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Effect of pH on DNA Alkylation by Enzyme-Activated Mitomycin C and Porfiromycin

FANG YU and SU-SHU PAN

Division of Developmental Therapeutics, University of Maryland Cancer Center, and Department of Pharmacology and Experimental Therapeutics, School of Medicine, University of Maryland, Baltimore, Maryland 21201

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SUMMARY

DNA adduct formation by enzyme-activated antibiotics, mitomycin C (MMC) or porfiromycin (PFM), at pH 7.6 or pH 6.0 under anaerobic conditions was analyzed by a ^{32}P-postlabeling method. Antibiotic activation by rat liver NADPH-cytochrome P-450 reductase (EC 1.6.2.4) and bovine milk xanthine oxidase (EC 1.2.3.2) produced similar results. Five ^{32}P-labeled MMC adducts were separated by thin layer chromatography and high performance liquid chromatography from DNA alkylated at either pH. Four of the radioactive spots separated by thin layer chromatography were identified as two monofunctional monoadducts [1″α and 1″β forms of N^2-(2″β,7″-diaminomitosen-1″-yl)-2′-deoxyguanylic acid], one bifunctional monoadduct [N^2-(10″-decarbamoyl-2″,7″-diaminomitosen-1″α-yl)-2′-deoxyguanylic acid], and one cross-linked adduct [N^2-(2″β,7″-diamino-10″-deoxyguanyl-N^2-yl-mitosen-1″α-yl)-2′-deoxyguanylic acid]. One minor radioactive spot was not identified. By comparing DNA alkylated at the two pH values, based on equal amounts of ^{32}P radioactivity, similar amounts of cross-links were detected. However, the DNA showed different ratios of the α and β isomers of the monofunctional monoadduct. Furthermore, the DNA alkylated at pH 6.0 showed more bifunctional monoadducts than did the DNA alkylated at pH 7.6. Analysis of alkylated DNA by enzyme-activated PFM showed a similar spectrum of DNA adduct formation. The effect of pH on the distribution of the five PFM-DNA adducts was similar to that observed for the five MMC-DNA adducts. The distribution of adducts in DNA alkylated at the same pH was similar irrespective of which enzyme activated MMC or PFM. The pH of the reaction during DNA and MMC interaction was the determining factor for the quantitative distribution of the adducts. This pH effect may be important for the cytotoxicity of MMC and PFM in tumor cells that have high levels of reductive enzymes with low optimal pH values.

The reactions between reductively activated MMC and DNA have been under investigation for many years. In recent years, several covalently linked DNA adducts have been isolated and identified (Fig. 1). A major monofunctional adduct was first isolated by Tomasz *et al.* (1) from chemically and enzymatically activated MMC-alkylated dinucleotide, d(GpC). Later they identified its full structure as 1″αN^2-G-MMC (2). The same adduct was also isolated by Pan *et al.* (3) and Tomasz *et al.* (4) from calf thymus DNA alkylated by enzyme-activated MMC. Subsequently, an interstrand cross-linked bifunctional adduct of two deoxyguanosines [(N^2-G)$_2$-MMC], a minor monofunctional monoadduct (1″βN^2-G-MMC), a bifunctional monoadduct (N^2-G-DMC), and an intrastrand cross-linked bifunctional adduct were also identified by Tomasz and co-workers (5–7). Studies of interaction of DNA and PFM, an *N*-methylated analogue of MMC, showed production of adducts similar to those formed with MMC (8). A cross-linked PFM adduct was identified (9).

Tomasz *et al.* (10) used a number of different reductive methods for investigating DNA adduct formation by MMC. They reported that the conditions under which reductive activation occurred influenced the ratio of monoadducts to bifunctional adducts formed along DNA strands. It was observed that MMC reduction with low concentrations of H_2/PtO_2 or enzymatic reduction produced mainly monoadducts, whereas reduction with $Na_2S_2O_4$ or high concentrations of H_2/PtO_2 produced more bifunctional adducts (including N^2-G-DMC and the cross-linked adduct). These observations led to the conclusion that the kinetics of MMC reduction determined the ratio of monofunctional and bifunctional activation and thus the formation of monoadducts and bifunctional adducts.

Previous research has shown that reaction pH is a major

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ABBREVIATIONS: MMC, mitomycin C; PFM, porfiromycin; pH 7.6 (or pH 6.0) MMC-DNA (or PFM-DNA), DNA alkylated at pH 7.6 (or pH 6.0) by enzyme-reduced mitomycin C (or porfiromycin); α (or β)N^2-G-MMC, N^2-(2″β,7″-diaminomitosen-1″α (or β)-yl)-2′-deoxyguanylic acid; N^2-G-DMC, N^2-(10″-decarbamoyl-2″,7″-diaminomitosen-1″α-yl)-2′-deoxyguanylic acid; (N^2-G)$_2$-MMC, N^2-(2″β,7″-diamino-10″-deoxyguanyl-N^2-yl-mitosen-1″α-yl)-2′-deoxyguanylic acid (adduct of two deoxyguanylic acids cross-linked at the N^2-position by mitomycin C); SVPDE, snake venom phosphodiesterase I; MES, 2-(*N*-morpholino)ethanesulfonic acid; TLC, thin layer chromatography; HPLC, high performance liquid chromatography.

determinant of MMC metabolite formation by both chemical activation (11, 12) and enzymatic activation by NADPH-cytochrome P450 reductase and xanthine oxidase (13), DT-diaphorase (14), and xanthine dehydrogenase (15). The amount of DNA cross-linking was also determined by pH. This was shown with chemically activated MMC in cell-free systems (16) and DNA in EMT6 cells (17). However, limited information has been reported regarding the effect of pH on the types of MMC-DNA adduct formed, because most studies were conducted at physiological pH.

