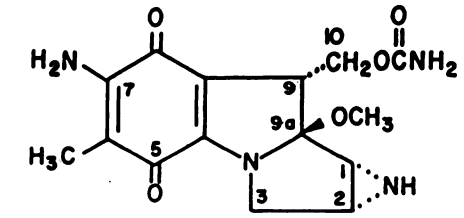
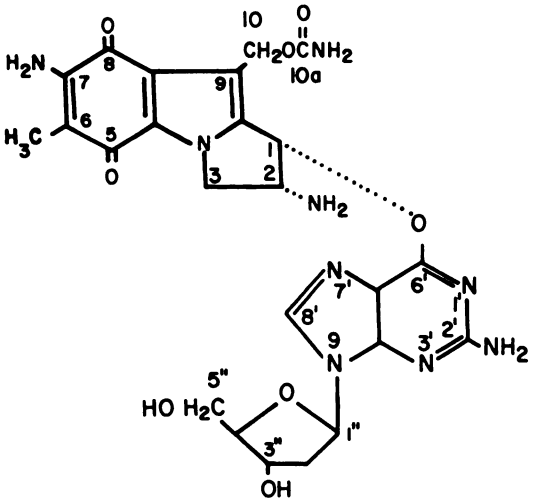
ABBREVIATIONS: MC, mitomycin; MC-DNA, mitomycin-alkylated DNA; HPLC, high pressure liquid chromatography.



1 MC



2 MC-Adduct

Fig. 1. Chemical structure of MC (*1*) and a major MC adduct (*2*).

liver NADPH cytochrome P-450 reductase (EC 1.6.2.4) were isolated according to the method of Nelson and Handler (14) and Yasukochi and Masters (15), respectively. Proteins were determined by a modified method of Lowry *et al.* (16). The sources for other enzymes and compounds are as follows: snake venom phosphodiesterase (EC 3.1.4.1) and alkaline phosphatase (EC 3.1.3.1) from Worthington (Freehold, NJ); p_1 nuclease (EC 3.1.30.X) and S_1 nuclease (EC 3.1.30.1) from P-L Biochemicals (Milwaukee, WI); calf thymus DNA, *Micrococcus lysdeikticus* DNA, and pancreatic DNase (EC 3.1.4.5) from Sigma (St. Louis, MO); pyridine nucleotides from Boerhinger Mannheim Biochemicals (Indianapolis, IN).

Preparation of MC-DNA

Calf thymus or other DNA was dissolved in 80 mM NaCl at 2 mg/ml and sheared by passing the solution twice through a 22 gauge needle (1 in.). Reaction mixture included 20 mM NaCl, 1 mM MC, 1.5 mM NADH (or NADPH), and DNA at 0.5 mg/ml (1.44 mM) in 50 mM Tris-HCl at pH 7.3–8.5, or in 50 mM imidazole-HCl at pH between 6.0 and 7.0. The mixture was sealed in a flask or tube with a serum stopper, and air was removed by flushing with N_2 gas for 1 min/ml of solution. The reaction was initiated by injecting xanthine oxidase at 30 μg/ml or NADPH cytochrome P-450 K reductase at 3 μg/ml; incubation was then carried out at 37° in a shaing water bath for the designated time periods. Controls were carried out precisely in the same manner without xanthine oxidase or NADPH cytochrome P-450 reductase. At the end of incubation, DNA was collected by precipitating with 2 volumes of cold 95% ethanol for 2 hr at −20°, then centrifuging at 5000 × *g* for 5 min. The pellet was redissolved in 2 M NaCl and reprecipitated with ethanol, and then redissolved a second time in 50 mM Tris-HCl and 20 mM NaCl at pH 7.5 and reprecipitated. The final MC-DNA was dissolved in the buffer systems specified for nuclease digestion.

Estimation of MC Bound to DNA

Quantitative estimation of the amount of MC covalently bound in MC-DNA was based on the molar absorptivities of native DNA (7000 at 260 nm), and MC adduct, O^6-deoxyguanosyl-2,7-diaminomitosene (6030 at 312 nm) reported by Tomasz *et al.* (11). Measurements of MC modified DNA were determined at three stages. 1) Estimations at 312 nm of the MC-DNA taken before nuclease digestion reflect the total bound MC (assuming the molar absorptivity of DNA bound mitosene to be the same as the adduct). 2) After nuclease digestion and the removal of the nuclease-resistant insoluble fraction, the soluble fraction was measured at 312 nm to calculate the nuclease-solubilized MC adducts. 3) The MC adducts resolved by HPLC were calculated with the purified major adduct O^6-deoxyguanosyl-2,7-diaminomitosene as external standard. Results were presented as μmol of HPLC-detected MC adducts per mmol of DNA.

