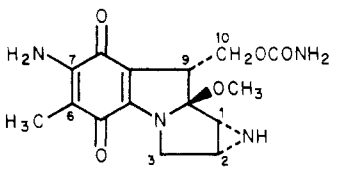
Reductive Metabolism and Alkylating Activity of Mitomycin C Induced by Rat Liver Microsomes†

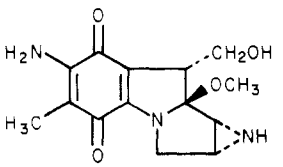
Maria Tomasz* and Roselyn Lipman

ABSTRACT: Mitomycin C, an antitumor antibiotic, is rapidly metabolized in the presence of rat liver microsomes. NADPH and anaerobic conditions are required for the process. The products isolated after reexposure to air are 2,7-diaminomitosene derivatives. Specifically, in the presence of inorganic phosphate, 1,2-*cis*- and -*trans*-2,7-diaminomitosene 1-phosphates, 1,2-*cis*- and -*trans*-2,7-diamino-1-hydroxymitosenes, and 2,7-diaminomitosene are formed. The last substance is a new mitomycin C derivative, and proof for its structure is presented. Mytomycin C has been previously postulated to be an alkylating agent requiring reduction for activity [Iyer, V. N., & Szybalski, W. (1964) *Science (Washington, D.C.) 145*, 55]. The 1-phosphates above represent the first chemically characterized bioreductive alkylation products of the drug. 5′-Uridylic acid is alkylated analogously under these conditions, to give *cis*- and *trans*-2,7-diaminomitosene 1-(5′-uridylate), while the phosphodiester UpU and uridine itself are inert. Hydrogen gas/PtO_2 gives the same results as microsomes/NADPH. The formation of the observed compounds indicates that enzymatic (or chemical) reduction of the quinone system of mitomycin C induces ring opening of the aziridine function, generating a reactive center at the C_1 position as previously postulated by others (ibid.). The second alkylating center, also postulated, is not evident, however, under the conditions tested, indicating that the aziridine is the primary bioreductive alkylation function of mitomycin C. Identification of the products and mechanism of the microsomal anaerobic metabolism of mitomycin C are significant in view of the reported toxicity of the drug to anaerobic cancer cells.

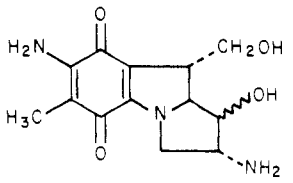
Mitomycin C (MC, I),[1] the potent antibiotic and clinically useful antitumor agent, binds covalently to cellular DNA



I (MC)



II



III

(Szybalski & Iyer, 1964) and induces covalent cross-links between the complementary strands of the nucleic acid (Iyer & Szybalski, 1963; Matsumoto & Lark, 1963). These direct damages to DNA are believed to be essential for the cytotoxicity of the drug (Iyer & Szybalski, 1963; Small et al., 1976; Mercado & Tomasz, 1972; Weiss et al., 1968). While this type of activity is similar to that of synthetic antitumor alkylating agents, MC is unique in that metabolic activation is required for its interactions with DNA (Iyer & Szybalski, 1964). The nature of the activation differs from the widely studied liver microsomal oxidative activation of various procarcinogens to DNA alkylating agents (Miller & Miller, 1976) by being a *reductive* process. MC, therefore, has been termed the prototype of "bioreductive alkylating agents" (Lin et al., 1976) and it is presently the only known natural product member as well as the best antitumor agent of this class.

The binding and cross-linking action of MC on DNA can be readily observed in vitro when certain reducing agents are added for activation of MC in situ (Iyer & Szybalski, 1964). The chemical structural details of the MC–nucleic acid complex are not known yet. The active form(s) of MC itself is also unknown due to its extreme instability, as indicated by reports that chemical reduction of MC followed by exposure to air generates complex mixtures of autoxidized quinones (Patrick et al., 1964; Hornemann et al., 1976).