Detection of MMC-DNA adducts by HPLC has relied on the optical absorbance of nucleotides and mitosene. However, investigation of DNA alkylation in cells and *in vivo* has been limited by the lack of radioactive MMC. To overcome these difficulties, we have used radioactive PFM to study DNA adduct formation in murine leukemia P388 cells (18); we found a major monoadduct, the equivalent of 1″αN^2-G-MMC, and possibly a cross-linked adduct. In recent years, the highly sensitive ^{32}P-postlabeling technique (19–22) has allowed the study of MMC-DNA adduct formation in *in vivo* systems without using large doses of MMC (23, 24). We have now used the ^{32}P-postlabeling technique to investigate the effect of pH on DNA adduct formation by enzyme-activated MMC and PFM. TLC and HPLC separations of individual adducts were used in combination to identify the labeled MMC-DNA and PFM-DNA adducts. The information obtained from this study provides the foundation to investigate the relationship between DNA adduct formation by MMC and pH modulation of reductive enzyme activity in tumor cells.

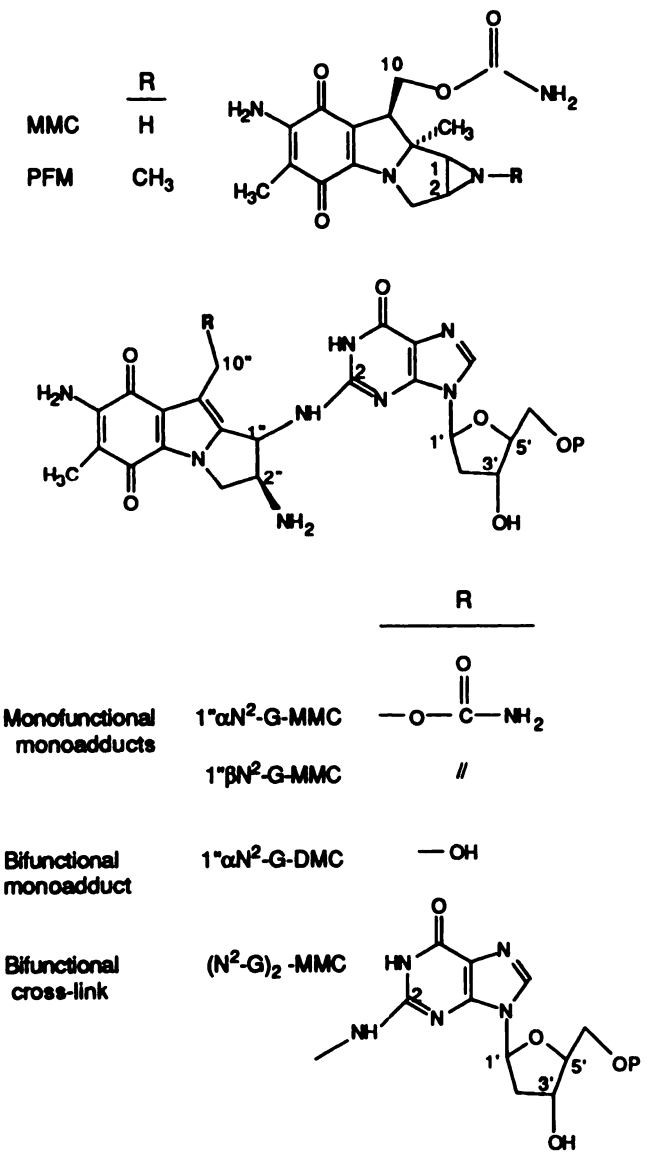

Fig. 1. Structures of MMC, PFM, and DNA adducts of MMC.

Materials and Methods

Reagents. MMC and PFM were kindly supplied by the Natural Products Branch, Division of Cancer Treatment, National Cancer Institute (Bethesda, MD). Bovine milk xanthine oxidase (EC 1.2.3.2) and NADPH-cytochrome P-450 reductase (EC 1.6.2.4) were purified according to the method of Nelson and Handler (25) and the method of Yasukochi and Masters (26), respectively. T_4 polynucleotide kinase (EC 2.7.1.78) was from United States Biochemical Corporation (Cleveland, OH). CN-HPTLC plates were from EM Industries (Gibbson, NJ). [γ-^{32}P]ATP was from ICN Biomedicals (Costa Mesa, CA). Nuclease P1 (EC 3.1.30.1), SVPDE (EC 3.1.4.1), DNase I (EC 3.1.21.1), apyrase (EC 3.6.1.5), prostatic acid phosphomonoesterase (EC 3.1.3.2), and other biochemicals were purchased from Sigma Chemical Co. (St. Louis, MO).

Preparation of alkylated DNA. Calf thymus DNA was alkylated by MMC or PFM according to a modification of our previously reported method (3, 8). The 1-ml reaction mixture contained 20 mM NaCl, 0.5 mM MMC (or PFM), 1.0 mM NADH (or NADPH), and 0.5 mg/ml calf thymus DNA in either 50 mM Tris·HCl at pH 7.6 or 50 mM MES·HCl at pH 6.0. Xanthine oxidase (80 μg/ml) or NADPH-cytochrome P450 reductase (3 μg/ml) was used to activate MMC or PFM. Activation at both pH values was conducted anaerobically at 37° for 60 min. DNA for controls was treated identically except that MMC (or PFM) was not included. Termination of the reaction and recovery of the alkylated DNA were carried out as described previously (3, 8). Recovered DNA was dissolved in 10 mM Tris at pH 7.5 for ^{32}P-postlabeling and preparation of unlabeled DNA adducts.