DNA Digestions

DNA digestion was carried out to the nucleotide stage by one of three methods. A modification of the procedure of Baird and Brookes (17) was used for the digestion by pancreatic DNase and snake venom phosphodiesterase. Each mg of MC-DNA was dissolved in 1.0 ml of 50 mM Tris-HCl, 5 mM $MgCl_2$ at pH 7.0. DNase at 200 kallikrein units/mg of DNA was added and the mixture was incubated at 37° for 30 min. The pH of the solution was then raised to 8.9 by the addition of 4 M Tris, and snake venom phosphodiesterase was added at 0.04 unit/mg of DNA; then, the incubation was continued at 37° for 2 hr. P_1 nuclease digestion was by a method similar to that of Fujimoto *et al.* (18). Washed MC-DNA was dissolved in 50 mM sodium acetate buffer, pH 5.0, at 1.0 mg of DNA/ml. Digestion was carried out at 55° for 2 hr with 20 units of P_1 nuclease/mg of DNA. S_1 nuclease digestion was performed according to the method of Vogt (19). Further digestion to nucleoside stage was carried out by the addition of *E. coli* alkaline phosphatase (0.1 unit/mg of DNA) at pH 8.9, and incubation at 37° for 2 hr. At the end of the designated digestions, each solution was centrifuged at 20,000 × *g* for 10 min to separate the insoluble debris. The soluble fractions containing either nucleotides or nucleosides were analyzed by HPLC.

Detection and Isolation of MC Adducts by HPLC

A reverse phase HPLC method published earlier (20) was used with slight modification. Radial-PAK C_{18} (8 mm × 10 cm) cartridges (10 μm) (Waters Associates, Milford, MA) were used for both analytical and preparative columns. Preparative separations were effected with a linear gradient from 0 to 50% methanol in 10 mM ammonium formate at pH 6.4 in 13 min at a flow rate of 3 ml/min. Dual detectors at 313 and 254 nm were used. Aliquots of analytical samples were injected directly for analysis. MC adducts give high absorbance at both 313 and 254 nm. Peak area or height was used to calculate the amount of HPLC-detectable MC adducts using purified adduct as an external standard. For the isolation of adducts, products were collected from the column manually, concentrated on a flash evaporator, and then lyophilized. The purity of each product was confirmed by analytical HPLC with two solvent systems, the gradient system published previously (20), and an isocratic system including acetyl nitrile/10 mM ammonium formate at pH 6.4 (15:85).

Detection and Isolation of MC Adducts by Gel Filtration

Sephadex G-25 (fine) columns (1.5 × 26 cm) were equilibrated in 50 mM ammonium bicarbonate. The digested DNA-soluble fraction was concentrated by flash evaporator, and 1.5 ml, containing material equivalent to 8 mg of DNA, was placed on the column for fractionation. Peaks with high absorbance at 312 nm were pooled and concentrated. Each peak was then analyzed by HPLC.

Spectroscopic Analysis

UV and visible absorbance. UV absorbance spectra were obtained with a Cary 118 spectrophotometer (Varian, Palo Alto, CA). The spectra of MC adducts were taken in 0.1 M Tris-HCl at pH 7.5. The aqueous insoluble precipitate, the nuclease-resistant fraction, was solubilized by dimethyl sulfoxide for UV scan.

NMR spectrum. Proton NMR spectra were obtained with a Nicolet NT360 NMR spectrometer (Concord, CA) operated in the FT mode. Samples of 300 mg were lyophilized three times. Accumulation of 1024 scans was taken in 0.5 ml of deuterated dimethyl sulfoxide.

Results

Kinetics of Alkylation

Incubation time effect. The amount of MC bound in MC-DNA and the products generated from nuclease digestion were evaluated through timed incubations (Fig. 2). We conducted the studies at pH 7.5 and 8.5 at an initial MC/DNA concentration ratio of 1 (1 mM) with NADPH cytochrome P-450 reductase serving as the activating enzyme.

At pH 7.5, total bound MC increased nearly linearly with time until 90 min, the last assay point. In contrast, nuclease-solubilized MC adducts and the HPLC-detected MC adducts increased linearly for about 20 min and began to plateau. At pH 8.5, the formation rates for total bound MC, nuclease-digestible MC, and HPLC-detected MC adducts were about 2-fold greater than the rates at pH 7.5 during the first 20 min. The increase of each type began to plateau at 20 min and did not reach the total bound MC formed at pH 7.5.