We investigated the enzymatic reduction of MC in a rat liver microsomal system and succeeded in isolating and identifying all major metabolites and alkylation products of the drug formed under these conditions. The results represent characterization of bioreductive metabolites and bioreductive alkylation products of MC for the first time.

Experimental Procedures

Materials. The sources of mitomycin C, bacterial alkaline phosphatase, snake venom diesterase, 5′-UMP, UpU, and uridine were given previously (Tomasz & Lipman, 1979). NADPH was purchased from Boehringer. Rat liver microsomes (male Sprague-Dawley rats, 200–250 g weight, not fasted) were prepared by the method of Cederbaum et al. (1976) and were a kind gift from Dr. Arthur Cederbaum, Mt. Sinai School of Medicine, New York. The pelleted preparation was suspended in 0.125 M KCl and stored at −80 °C. Protein content (11.2 mg/mL) was determined by the Lowry method. MC reducing activity noticeably decreased after several weeks of storage.

Decarbamoyl mitomycin C (II) was synthesized by a published procedure (Kinoshita et al., 1971) and purified by

† From the Department of Chemistry, Hunter College, The City University of New York, New York, New York 10021. *Received February 3, 1981.* This work was supported by grants from the National Science Foundation (PCM 7922891) and the U.S. Public Health Service (CA 28681) and a CUNY Faculty Research Award. Presented at the 180th National Meeting of the American Chemical Society, Las Vegas, NV, Aug 1980, Abstract MEDI 59. NMR facilities at The Rockefeller University are supported by National Science Foundation Grant PCM 77-07671.

[1] Abbreviation used: MC, mitomycin C.

0006-2960/81/0420-5056$01.25/0

Table I: Analytical Properties of New MC Derivatives

compd	elution vol (mL, at peak)[a]	R_f	electrophoretic mobility (cm)[b]	λ_{max} (nm) ($\epsilon \times 10^{-3}$)[c]
III	600	0.74[d,f]	−7.2 (pH 3.5)	313, 249 (pH 11);
			−1.6 (pH 7.2)	311, 248, 205 (pH 7); 309, 249 (pH 1.7)
VIII	1030	0.85[d,f]	−5.0 (pH 3.5)	317, 243 (pH 11);
		0.13[d,g]	−2.8 (pH 7.2)	314 (10.3), 243 (15.7) (pH 7);
		0.55[e,h]		312, 242 (pH 1.7)

[a] On Sephadex G-25 column chromatography; see Figure 1 for conditions. [b] Paper electrophoresis (Whatman 3 MM), 22 V/cm, 2 h; pH 3.5 buffer is 0.02 M sodium citrate, pH 7.2 buffer is 0.02 M sodium phosphate. [c] Determined in 0.1 M sodium phosphate buffers (pH 11 and 7) and in 0.1 M H_3PO_4 (pH 1.7). [d] TLC plates (silica, Macherey-Nagel). [e] TLC plates (cellulose, Macherey-Nagel). [f] Isopropyl alcohol–1% NH_4OH, 2:1 (v/v). [g] Acetonitrile–1-butanol–acetone, 2:1:1 (v/v). [h] 1-Butanol–acetic acid–H_2O, 4:1:5 (v/v), upper layer.

preparative TLC [silica gel; $CHCl_3$–acetone, 1:1 (v/v)].

Methods. Proton NMR spectra in the FT mode were obtained by using a 220-MHz Varian instrument. Field desorption mass spectroscopy was kindly performed at the National Science Foundation Regional Mass Spectroscopy Facility at The Johns Hopkins University, Baltimore, by Dr. Robert J. Cotter, Facility Manager. Infrared spectra were obtained on KBr micropellets (200-μg sample) by Kendall Infrared Laboratories, Plainfield, NJ. Cary 219 and Gilford 250 instruments were used for ultraviolet spectra and absorbance determinations, respectively.