Isolation of unlabeled DNA adducts. Alkylated DNA was digested with DNase I and SVPDE according to the method of Pan and co-workers (3, 8). MMC (or PFM)-modified nucleotides in this DNA digest were separated and isolated by the HPLC method of Tomasz *et al.* (5, 6). The adducts isolated were monophosphates instead of nucleosides. To confirm their identity, individual isolates (10 μg) were dissolved in 50 mM Tris·HCl at pH 8.5 and digested with alkaline phosphatase (1 unit). The resulting nucleosides were analyzed by thermospray mass spectrometry, as described by Musser *et al.* (27).

^{32}P-postlabeling. The ^{32}P-postlabeling procedure developed by Randerath *et al.* (19), using nuclease P1 and prostatic acid phosphatase, was followed with some modification. Alkylated DNA (12 μg/6 μl) was digested at 37° for 60 min with nuclease P1 (1.5 μg) and acid phosphatase (200 milliunits) in the presence of 0.15 mM $ZnCl_2$ and acetate buffer, pH 5.0, in a total volume of 10 μl. After this initial digestion, the reaction mixture was adjusted to pH 7.8 with 1.4 μl of 0.29 M Tris. Prostatic acid phophatase was destroyed by heating the mixture at 65° for 10 min. A 2-μl aliquot was taken to monitor DNA digestion, by absorbance at 254 nm. To the rest of the DNA digest, a 7.3-μl solution containing 50 μCi of carrier-free [γ-^{32}P]ATP, 7.2 units of T_4 polynucleotide kinase, and 2.0 μl of polynucleotide kinase buffer (0.5 M Tris·HCl, pH 7.6, 100 mM $MgCl_2$, 100 mM 2-mercaptoethanol) was added. Incubation was resumed at 37° for 60 min. To demonstrate that SVPDE is required for complete digestion of adducts, the reaction mixture was divided into two equal aliquots. One aliquot was further digested at 37° for 60 min with 40 milliunits of potato apyrase and 9.0 milliunits of SVPDE. The other aliquot was digested with only 40 milliunits of

potato apyrase. Final products of the labeled DNA digests were analyzed by TLC and HPLC.

TLC analysis of labeled products. The two-dimensional TLC system developed to analyze the labeled DNA adducts of MMC and PFM used a reverse phase, low ion exchange, CN-HPTLC silica gel plate. A 1-μl DNA sample (0.3 μg of DNA) was spotted for each analysis. The first dimension (D1) was developed overnight at 4° with a solvent system of 0.4 M NH_4HCOOH. During D1 development, a Whatman paper wick (1 mm × 10 cm × 25 cm) was attached to the top of the plate to remove orthophosphate, which was the product of hydrolysis of excess [^{32}P]ATP. After D1 development, the plates were dried at room temperature for 2 hr. The plate was rotated 90° clockwise, and the second dimension (D2) was developed with 0.75 M KH_2PO_4 at room temperature until the solvent front reached the top of the plate. Finally, the plates were thoroughly dried at room temperature before exposure to film.

HPLC separation of MMC-5′-nucleotides. Before HPLC analysis, labeled DNA digests were purified with Waters SEP-PAK C_{18} cartridges (Millipore Corporation, Milford, MA) to remove normal nucleotides and inorganic phosphates. Cartridges were first primed with methanol and washed with water. A 10-μl aliquot of DNA digest was loaded onto a cartridge, which was subsequently washed with 10 ml of water and then eluted with 2 ml of methanol. The methanol eluate was concentrated and redissolved in water for HPLC analysis by the procedure developed by Tomasz *et al.* (4–6). The HPLC method of Pan *et al.* (3) was used to confirm the separation and identity of adducts. All procedures were followed accordingly, except that a Nova-Pak C_{18} 4-μm cartridge (8 × 100 mm; Millipore) was used for both methods. To prepare internal standards, alkylated DNAs were digested to nucleotides with DNase I and SVPDE, according to the method from an earlier publication (3). This digested homogenate was also purified with a SEP-PAK C_{18} cartridge as described above for the labeled DNA sample. To establish the HPLC separation profiles, the unlabeled DNA digest was analyzed alone by the two HPLC methods (3–6). Authentic adducts, 1″αN^2-G-MMC, 1″βN^2-G-MMC, αN^2-G-DMC, and (N^2-G)$_2$-MMC, were chromatographed separately as external standards. The ^{32}P-postlabeled DNA digest (4.5 × 10^6 dpm) was then co-chromatographed with the unlabeled DNA digest of the same pH (100 μg of DNA). Absorbance at 312 and 254 nm was used to detect unlabeled MMC-modified nucleotides. Fractions (0.3 ml) of eluate were collected and counted with a Beckman 6000 scintillation counter (Beckman Instruments, Fullerton, CA). Individual radioactive fractions were concentrated and reconstituted to 5 μl. Aliquots with an equivalence of 5000 dpm were analyzed by TLC.

Autoradiography. Radioactive TLC plates were exposed to XAR-5 or XRP-5 film (Eastman Kodak, Rochester, NY) for different time periods, depending on the intensity of the labeled DNA digest. Exposed films were developed with X-OMAT (Eastman Kodak).

Results

Requirement for SVPDE for MMC-DNA adduct digestion. The necessity of including SVPDE digestion after nuclease P1 digestion of DNA in the ^{32}P-postlabeling procedure was first verified. When SVPDE digestion was omitted from the preparation of DNA samples, TLC separation produced large, poorly resolved, radioactive clusters with high mobility (Fig. 2, A and B). In the aliquot that included SVPDE digestion (Fig. 2, C and D), clusters were not detected and radioactive spots were resolved clearly by TLC. Based on this evidence that SVPDE is required for complete digestion of MMC-modified DNA, all subsequent experiments performed in this study included SVPDE digestion in each preparation.