In these experiments, the nuclease-resistant insoluble fraction, which is the total bound MC minus the nuclease-digestible MC, showed interesting formation rates. This fraction is the first product seen at both pH 7.5 and 8.5 and remains a significant amount of product at pH 7.5. At pH 7.5, after 2–6 min incubation, the nuclease-resistant insoluble fraction appears to be formed at a very rapid or "burst" rate. Over 75% of the total bound MC at this early reaction time is nuclease resistant. For the next 15 min the rate of formation of nuclease digestible equals nuclease resistant. After 20 min, the nuclease-digestible rate lessens, and the formation of the nuclease-resistant fraction increases over 90 min. The nuclease-resistant fraction is less prominent at pH 8.5 than at pH 7.5 except at the early time. For the times incubations, at both pH 7.5 and 8.5, the increase of the nuclease-resistant fraction never corresponded to a decrease of HPLC-detected MC adducts. Data obtained with xanthine oxidase as catalyst were very similar to those obtained with NADPH cytochrome P-450 reductase data (data not shown).

Concentration effect. The kinetics of DNA alkylation were examined further by varying the initial concentration ratio of MC and DNA (Fig. 3). Reactions were performed at pH 7.5 for 60 min with 1 mM DNA while increasing concentrations of MC from 10 μM to 2.0 mM. At low MC concentration (10 μM), which is pharmacologically significant, almost 90% of MC is bound as nuclease-resistant insoluble (Fig. 3, *inset*). This preferential reaction of MC to nuclease-resistant adduct is sustained from 10 μM MC to about 0.3 mM when the nuclease-digestible fraction approaches 50% of the total MC product. A linear increase of bound MC in the nuclease-digestible and HPLC-detected MC adducts occurs as the concentration of MC is increased from 0.1 mM to 2.0 mM. However, even at 2.0 mM, saturation of MC binding was not achieved at a ratio of 125 μmol of MC/1 mmol of DNA.