Activation of MC in a Microsomal System. In two separate vessels 0.5 mL of the microsomal preparation at 0 °C and a mixture of 13.3 μmol of MC and 13.8 μmol of NADPH in 10.0 mL of 0.2 M sodium phosphate buffer, pH 7.1, at 37 °C were deaerated by bubbling helium gas (chromatographic grade) for 15 min; then 50 μL of the microsomal preparation (0.56 mg of protein) was transferred to the MC solution. Helium bubbling continued for 15 min. A color change from blue (MC) to deep purple was noticeable. The mixture was exposed to air, filtered rapidly, then applied immediately to a Sephadex G-25 column, and eluted with 0.02 M NH_4HCO_3.

Activation of MC by catalytic hydrogenation was accomplished by first deaerating a solution with helium containing 13.3 μmol of MC and 1.6 mg of PtO_2 in 10.0 mL of 0.34 M sodium phosphate, pH 7.05, followed by bubbling H_2 gas for 3 min and then again helium for 5 min, at room temperature. The same color change occurred as above. The mixture was exposed to air and filtered and the filtrate chromatographed as above.

Activation of MC by $Na_2S_2O_4$. MC (6.5 μmol) in 5.0 mL of 0.34 M sodium phosphate, pH 7.05, was deaerated as above; then 44 μL of 0.29 M $Na_2S_2O_4$ (12.6 μmol) (freshly prepared under anaerobic conditions) was added at room temperature. After 10-min incubation, the mixture was exposed to air (5-min bubbling) and chromatographed as above.

Quantitation of MC and its products was based on UV spectrophotometry in aqueous media as described previously (Tomasz & Lipman, 1979). Spectral properties of additional compounds (III, VIII) are given in Table I.

Alkaline phosphatase and snake venom diesterase treatments of phosphate derivatives of MC as well as separation and identification of the products of enzyme treatments were described previously (Tomasz & Lipman, 1979).

Synthesis of III.[2] This new MC derivative was obtained by hydrolysis of II in 0.05 N HCl for 20 min at 25 °C, in complete analogy to the conversion of I to a mixture of VI and VII (Stevens et al., 1964; Taylor & Remers, 1974), and purified by Sephadex G-25 chromatography. The single peak obtained from the column behaves as a homogeneous compound in all systems tested (Table I). Its structure as III is proven by its 7-aminomitosene[3] spectra at three pH values (Table I) and its conversion to the 7-OH analogue of III, a known compound (Webb et al., 1962; Stevens et al., 1964), by treatment with 0.2 N HCl for 7 h. These conditions hydrolyze the 7-NH_2 to the 7-OH group in mitosenes but are too mild to hydrolyze the 10-carbamate if present (same references). The stereochemistry of III at C_1 remains undefined by these experiments.