Separation of MMC-DNA adducts by TLC. Alkylated DNA prepared at the same pH with either NADPH-cytochrome P450 reductase or xanthine oxidase as the enzyme for MMC activation showed similar results. The results obtained for the DNA produced with xanthine oxidase as the catalytic enzyme are presented. At pH 7.6, one major (**b**) and three minor (**a**, **d**, and **f**) radioactive spots were separated by TLC from the alkylated DNA (Fig. 2C). In contrast, after alkylation at pH 6.0 six radioactive spots (**a**, **b**, **d**, **e**, **f**, and **i**) were resolved from the DNA (Fig. 2D). Although four of the spots (**a**, **b**, **d**, and **f**) were common to DNAs alkylated at the two different pH values, the relative intensities of these four spots were different. Control DNA did not show any radioactive spots (Fig. 2E).

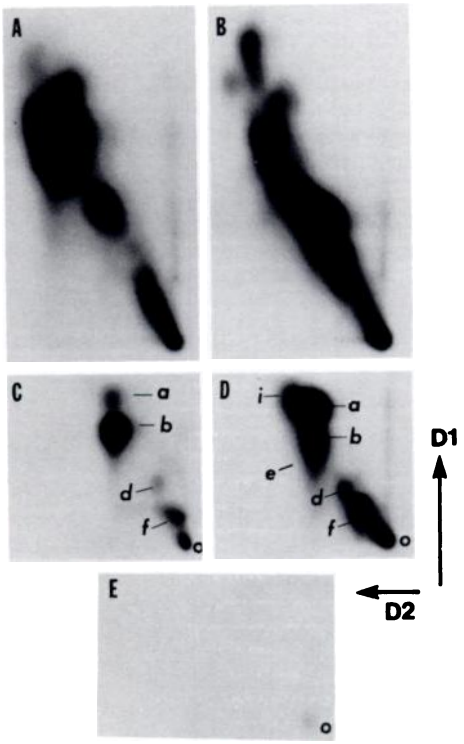

Fig. 2. Comparison of ^{32}P-labeled nucleotides from pH 6.0 and pH 7.6 MMC-DNA, by two-dimensional TLC. The preparation of MMC-DNA, the procedure for ^{32}P-labeling, and the two-dimensional TLC development were as described in the text. Equal amounts of DNA (0.3 μg) from five different treatments were spotted. A, Digest of pH 7.6 MMC-DNA without SVPDE digestion; B, digest of pH 6.0 MMC-DNA without SVPDE digestion; C, digest of pH 7.6 MMC-DNA with SVPDE digestion; D, digest of pH 6.0 MMC-DNA with SVPDE digestion; E, control digest of DNA that was treated the same way as the pH 6.0 MMC-DNA except that MMC was omitted. *o*, Origin.

Separation of MMC-DNA adducts by HPLC. By ^{32}P radioactivity detection (Fig. 3, *upper*), HPLC separation of the pH 7.6 MMC-DNA digest revealed five peaks (**A**, **B**, **D**, **E**, and **F**), and separation of the pH 6.0 MMC-DNA digest revealed six peaks, with five (**A**, **B**, **D**, **E**, and **F**) showing the same retention times as the five peaks of the pH 7.6 MMC-DNA. Seven peaks (**1**, **2**, **3**, **4**, **5**, **6**, and **7**) absorbing at 313 nm were detected from digests of MMC-DNA produced at both pH values without ^{32}P label (Fig. 3, *lower*; Table 1). Absorbance peaks **1**, **2**, **3**, **4**, **6**, and **7** co-chromatographed with ^{32}P-radioactive peaks **A**, **B**, **C**, **D**, **E**, and **F**, respectively. Absorbance peak **5** did not co-chromatograph with any radioactive peak. Absorbance peaks **1**, **2**, **6**, and **7** showed the same retention times as authentic adducts N^2-G-DMC, 1″αN^2-G-MMC, (N^2-G)$_2$-MMC (the cross-linked adduct), and 1″βN^2-G-MMC, respectively.