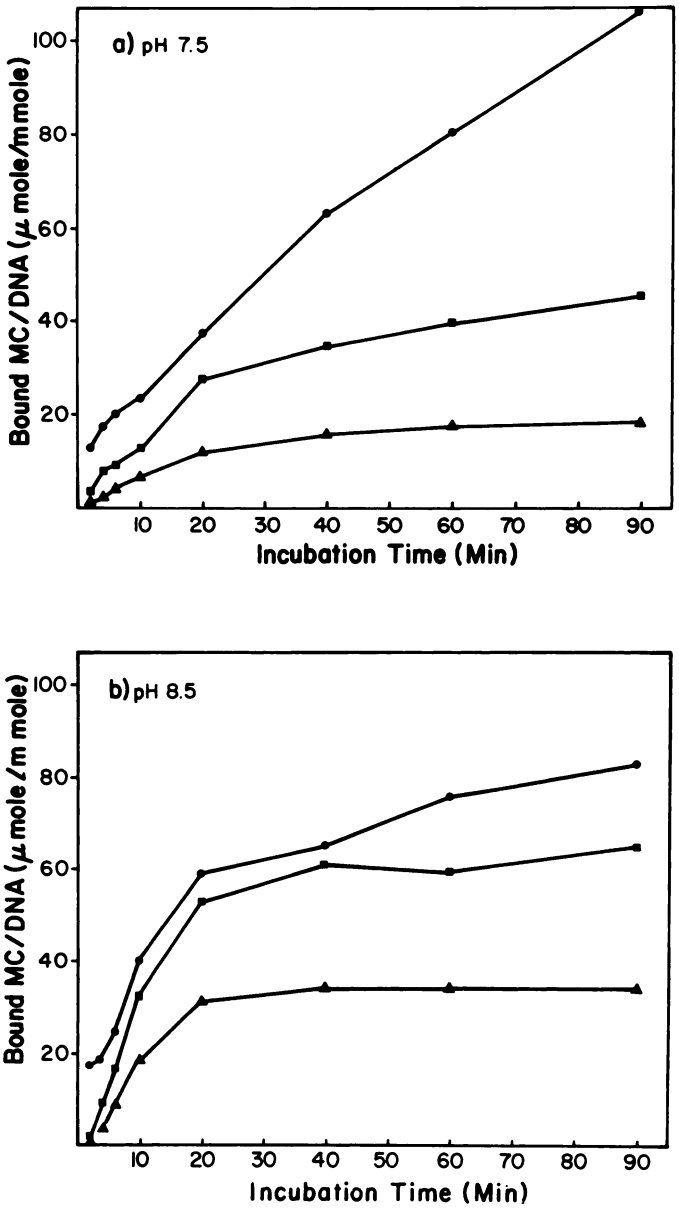


Fig. 2. Effect of incubation time on MC alkylation of DNA at pH 7.5 (a) and pH 8.5 (b). NADPH cytochrome P-450 reductase was the activating enzyme. The initial MC/DNA concentration ratio was 1 (1 mM). Details of preparation, nuclease digestion of MC-DNA, and the HPLC method are described in the text. Amount of MC bound in MC-DNA was measured by absorbance at 312 nm, taken before nuclease digestion as total alkylation (●) and after nuclease digestion and the removal of nuclease-resistant insoluble fraction as nuclease-solubilized MC adducts (■). Calculation was based on the molar absorptivity of O^6-deoxyguanosyl-2,7-diaminomitosene (6030 at 312 nm). HPLC-detected MC adducts (▲) were monitored by absorbance at 313 nm with purified O^6-deoxyguanosyl-2,7-diaminomitosene as external standard. DNA was estimated by absorbance at 260 nm (molar absorptivity 7000).

pH Effect. While varying pH from 6.0 to 8.5, the MC-alkylated DNA products differed significantly in their response to nuclease digestion. Reaction mixtures at an initial MC and DNA concentration ratio of 1 (1.0 mM), with xanthine oxidase as the activating enzyme, were chosen for the study of pH effect at two incubation periods, 15 min and 60 min (Fig. 4). At pH 6 and 6.5 (Fig. 4a), due to the unfavorable pH for enzyme activity,

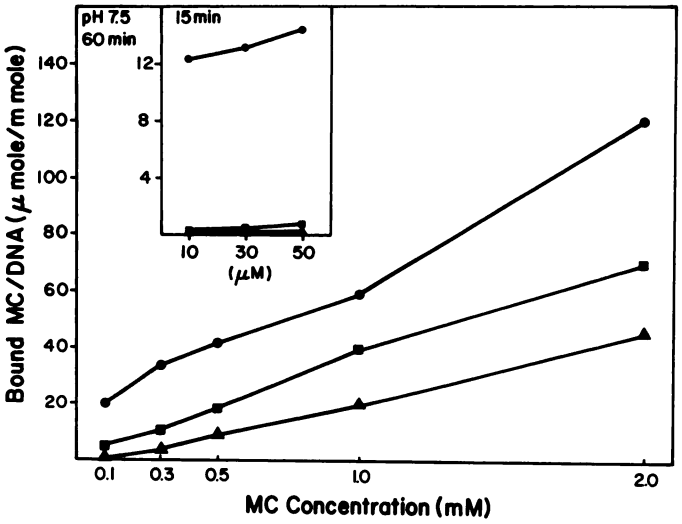


Fig. 3. Effect of the concentration of MC on DNA alkylation. Xanthine oxidase was the activating enzyme and incubation times were 15 and 60 min. Total amounts of alkylation (●), nuclease-solubilized MC adducts (■), and HPLC-detected MC adducts (▲) were estimated as described in Fig. 2.

the amount of MC bound to DNA as total MC-bound, nuclease-digestible, and HPLC-detected MC adducts was low after 15 min, but increased sharply at higher pH. HPLC-detected MC adducts accounted for most of the alkylation at higher pH. For instance, there were 2 times as much total alkylation at pH 8.5 as at pH 7.0, yet the alkylated DNA product at pH 7.0 was more resistant to nuclease digestion. During this 15-min period, sufficient substrates were available.

When the alkylation time was extended to 60 min, several noticeable changes took place (Fig. 4b). MC became exhausted at pH 7.5 and higher as seen by HPLC analysis of the reaction mixture, whereas sufficient MC still remained in the reaction mixtures of pH 6.0 and 6.5 due to the unfavorable pH for enzyme activity. Substantially less MC is bound in MC-DNA at pH 6.0 and 6.5 than at the higher pH. The proportions of MC bound in the total and in the fractions showed obvious differences among the MC-DNA from different pH. The proportion of nuclease-solubilized MC-DNA adducts increased with increasing pH. DNA alkylated at pH 7.5 or lower contained more nuclease-resistant products, and the HPLC-detectable MC adducts were only the smaller proportion of the total bound MC. In DNA alkylated at pH 8.0 and 8.5, the HPLC-detectable products are the major type of total bound MC. Results obtained with the use of NADPH cytochrome P-450 reductase were very similar, except that this enzyme was less stable than xanthine oxidase at pH 6.5 and lower (data not shown).