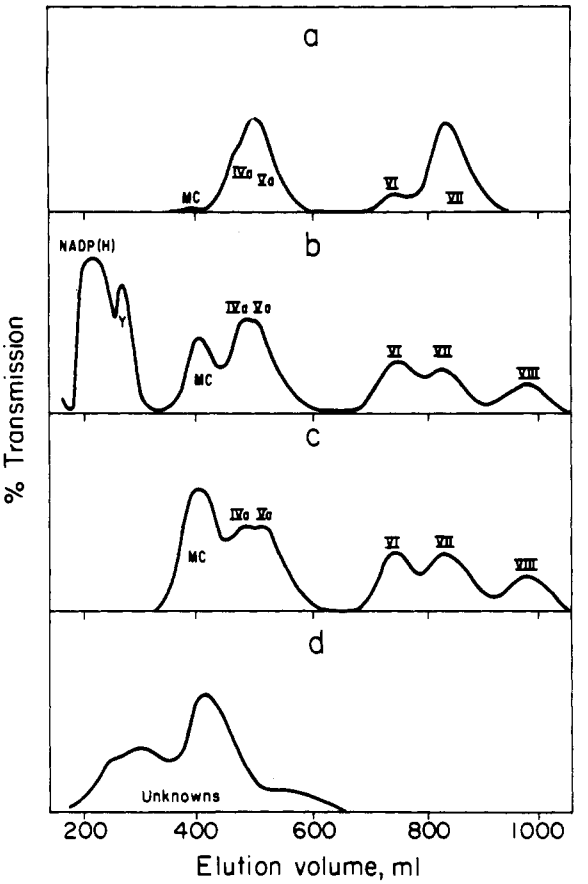


FIGURE 1: Separation patterns of products of MC activated under various conditions in the presence of inorganic phosphate (see Methods). Sephadex G-25 (fine) column chromatography (2.5 × 56 cm, 0.02 M NH_4HCO_3 eluant, void volume 131 mL, inclusion volume 220 mL) was used. (a) Products of incubation of MC in 0.34 M sodium phosphate, pH 3.5, 2 h, room temperature (Tomasz & Lipman, 1979). (b) Products of microsomal activation. (c) Products of catalytic hydrogenation. (d) Products of $Na_2S_2O_4$ activation.

[2] This part of the work was skillfully carried out by Lydia Koppel.

[3] 7-Aminomitosenes are exemplified by IV–VIII, while a 7-aminomitosane is exemplified by I. The UV spectra of these two classes are highly distinguishable (Webb et al., 1962). 7-Hydroxy analogues can also be distinguished from the above by their UV spectra (Webb et al., 1962; Stevens et al., 1964).

Results

Activation of MC by Microsomes: Reduction and Alkylation Products. The chromatography pattern (Figure 1b) indicates the following products: microsomal protein peak at 131 mL (not shown); NADP + NADPH mixture at 205 mL (same as that of an authentic mixture); a purple peak Y, 303 mL, showing a composite UV spectrum of NADP, NADPH, and 7-aminomitosene.[3] The peaks marked MC, IVa, Va, VI, and VII were identified by comparison of various properties with those of the authentic compounds described previously (Tomasz & Lipman, 1979). The last peak, VIII, is a previously unknown derivative of MC. Proof of its structure as VIII will be described below. The relative quantities of the various fractions are the following (in mol %): MC, 9; IVa, 25; Va, 21; VI, 17; VII, 16, VIII, 11.

Control Experiments. When either NADPH or microsomes were omitted or when air instead of helium was bubbled through the complete microsomal incubation system, all conversion products of MC were completely absent from the column pattern. Incubation of MC with NADPH alone overnight gave similar negative results.

Catalytic Hydrogenation: Reduction and Alkylation Products of MC. The chromatographic pattern (Figure 1c) yielded the same products as that of the microsomal reduction except that the protein and NADP(H) peaks were absent. The mol % distribution was the following: MC, 42; IVa, 15; Va, 15; VI, 9.7; VII, 10.2; VIII, 8. Overall recovery of material based on initial amount of MC was usually 80–90%. The balance of the material may be accounted for by a precipitate filtered out together with the PtO_2 before chromatography. This precipitate was dissolved in dimethyl sulfoxide, diluted carefully with column buffer, filtered from PtO_2, and chromatographed as above, yielding VI, VII, and VIII in about the same ratios as above.

Alkylation products of 5′-UMP (IVb and Vb) were obtained by both microsomal and H_2/PtO_2 activation of MC by the methods above, except that 0.02 M 5′-UMP, pH 7.4, was substituted for the sodium phosphate. The alkylated UMPs (IVb, Vb) coeluted with MC from the usual column. Their presence was verified in the mixed fractions by paper electrophoresis and TLC by use of direct comparison with authentic standards obtained previously by other means (Tomasz & Lipman, 1979). In addition, snake venom diesterase treatment of the mixed column fractions from the microsomal experiment followed by chromatography on the Sephadex G-25 column yielded 5′-UMP (0.9 μmol), unchanged MC, VI (0.35 μmol), and VII (0.45 μmol). These data indicate that in the original mixture of the trans and cis isomers IVb and Vb, their molar ratio was approximately 0.8:1. Their combined yield was 6%, based on original MC.