TLC analysis of HPLC-separated components. One-

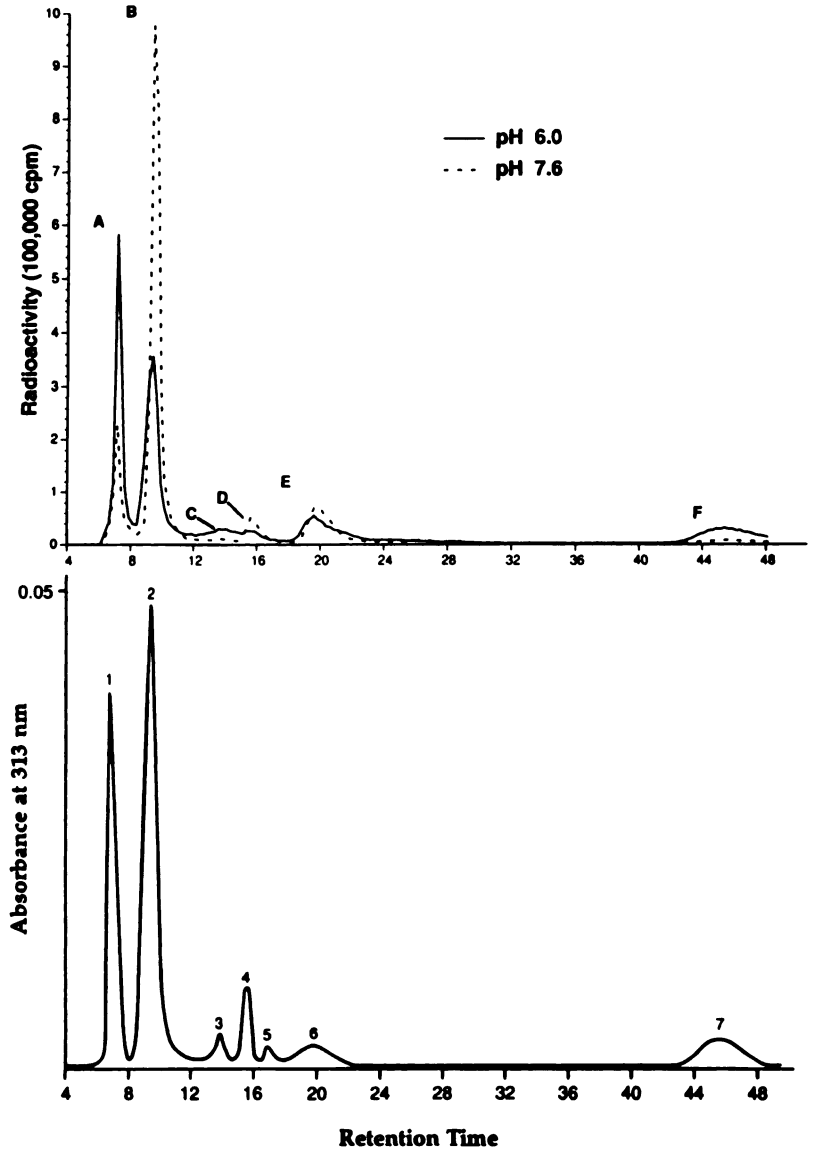

Fig. 3. HPLC separation of ^{32}P-postlabeled DNA adducts. Alkylated DNA prepared at pH 6.0 was digested with DNase I and SVPDE and prepurified as described in Materials and Methods. An aliquot of this unlabeled digest (100 μg of DNA) and ^{32}P-postlabeled MMC-DNA digest (4.5 × 10^6 dpm) were co-chromatographed by the HPLC method of Tomasz *et al.* (5–7). Fractions (0.3 ml) were collected and counted for radioactivity. *Upper*, profile of radioactivity for digests of pH 7.6 MMC-DNA (– – –) and pH 6.0 MMC-DNA (——). *Lower*, profile of absorbance at 313 nm for digest of pH 6.0 MMC-DNA (data for pH 7.6 MMC-DNA are not shown).

TABLE 1

Identification and assignment of radioactive and absorptive peaks separated by HPLC and radioactive spots separated by TLC

Radioactive HPLC peaks	HPLC peaks absorbing at 313 nm	Radioactive TLC spots	Identification
A	1	a	N^2-G-DMC
B	2	b	αN^2-G-MMC
C	3		Residue of B
D	4	d	Unknown
	5		
E	6	e	$(N^2\text{-G})_2$-MMC
F	7	f	βN^2-G-MMC
		g[a]	Unknown
		h[a]	Unknown
		i	Unknown

[a] These spots appeared only with samples containing large amounts of radioactivity.

dimensional TLC chromatograms showed that the five radioactive peaks (**A**, **B**, **D**, **E**, and **F**) isolated by HPLC from the pH 6.0 MMC-DNA digest had different R_f values (Fig. 4, *1*). Peak **C** had a R_f between those of peaks **B** and **D** and probably was a mixture of peaks **B** and **D**. Two-dimensional TLC (Fig. 4, *2-5*) of individual radioactive peaks isolated by HPLC was used to identify their positions in the total DNA digest shown in Fig. 2. Correlation of these HPLC peaks with TLC spots is shown in Table 1 and is as follows: peak **A** = peak **1** = spot **a**, peak **B** = peak **2** = spot **b**, peak **D** = peak **4** = spot **d**, peak **E** = peak **6** = spot **e** (which was overshadowed by spot **b** in the plate of whole-DNA digests due to low content), and peak **F** = peak **7** = spot **f**. HPLC separation did not reveal any detectable radioactive peak for spot **i**, which appeared frequently with the pH 6.0 MMC-DNA. It was probably a di- (or tri-)nucleotide that resisted SVPDE digestion or the intrastrand cross-linked

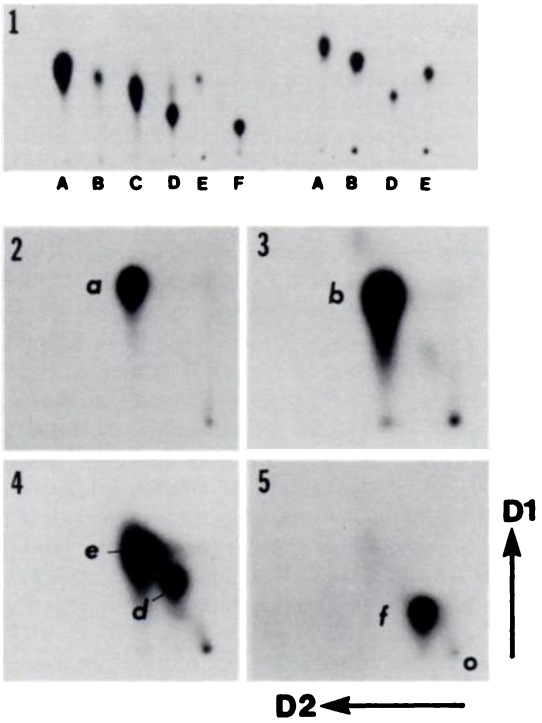

Fig. 4. TLC analysis of radioactive peaks separated by HPLC. The fraction with the highest level of radioactivity in each HPLC peak was concentrated and redissolved in 5 μl of water. An aliquot containing 4000–6000 dpm was spotted on TLC plates and developed as described in Materials and Methods. *1*, One-dimensional TLC with D1 solvent of peaks separated by HPLC from pH 6.0 MMC-DNA (*left*) and pH 7.6 MMC-DNA (*right*). *2-5*, Two-dimensional TLC of radioactive peaks isolated by HPLC from digest of pH 6.0 MMC-DNA. *2*, Peak **A**; *3*, peak **B**; *4*, peaks **D** and **E**; *5*, peak **F**. *o*, Origin.

adduct. TLC analysis also confirmed that peaks **A**, **B**, **D**, **E**, and **F** from the pH 7.6 MMC-DNA were identical to peaks **A**, **B**, **D**, **E**, and **F**, respectively, in the pH 6.0 MMC-DNA (two-dimensional TLC data for pH 7.6 MMC-DNA are not shown).