Separation of MC Adducts by HPLC

When MC-DNA was digested by DNase and snake venom phosphodiesterase, HPLC analysis of the soluble fraction revealed one major peak at $k' = 9.4$ and two less prominent peaks at $k' = 10.6$ and 11.2 (Fig. 5a). In all cases, the second and the third products that were detected by HPLC amounted to approximately 3–5% and 8–10% of the main product, respectively. When the same MC-DNA was also subjected to either P_1 nuclease or S_1 nuclease digestion alone, HPLC analysis yielded different results. Four peaks were separated at $k' = 7.4$, 7.9, 8.2 and 8.6 (Fig. 5b). When the P_1 or S_1 nuclease-digested homogenate was digested further by DNase and phosphodiesterase,

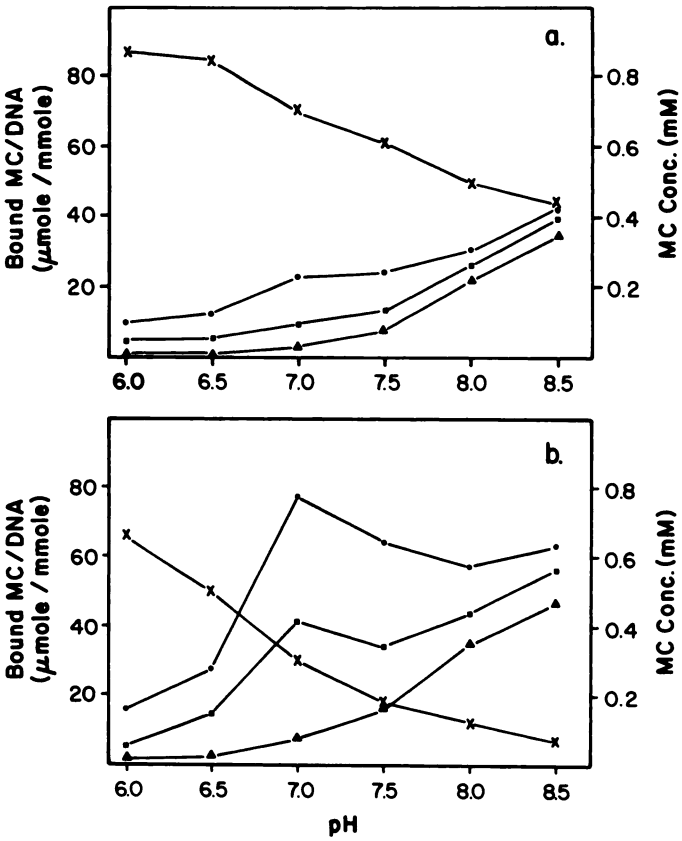


Fig. 4. Effect of buffer pH on MC alkylation of DNA. Xanthine oxidase was the activating enzyme. Incubation periods were 15 min (a) and 60 min (b). The initial MC/DNA concentration ratio was 1 (1 mM). Total amounts of alkylation (●), nuclease-solubilized MC adducts (■), and HPLC-detected MC adducts (▲) were estimated as described in Fig. 2. MC that remained in the reaction mixture was monitored at 365 nm (x).

the final product exhibited an HPLC profile revealing precisely the same peaks as the one that was produced by direct digestion with DNase and phosphodiesterase. A reciprocal experiment demonstrated no changes on HPLC profile. Therefore, we decided that neither P_1 nuclease nor S_1 nuclease was suitable for the digestion of MC-alkylated DNA. DNase and phosphodiesterase digestion systems were used throughout the study, unless indicated otherwise.

HPLC analysis of the material obtained after hydrolyses by alkaline phosphatase revealed one major peak ($k' = 14.0$) and two less prominent products ($k' = 14.2$ and 15.1), and no alteration of the ratio of their absorbance at 313 and 254 nm (Fig. 5c).

Separation of MC Adducts by Gel Filtration

Sephadex G-25 column chromatography separated the nuclease-solubilized MC-DNA into two fractions of unalkylated mononucleosides and three fractions (A, B, and C) that showed high absorption at both 312 and 254 nm. Fraction C was eluted between 72 and 90 ml, concentrated, and analyzed by HPLC. It was found to contain three peaks with the same k' value as the total soluble homogenate. By contrast, fractions A and B, which were eluted at void volume and at 30 ml, respectively, did not reveal any peaks with signifiant absorbance at both 313 and 254 nm by HPLC analysis.