UpU or uridine was not alkylated under conditions analogous to those above (i.e., UpU or uridine substituted for 5′-UMP).

Activation of MC by $Na_2S_2O_4$ yielded a column pattern (Figure 1d) strikingly different from the previous ones. No trace of IVa, Va, VI, VII, or VIII was evident. The overlapping peaks were pooled into four separate fractions; each was rechromatographed in order to obtain a reference elution pattern and then treated with alkaline phosphatase. Chromatography indicated no change in the elution patterns in any of the four fractions as a result of the phosphatase treatment. These results suggested that no phosphate alkylation products of MC were formed in the original reaction. This was corroborated by a control reduction of MC by $Na_2S_2O_4$ in the absence of sodium phosphate which gave the same characteristic pattern of products as that in the presence of phosphate (i.e., Figure 1d).

Table II: 220-MHz ^{1}H NMR Data[a] for VIII and N^2-Acetyl-VIII

VIII		N^2-acetyl-VIII
in Me_2SO-d_6[b]	in 50% acetone-d_6–D_2O	in Me_2SO-d_6[b]
6.47 (br s,[c] 4, C_7NH_2, $CONH_2$)	5.25 (s, 2, $C_{10}H_2$)	8.44 (d, 1, J = 6.6 Hz, C_2NHOAc)
5.00 (s, 2, $C_{10}H_2$)	4.44[d] (dd, 1, C_3H_α)	6.51 (br s,[c] 4, C_7NH_2, $CONH_2$)
4.23 (dd, 1, J = 12.9, 6.5 Hz; C_3H_α)	4.28 (ragged s, 1, C_2H)	5.01 (s, 2, $C_{10}H_2$)
4.02 (ragged s, 1, C_2H)	3.92 (dd, 1, C_3H_β)	4.79 (m, 1, C_2H)
3.76–3.69[d] (dd, C_3H_β)	3.25 (dd, J = 16.6, 6.9 Hz, C_1H_α)	4.34 (dd, 1, J = 13.0, 7.5 Hz, C_3H_α)
3.03 (dd, 1, J = 16.6, 6.9 Hz, C_1H_α)	2.70 (dd, 1, J = 16.6, 5.4 Hz, C_1H_β)	4.00 (dd, 1, J = 13.0, 4.1 Hz, C_3H_β)
2.5–2.44[e] (m, C_1H_β)	1.81 (s, 3, C_7CH_3)	3.13 (dd, 1, J = 13.7, 7.5 Hz, C_1H_α)
1.73 (s, 3, C_7CH_3)		2.70[f] (dd, C_1H_β), 1.81 (s, 3, $NCOCH_3$), 1.73 (s, 3, C_7CH_3)

[a] In δ; Me_4Si is used as internal standard. [b] Protons exchangeable in D_2O are not listed, unless noted otherwise. [c] Exchangeable in D_2O. [d] Distorted by HDO side band. [e] Distorted by Me_2SO band. [f] Distorted by Me_2SO side band.

Proof of Structure of VIII. Properties of VIII are listed in Table I. The UV data indicate the 7-aminomitosene chromophore.[3] The IR spectrum shows a strong band at 1715 cm^{-1}, known to specify the C_{10} carbamate group in mitomycin derivatives [e.g., Stevens et al. (1964)]. Field desorption mass spectra show only two peaks, m/e 304 (100%) and 305 (30%), assigned M^+ and MH^+ ions, respectively (calculated M = 304). Electrophoresis indicates it is a base (Table I), moving about as fast as VI (faster than VII) at pH 3.5 (Tomasz & Lipman, 1979). The NMR spectra (Table II) were analyzed in comparison to the known NMR spectra of VI and VII (Taylor & Remers, 1975; also determined by ourselves for direct comparison). The main supporting features of the NMR for structure VIII in Me_2SO-d_6 are the following: (a) The C_{10} protons form a sharp singlet rather than an AB quartet as in VI and VII, J = 12.0 and 12.7 Hz, respectively [M. Tomasz, unpublished experiments; see also Hornemann et al. (1979)]. This indicates that the asymmetry of C_1, responsible for the AB quartet type splitting in VI and VII, is not present in VIII. (b) The C_1 protons shifted far upfield, from δ 4.60 in VI and 4.67 in VII to δ 3.03 and 2.5, as two double doublets,[4] consistent with the $C(OH)H \rightarrow CH_2$ change in VIII. As further proof, VIII was acetylated (4 mg of VIII, 0.3 mL of acetic anhydride, 0.8 mL of anhydrous pyridine, ice or room temperature, 10 min to 17 h), giving a single compound, R_f 0.73 in systems d and g of Table I, which crystallized from water. The NMR spectrum (Table II) shows one acetyl group (δ 1.81) and a low-field doublet (δ 8.44), observed in N^2-acetylmitosenes (Taylor & Remers, 1975). These and all other features of the spectra are fully consistent with N^2-acetyl-VIII as the sturucture. This fact, in turn, further confirms the structure of VIII.