Chemical identity assignment of TLC spots. The identities are as listed in Table 1 and are as follows: TLC spot **a** (HPLC peaks **A** and **1**) is N^2-G-DMC, spot **b** (peaks **B** and **2**) is 1″αN^2G-MMC, spot **e** (peaks **E** and **6**) is the cross-linked adduct (N^2-G)^2-MMC, and spot **f** (peaks **F** and **7**) is 1″βN^2-G-MMC. The identity of TLC spot **d** remains unknown. Occasionally, spots **g** and **h** were separated by TLC when samples with large amounts of radioactivity were used, as listed in Table 1. Corresponding peaks for **g** and **h** were not found by HPLC. The identities of these spots remain unknown.

Quantitative estimation of adducts. Assuming each monoadduct is labeled with one [^{32}P]phosphate and each cross-linked adduct is labeled with two [^{32}P]phosphates (see Discussion), the radioactivity of each peak separated by HPLC was tabulated (Table 2). The distribution of the three monoadducts was consistently different between DNA alkylated at pH 7.6 and DNA alkylated at pH 6.0. αN^2-G-MMC was always the major monoadduct in the pH 7.6 MMC-DNA, whereas αN^2-G-MMC and N^2-G-DMC were generated in almost equal amounts in the pH 6.0 MMC-DNA. N^2-G-DMC and βN^2-G-MMC were always produced in larger amounts in the pH 6.0 MMC-DNA than in the pH 7.6 MMC-DNA. However, the amount of cross-linked adduct was similar in the pH 6.0 and 7.6 samples.

Separation of PFM-DNA adducts by TLC. TLC separation of ^{32}P-labeled adducts resulting from the alkylation of DNA by PFM showed a separation pattern similar to that found with DNA alkylated by MMC. This implied that the *N*-methylation of MMC at the C-1 position to produce PFM did not alter the pattern of alkylation of DNA. DNA adducts produced by enzyme-activated PFM at pH 7.6 and 6.0 were similar to DNA adducts produced by MMC at the same pH values (Fig. 5). The same pH effect and similar patterns of TLC separation of ^{32}P-labeled adducts were observed. Adduct spots **a′**, **b′**, **d′**, and **f′** separated after reaction of PFM with DNA were equivalent to adduct spots **a**, **b**, **d**, and **f**, respectively, separated after alkylation of DNA by MMC. The ratio difference of adducts **a′**, **b′**, and **f′** in pH 6.0 PFM-DNA and pH 7.6 PFM-DNA was similar to the ratio difference of adducts **a**, **b**, and **f**, respectively, in pH 6.0 MMC-DNA and pH 7.6 MMC-DNA. Spot **e′** was overshadowed by spot **b′**, but its existence was confirmed by HPLC. Occasionally, spots **g′** and **h′** were separated by TLC for PFM-DNA as well as MMC-DNA. These spots are not detected by HPLC, and their identities are unknown.

TABLE 2

Effect of pH on the distribution of different DNA adducts produced by MMC

Alkylated DNA was prepared with xanthine oxidase-activated MMC at pH 6.0 and pH 7.6 as described in the text. Procedures for ^{32}P-postlabeling, purification of DNA digests by SEP-PAK C_{18} cartridge, and HPLC separation of adducts were as described in Materials and Methods. Equal amounts of radioactivity of each labeled DNA digest (4.5×10^8 dpm) were analyzed by HPLC. Fractions of individual radioactive peaks were combined and tabulated. Data represent the average of two experiments.

HPLC peaks	Identification	MMC-alkylated DNA			
		pH 7.6		pH 6.0	
		Radioactivity	Percentage	Radioactivity	Percentage
		×10³ dpm		×10³ dpm	
A	N^2-G-DMC	556	14.2	1372	34.3
B	αN^2-G-MMC	2693	68.6	1582	39.5
D	Unknown	215	5.5	144	3.6
E	(N^2-G)$_2$-MMC[a]	505	6.4	532	6.6
F	βN^2-G-MMC	126	3.2	508	12.7
Others		81	2.0	130	3.2

[a] Labeled (N^2-G)$_2$-MMC contains one $^{32}PO_4$ for each of the two deoxyguanines. Therefore, the actual quantity of (N^2-G)$_2$-MMC is half of the radioactivity; this was considered during calculation of percentage.

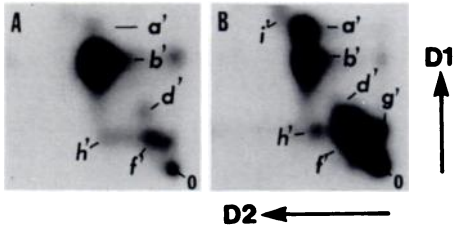

Fig. 5. Comparison of ^{32}P-labeled nucleotides from pH 6.0 and pH 7.6 PFM-DNA, by two-dimensional TLC. The preparation of PFM-DNA, the procedure for ^{32}P-labeling, and the two-dimensional TLC development were as described in the text. Equal amounts of DNA (0.5 μg) were spotted. A, Digest of pH 7.6 PFM-DNA; B, digest of pH 6.0 PFM-DNA. *O*, Origin.