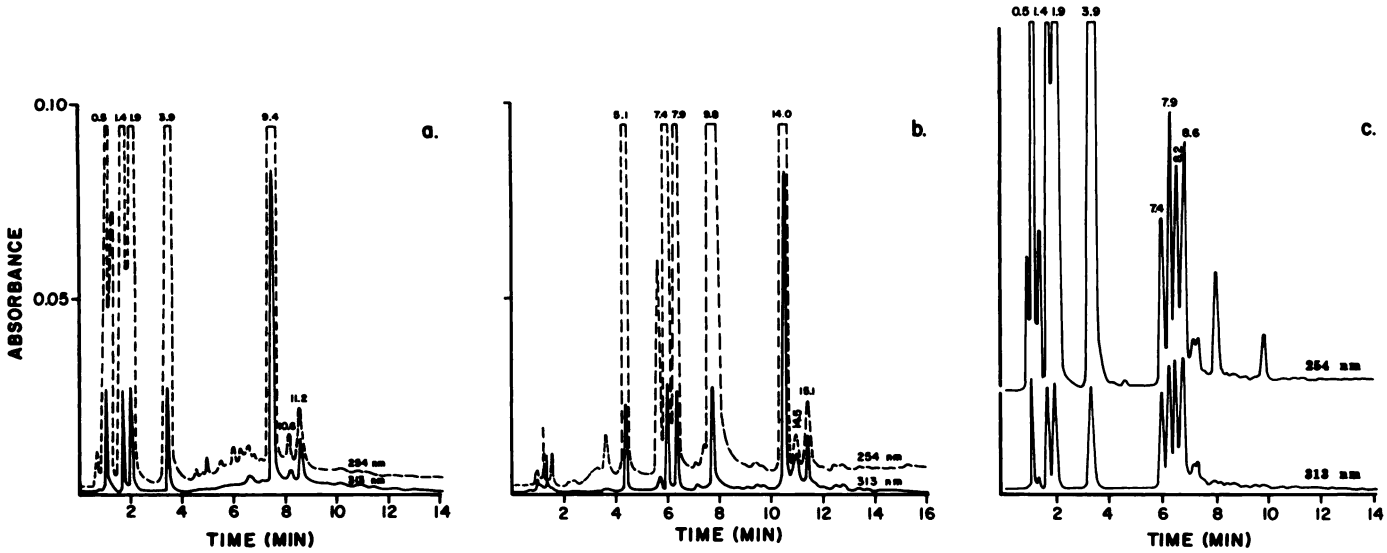


Fig. 5. HPLC profile of MC-DNA after digestion by nucleases. DNA aklylation was performed at pH 7.5 with xanthine oxidase as MC-activating enzyme. MC-DNA was digested by DNase and snake venom phosphodiesterase (a), DNase, snake venom phosphodiesterase and alkaline phosphatase (b), and P_1 nuclease (c). Details are described in the text.

Spectrophotometric Analysis of the Isolated Adducts

UV and visible absorbance. The main nucleotide product (k' = 9.4) showed an absorbance spectrum with maximum absorbance at 312, 252 with shoulders at 360 and 284 nm (Fig. 6). The minor products showed similar absorbance spectra. Conversion of nucleotide product to nucleoside by alkaline phosphotase did not alter the characteristics of the absorbance spectra. Absorbance ratios at 312 and 254 nm of the three separated products were between 0.4 and 0.5.

NMR spectra. Proton NMR spectrum of the major nucleoside product (k' = 14.0) was obtained in deuterated dimethyl sulfoxide (Fig. 7). It showed the 6-methyl peak at δ1.75, the 10-methylene protons at δ4.97, the 2-H proton as a multiplet at δ4.45, and the 7-amino proton as a singlet at δ6.58. These signals were similar to the NMR spectra of 6-methyl-7-amino-10-carbomoyl mitosenes such as the 2,7-diamino-1-hydroxyl mitosene which we isolated from electrochemical reduction of MC (21). The spectrum also showed a triplet at δ6.14 which was assigned to the anomeric 1-H proton. Anomeric 5-OH may have been overlapped by the 10-methylene of mitosene, and 3-OH was unclear. The singlets at δ6.32 and δ7.94 were assigned to the 2-amino protons and the 8-H proton of the purine.