Chemical Stability of VIII. Treatment with 0.01 N HCl at room temperature for 2 h converted ca. 50% of VIII to a

[4] NMR spectra in acetone-d_6/D_2O allowed observation of C_1H_β free from the overlap with the solvent peak observed in Me_2SO-d_6; see Table II.

Scheme I

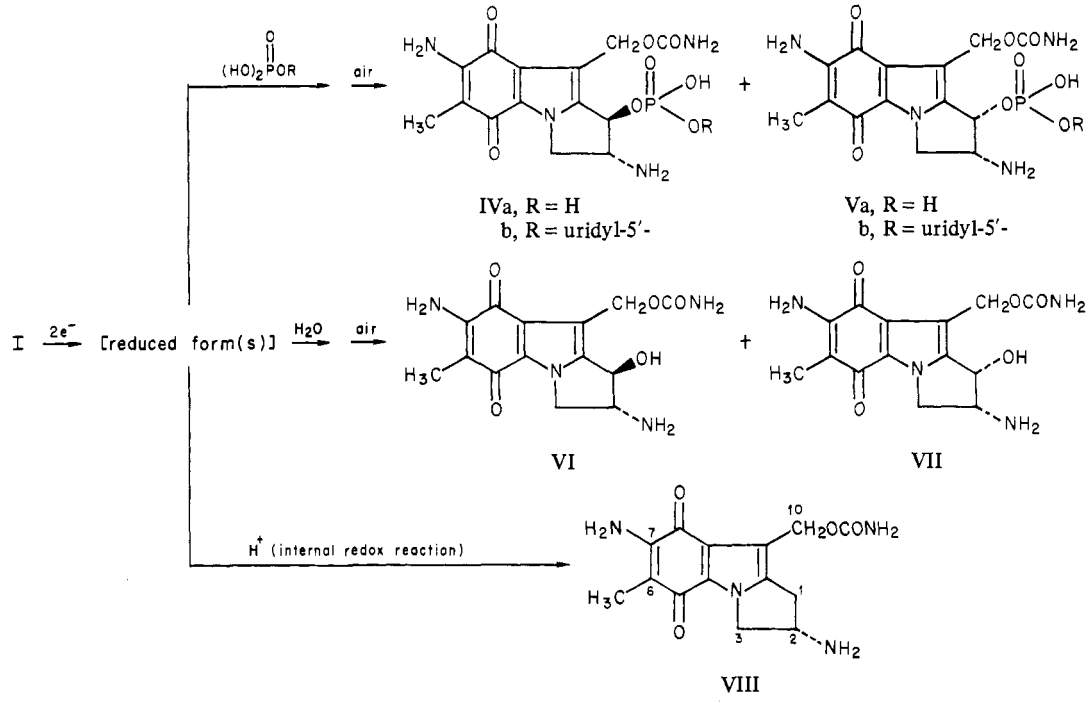


Table III: Yield of VIII at Various pHs[a]

pH	yield (%)[b]
5.1	52
7.0	15
9.0	0

[a] MC was activated by catalytic hydrogenation in the presence of 0.34 M sodium phosphate as under Methods. The pH of the phosphate buffer was varied as indicated. [b] Based on the total amount of MC converted.