Discussion

Randerath *et al.* (19) recently reported a method, using nuclease P1 and prostatic acid phosphatase for the initial DNA digestion, to study bulky DNA adducts. This method was

designed to take advantage of the fact that the nucleotide on the 3′ side of the bulky adducts resisted digestion by nuclease P1. Our earlier work (3) and a publication by Tomasz *et al.* (4) showed that MMC-modified nucleotides also protected the adjacent nucleotide from digestion by nuclease P1. This led us to choose the nuclease P1 version of the ^{32}P-postlabeling method for the current study. Nuclease P1 digestion of MMC-DNA produced normal mononucleotides (pN), modified dinucleotides (pXpN) containing a 5′-phosphate MMC-modified nucleotide (pX) and a normal nucleotide (pN), and tetranucleotides consisting of two dinucleotides with 5′-phosphates cross-linked by mitosene [(pXpN)-pXpN]. The 5′-phosphate of all nucleotides was removed by acid phosphatase to produce N, XpN, and (XpN)-XpN. Then, only the 5′ end of XpN and (XpN)-XpN was labeled with [^{32}P]phosphate by polynucleotide kinase. Subsequent SVPDE digestion cleaved the normal nucleotide from the dinucleotides and the cross-linked tetranucleotides to yield ^{32}P-labeled mononucleotide adducts (^{32}PX) and mitosene-cross-linked dinucleotide adducts (^{32}PX-X^{32}P). Our data confirmed that SVPDE digestion was necessary to ensure complete digestion of MMC adducts and to avoid artifacts due to undigested ^{32}PXpN and cross-linked (^{32}PXpN)-^{32}PXpN.

We used a two-dimensional TLC system to analyze the ^{32}P-postlabeled MMC-DNA digest. Most ^{32}P-labeling analyses of DNA adducts employ three-dimensional TLC using two TLC plates, with a contact-transfer of sample from the first plate to a second plate being performed after the first-dimension development. The present procedure required only one TLC plate, thereby avoiding sample transfer and eliminating sample loss associated with such transfer. The separation of the cross-linked adduct (N^2-G)$_2$-MMC and the monoadduct αN^2-G-MMC was not resolved well with the present TLC system. Separation required HPLC to confirm the presence of both adducts. The identities of the four radioactive spots separated by TLC were assigned with the information attained by HPLC. To provide absolute chemical identification, analysis such as mass spectrometry or NMR spectroscopy of each spot is necessary. High yield of the bifunctional monoadduct N^2-G-DMC as a result of hydrolysis of the monofunctional adduct N^2-G-MMC has been shown to be unlikely (10). Corresponding peaks for TLC spots **g**, **h**, and **i** were not resolved by HPLC. Most likely, spots **g** and **h** were not detected during HPLC separation due to low yield and poor separations, because these spots appeared only when TLC plates were loaded with large amounts of radioactivity. Spot **i** may have been eluted with radioactive inorganic phosphate during HPLC separation.

Four MMC-DNA adducts that have been reported in previous publications (1–8) were identified in our study. Intrastrand cross-linked adduct was not detected in the present system. The effect of pH on the formation of these DNA adducts by enzyme-activated MMC and PFM can be envisioned in two ways. First, pH affects the enzymatic activity. Earlier reports showed that the kinetics of xanthine oxidase and NADPH-cytochrome P450 reductase in reducing MMC were different, and the optimal pH of both enzymes was in the higher pH range (3, 8). Two other reductive enzymes, DT-diaphorase reported by Siegel *et al.* (14) and xanthine dehydrogenase reported by Gustafson and Pritsos (15), were shown to have an optimal pH for reducing MMC in the lower range. As a result, pH controls the rate of MMC activation and thus the kinetics of DNA alkylation. MMC- and PFM-modified DNA prepared in this study were extensively alkylated. Therefore, the rates of alkylation catalyzed by NADPH-cytochrome P450 reductase and xanthine oxidase were not reflected by the current data. Second, our data demonstrated that pH affects the quantitative distribution of the monofunctional monoadducts and the bifunctional adducts. Tomasz *et al.* (10) proposed a mechanistic scheme for DNA adduct formation by MMC under different reductive conditions. They suggested that monofunctional monoadducts (1″α- and 1″βN^2-G-MMC) are the direct result of monofunctional activation and that the bifunctional monoadduct (N^2-G-DMC) and cross-linked adducts are co-products of bifunctional activation of MMC. According to this scheme, the higher yield of N^2-G-DMC observed in pH 6.0 MMC-DNA (Table 1) may be an indication that acidic pH is more favorable for the bifunctional activation of MMC. Bifunctional activation of MMC should also favor the formation of cross-linked adducts, yet our data did not show any differences in cross-linked adducts in alkylated DNA at pH 6.0 and 7.6. One reason for failing to demonstrate a difference was probably the result of the extensive alkylation of the DNAs at both pH values. Because of the extensive alkylation of both DNA samples, probably all sites for cross-linking were alkylated. Therefore, we were unable to detect any difference. Direct evidence to link any individual MMC monoadduct to its significance for cytotoxicity has never been reported. However, our two-dimensional TLC mapping and identification of DNA adducts of MMC and PFM provide the foundation for investigating the relationships of DNA adduct formation and reductive enzymes in tumor cells.