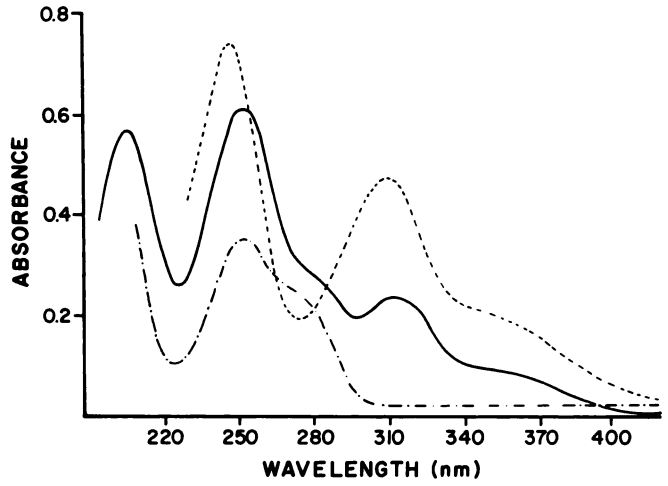


Fig. 6. Absorbance spectra of *trans*-2,7-diaminomitosene (– – –), deoxyguanosine (— · —), and the major MC-adduct (—). Spectra were taken in 0.1 M potassium phosphate at pH 7.5.

Discussion

DNA is readily alkylated by enzyme-activated MC under anaerobic conditions. The MC-alkylated DNA in general became more resistant to the digestion by nucleases. Assuming that MC-DNA is alkylated in monofunctional and bifunctional linkages, digestion by DNase and snake venom phosphodiesterase of this MC-DNA should release the unalkylated nucleotides and, most likely, the monofunctionally modified nucleotides into soluble form. Dinucleotides with MC linked in between are the expected form for bifunctionally bound (cross-linked) MC nucleotides if they were released. In the present study, specific MC dinucleotides were not isolated and detected, whereas the mononucleotide adducts were isolated by HPLC (Fig. 5a) and by gel filtration in fraction C from the nuclease-solubilized MC-DNA. Our belief is that a small polynucleotide (fraction A) and an oligomeric nucleotide fraction (fraction B) resolved by gel filtration with high absorbance at 312 nm carries some of the cross-linked adducts, but the majority of the cross-linked adducts remained in the nuclease-resistant precipitate. Therefore, the degree of nuclease resistance of MC-DNA is probably indicative of the degree of cross-linking.

Quantitative calculations for MC bound to MC-DNA for this study may be somewhat tentative since molar absorptivity of MC bound in DNA is unknown. Tomasz *et al.* (8) determined the molar absorptivity at 310 nm to be 11,000 for porfiromycin bound to native DNA, and that value was applied for MC-DNA complex in studies by other (22, 23). In the present study we found it more appropriate to calculate the DNA-bound MC on the basis of the molar absorptivity of O^6-deoxyguanosyl-(2,7)-diaminomitosene (6030 at 312 nm) reported by Tomasz *et al.* (11) and Hashimoto *et al.* (9, 10).

The kinetics of DNA alkylation suggests that DNA cross-linking by MC is a reaction that occurs readily. Greater

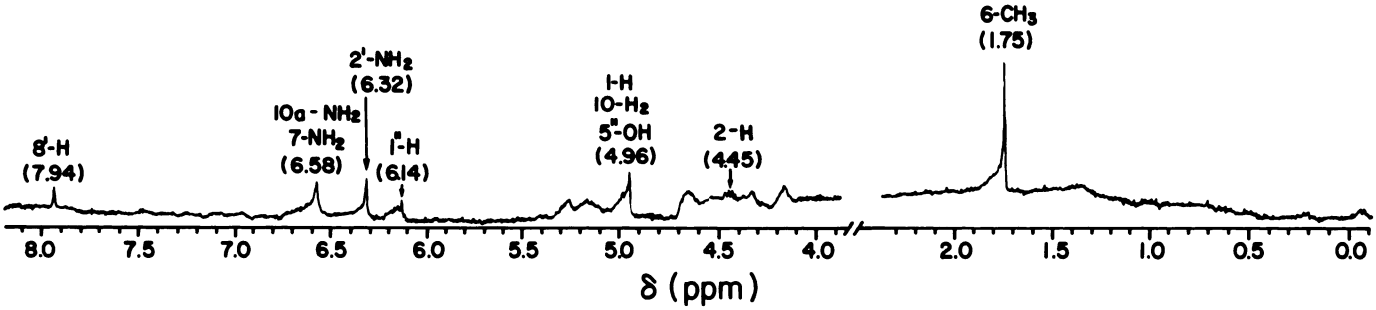


Fig. 7. Proton NMR spectrum of the major MC adduct. The 300-μg sample was lyophilized three times. A total of 1024 scans was obtained in deuterated dimethyl sulfoxide.