Table IV: Extent of Alkylation of Various Substrates by MC[a]

substrate	product	yield (%)[b]	1,2-cis/1,2-trans isomer ratio
P_i	IVa + Va	20–47	~1
5'-UMP	IVb + Vb	14	~1
UpU	none		
uridine	none		

[a] MC was activated by catalytic hydrogenation in the presence of either 0.34 M sodium phosphate (see Methods), 0.02 M 5'-UMP, 0.02 M UpU, or 0.02 M uridine, respectively. [b] Based on the total amount of MC converted.

7-hydroxymitosene, as evidenced by the indicator and UV spectral properties of the product[3] and by a lower elution volume from a Sephadex G-25 column; 0.2 N HCl (4 h) and 2 N HCl (2 h, 60 °C) effected partial and complete conversion, respectively, of this 7-hydroxymitosene to a new 7-hydroxymitosene having yet lower elution volume. These conversions are interpreted as acid hydrolysis of the 7-amino group followed by hydrolysis of the C_{10} carbamate under stronger acid conditions, in complete analogy to these steps known in the 2,7-diamino-1-hydroxymitosene series (Stevens et al., 1964), although VIII requires somewhat lower concentrations of HCl for these reactions.

Occasionally, partial conversion of VIII to its 10-decarbamoyl derivative was observed upon prolonged handling or storage in neutral solution at 5 °C. Assignment of structure of the conversion product was based on FD mass analysis [*m/e* 261 (M^+, 100%), 262 (MH^+, 12%)], lack of the 1715-cm^{-1} band (carbamate carbonyl) in the IR spectrum, and the presence of the 7-aminomitosene UV spectral characteristics.[3]

pH Dependence of Formation of VIII. The usual experiment of reduction of MC by H_2/PtO_2 (Methods) was modified by varying the pH of the phosphate buffer as 5.0, 7.0, and 9.0. A striking pH dependence of product distribution was observed, as seen in Table III, indicating that VIII is maximally produced at acid pH.

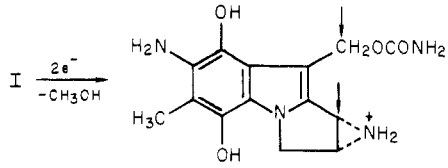
Discussion

Identification of the components of the complex reduction mixture of MC was greatly aided by our previous study of activation of MC by low pH (3–5) alone to alkylating agent (Tomasz & Lipman, 1979), which provided a series of rigorously characterized MC-alkylated phosphate derivatives, as well as the discovery that simple Sephadex G-25 gel filtration chromatography separates MC derivatives with extraordinary selectivity. For comparison, Figure 1 includes a representative product pattern of alkylation of P_i by low-pH-activated MC (Figure 1a).

The course of reductive activation of MC by microsomes is summarized by Scheme I, showing formation of the five major products (IV–VIII) isolated after exposure of the anaerobic reduction mixture to air (Figure 1b). Four of these are identical with the low-pH-activated products (Figure 1a), namely the 1,2-trans and -cis pairs of the mitosene 1-phosphates (IVa, Va) and of the hydrolysis products of MC (VI and VII). When P_i was replaced by 5'-UMP as the added nucleophile, the analogous phosphate alkylation products IVb and Vb were formed readily with no change otherwise in product pattern. In contrast, the phosphate group of UpU is not alkylated by MC (Table IV). Resistance of the phosphodiester group to alkylation was also observed in the case of low-pH-activated MC, and its rationale was discussed (Tomasz & Lipman, 1979).

VIII is a new 7-aminomitosene derivative, quite unusual in having no substituent in position 1. Its structure proof is discussed under Results. In addition to the identified products of Scheme I, NADP(H) itself also appears to be alkylated by MC, judging from the UV spectrum of the unknown peak Y (Figure 1b). This alkylation may occur on the monophosphate group of NADP(H), in analogy to alkylation of the phosphate

Scheme II



IX

of 5′-UMP above, although other possible positions are certainly not excluded.