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References

1. Tomasz, M., R. Lipman, J. K. Snyder, and K. Nakanishi. Full structure of a mitomycin C dinucleoside phosphate adduct: use of differential FT-IR spectroscopy in microscale structural studies. *J. Am. Chem. Soc.* **105**:2059–2063 (1983).
2. Tomasz, M., R. Lipman, G. L. Verdine, and K. Nakanishi. Reassignment of the guanine-binding mode of reduced mitomycin C. *Biochemistry* **25**:4337–4344 (1986).
3. Pan, S., T. Iracki, and N. R. Bachur. DNA alkylation by enzyme-activated mitomycin C. *Mol. Pharmacol.* **29**:622–628 (1986).
4. Tomasz, M., D. Chowdary, R. Lipman, S. Shimotakahara, D. Veiro, V. Walker, and G. L. Verdine. Reaction of DNA with chemically or enzymatically activated mitomycin C: isolation and structure of the major covalent adduct. *Proc. Natl. Acad. Sci. USA* **83**:6702–6706 (1986).
5. Tomasz, M., R. Lipman, D. Chowdary, J. Pawlak, G. L. Verdine, and K. Nakanishi. Isolation and structure of a covalent cross-link adduct between mitomycin C and DNA. *Science (Washington D. C.)* **235**:1204–1208 (1987).
6. Tomasz, M., R. Lipman, B. F. McGuinness, and K. Nakanishi. Isolation and characterization of a major adduct between mitomycin C and DNA. *J. Am. Chem. Soc.* **110**:5892–5896 (1988).
7. Bizanek, R., B. F. McGuinness, K. Nakanishi, and M. Tomasz. Isolation and structure of an intrastrand cross-link adduct of mitomycin C and DNA. *Biochemistry* **31**:3084–3091 (1992).
8. Pan, S., and T. Iracki. Metabolites and DNA adduct formation from flavoenzyme-activated porfiromycin. *Mol. Pharmacol.* **34**:223–228 (1988).
9. Tomasz, M., C. S. Hughes, D. Chowdary, S. R. Keyes, R. Lipman, A. C. Sartorelli, and S. Rockwell. Isolation, identification, and assay of [^{3}H]-porfiromycin adducts of EMT6 mouse mammary tumor cell DNA: effects of hypoxia and dicumrol on adduct patterns. *Cancer Commun.* **3**:213–223 (1991).
10. Tomasz, M., A. K. Chawla, and R. Lipman. Mechanism of monofunctional and bifunctional alkylation of DNA by mitomycin C. *Biochemistry* **27**:3182–3187 (1988).
11. Tomasz, M., and R. Lipman. Reductive metabolism and alkylating activity of mitomycin C induced by rat liver microsomes. *Biochemistry* **20**:5056–5061 (1981).
12. Andrews, P. A., S. Pan, and N. R. Bachur. Electrochemical reductive activation of mitomycin C. *J. Am. Chem. Soc.* **108**:4158–4166 (1986).

13. Pan, S., P. A. Andrew, C. J. Glover, and N. R. Bachur. Reductive activation of mitomycin C and mitomycin C metabolites catalyzed by NADPH cytochrome P450 reductase and xanthine oxidase. *J. Biol. Chem.* **259**:959–966 (1984).
14. Siegel, D., N. W. Gibson, P. C. Preusch, and D. Ross. pH-dependent metabolism of mitomycins by DT-diaphorase. *Proc. Am. Assoc. Cancer Res.* **32**:396 (1991).
15. Gustafson, D. L., and C. A. Pritsos. Bioactivation of mitomycin C by xanthine dehydrogenase from EMT6 mouse mammary carcinoma tumors. *J. Natl. Cancer Inst.* **84**:1180–1185 (1992).
16. Teng, S. P., S. A. Woodson, and D. M. Crothers. DNA sequence specificity of mitomycin cross-linking. *Biochemistry* **28**:3901–3907 (1989).
17. Kennedy, K. A., J. D. McGurl, L. Leondaridis, and O. Alabaster. pH dependence of mitomycin-induced cross-linking activity in EMT6 tumor cells. *Cancer Res.* **45**:3541–3547 (1985).
18. Pan, S. Porfiromycin disposition in oxygen-modulated P388 cells. *Cancer Chemother. Pharmacol.* **27**:187–193 (1990).
19. Randerath, K., E. Randerath, T. F. Danna, K. L. van Golen, and K. L. Putman. A new sensitive ^{32}P-postlabeling assay based on the specific enzymatic conversion of bulky DNA lesions to radiolabeled dinucleotides and nucleoside 5′-monophosphates. *Carcinogenesis (Lond.)* **10**:1231–1293 (1989).
20. Randerath, K., M. V. Reddy, and R. C. Gupta. ^{32}P-labeling test for DNA damage. *Proc. Natl. Acad. Sci. USA* **78**:6126–6129 (1981).
21. Reddy, M. V., R. C. Gupta, and K. Randerath. ^{32}P-base analysis of DNA. *Anal. Biochem.* **117**:271–279 (1981).
22. Gupta, R. C., M. V. Ruddy, and K. Randerath. ^{32}P-postlabeling analysis of nonradioactive aromatic carcinogen-DNA adducts. *Carcinogenesis (Lond.)* **3**:1081–1092 (1982).
23. Reddy, M. V., and K. Randerath. ^{32}P-analysis of DNA adducts in somatic and reproductive tissues of rats treated with the anticancer antibiotic, mitomycin C. *Mutat. Res.* **179**:75–88 (1987).
24. Kato, S., K. Yamashita, T. Kim, T. Tajiri, M. Onda, and S. Sato. Modification of DNA by mitomycin C in cancer patients detected by ^{32}P-postlabeling analysis. *Mutat. Res.* **202**:85–91 (1988).
25. Nelson, C. A., and P. Handler. Preparation of bovine xanthine oxidase and the subunit structures of some iron flavoproteins. *J. Biol. Chem.* **234**:5368–5373 (1968).
26. Yasukochi, Y., and B. S. S. Masters. Some properties of a detergent-solubilized NADPH-cytochrome c (cytochrome P-450) reductase purified by biospecific affinity chromatography. *J. Biol. Chem.* **251**:5337–5344 (1976).
27. Musser, S. M., S. Pan, and P. Callery. Liquid chromatography-thermospray mass spectrometry of DNA adducts formed with mitomycin C, porfiromycin and thioTEPA. *J. Chromatogr.* **474**:197–207 (1989).

Send reprint requests to: Su-shu Pan, Division of Developmental Therapeutics, University of Maryland Cancer Center, 655 W. Baltimore St., Baltimore, MD 21201.