amounts of cross-linked products than monofunctional adducts were seen (a) in the earliest product during time incubation for both pH 7.5 and 8.5 (Fig. 2), (b) at the lower MC concentrations (Fig. 3), and (c) at low pH when the rate of alkylation was low (Fig. 4). It seems that specific DNA sites for cross-links by activated MC are available in DNA, and these sites become the immediate and preferred target for MC alkylation. These results also suggest that DNA cross-linking which requires two reactive sites on the MC molecule is not a secondary reaction of monofunctional MC adducts. The effect of pH at this initial stage is mainly on the rate of alkylation since the optimal pH of NADPH cytochrome P-450 reductase and xanthine oxidase is at 8.2 (24, 25). The ratio of monofunctional adducts and cross-links by MC became more pH dependent after the specific sites became alkylated. Thus, monofunctional MC adducts did not appear to function as the intermediate for cross-linking, and DNA cross-linking by MC seems to be a rapid, concerted step reaction. The required active sites of MC for cross-linking remain to be determined.

Our identification of the major MC adduct is based primarily on NMR data (Fig. 7), which is similar to the spectrum of O^6-deoxyguanosyl-2,7-diaminomitosene published by Tomasz *et al.* (11). Five positions on the mitosene moiety are possible linking sites. C-10 carbamate, C-7 amino, and C-6 methyl are accounted for as unsubstituted positions. We were unable to obtain a spectrum under protonated conditions to show the 2-amino group as unsubstituted. However, we believe that the mitosene is linked at the C-1 position, since it has been shown by Tomasz and Lipman (26) and our laboratory (12) that nucleophilic attack on activated MC to open the aziridine ring always takes place at the C-1 position. As for the position on the deoxyribose portion, 3″-OH and 5″-OH should be unsubstituted, since both positions were linked to phosphate in the DNA skeleton and the removal of phosphate from nucleotide by alkaline phosphatase did not have any effect on the mitosene linkage. An anomeric 1″ proton was seen in the NMR spectrum which means it is unsubstituted. Therefore, the mitosene is not likely to be attached to deoxyribose and the linkage has to be on the purine moiety. The 2′-amino protons and 8′-H proton signals indicate no substitution at these two positions. Positon 7′ is ruled out due to the fact that the signal of 8′-proton is unshifted. The three positions that are left are N^1, N^3, and O^6 of the purine. We ruled out the N^3 position since it is known that N^3-alkylated products are relatively unstable. Tentatively, we assigned the linkage position at the O^6 of guanine and named the adduct O^6-deoxyguanosyl-2,7-diaminomitosene (Fig. 1).

UV and visible spectra of all three MC adducts that were separated by HPLC exhibited the characteristics of a composite of nucleotide and 7-aminomitosene. Similarity of the relative absorbance ratio at 312 and 254 nm of all three MC adducts indicates that the two minor MC adducts are most likely monofunctional adducts.

The proportions of the three soluble adducts were relatively consistent regardless of the conditions or extent of alkylation. Therefore, the O^6 position of guanine is definitely the preferential site for monofunctional linkage. This finding appears somewhat different from the report of Hashimoto *et al.* (10). They identified three monofunctional MC adducts with mitosene linked at O^6-guanine, N^2-guanine, and N^6-adenine. The yield of their three products indicated that alkylation took place almost in equal proportion for the three sites. But their results may be due to the chemical reductive conditions they used for MC activation. Their *in vivo* observation with rat liver DNA also showed a predominance of O^6-deoxyguanosyl-mitosene.

Various alkylating agents with cross-linking ability such as platinum compounds are widely used in cancer chemotherapy (27, 28). In the case of MC, it is not clear at the present time whether both types of its alkylating activities (monofunctional or bifunctional) are equally crucial for its antitumor activity. Our present work has shown that the initial burst of DNA alkylation by activated MC is pH independent and preferably results in cross-links. The limitation for initial cross-linking would depend on the efficiency of the activating process of MC. Enzymatically, it is obvious that higher pH is better for the activity of a number of flavoenzymes (24, 25). Kennedy *et al.* (5) demonstrated that MC-induced DNA cross-links were enhanced by lowering intracellular pH of EMT6 cells to 5.7 as compared to cells maintained at pH 7.5, which suggested that acid-catalyzed activation of MC could also be a major activating system intracellularly. It will be interesting and important to determine whether pH has an effect on the efficacy of MC as an antitumor drug.

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Send reprint requests to: Dr. Su-Shu Pan, University of Maryland Cancer Center, Bressler Research Building, Room 9-025, 655 West Baltimore Street, Baltimore, MD 21201.