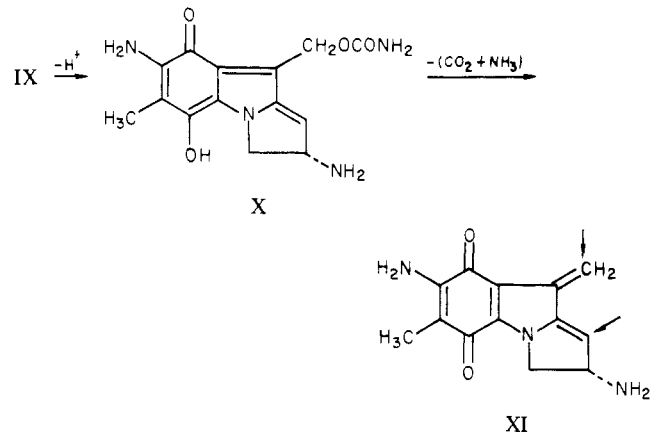
It was of considerable interest to us to compare the product pattern of microsomal activation of MC to that obtained upon chemical reduction. Finding a simpler system which gives the same products without the protein, NADP(H), and alkylated NADP(H) components present in the already complex product mixture of MC would facilitate studies of the alkylating properties of MC using more complex substrates. Brief catalytic hydrogenation (3 min, a trace of PtO_2) was found to be such a system (Figure 1c), reproducing the microsomal reactions with unexpected fidelity, both qualitatively and in relative distribution. Sodium dithionite ($Na_2S_2O_4$) reduction of MC, however, yielded an entirely different set of products (Figure 1d). The significance of this last finding will be discussed later.

Rat liver homogenate (Schwartz et al., 1963), bacterial lysates (Iyer & Szybalski, 1964), mouse liver microsomes and liver nuclear fractions (Kennedy & Sartorelli, 1979), and sonicated mouse tumor cell preparations (Kennedy et al., 1980a,b) have been shown to metabolize MC, as indicated by the decrease of absorbance of MC at its characteristics 367-nm UV maximum in the incubation mixtures. Kennedy & Sartorelli (1979) also noted that a certain color test for alkylating agents was positive in their microsomal system when MC was added. All of these systems required NADPH and anaerobic conditions for the above phenomena. Our results are consistent with these findings: in the absence of added NADPH or in the presence of air, all of the MC is recovered unchanged.

The microsomal reduction dependent formation of 1-phosphate derivatives of MC represents the first direct chemical evidence that MC is a bioreductive alkylating agent. In addition, these products, together with the other three identified metabolites, VI, VII and VIII, provide the first experimental insight into the activation mechanism of MC. A remarkable hypothetical mechanism was proposed by Iyer & Szybalski in 1964, and less directly by Patrick et al. (1964). The essential feature of the hypothesis (Scheme II) is that the biologically active form of MC is the hydroquinone IX, formed by reduction of the inert quinone I, followed by spontaneous loss of methanol. IX then functions as a bifunctional alkylating agent; the arrows indicate the postulated sites of attack by nucleophiles, resulting in displacement of the C_1 aziridine and C_{10} carbamate leaving groups. Formation of IV–VII indicates that in the reduced state the aziridine ring is indeed activated and opens, to give alkylation and hydrolytic products, with concomitant loss of methanol from positions 5 and 6, as postulated. The fact that enzymatic (microsomal) activation and catalytic hydrogenation yield identical products suggests that only the first step, i.e., reduction of the quinone to the hydroquinone form, is enzymatic (Scheme I). The hydroquinone forms of the products are probably reoxidized to quinone during isolation and/or by intermolecular electron transfers between various quinones, semiquinones, and hydroquinones present during the reaction.

In a theoretical paper, Moore (1977) proposed further details of the basic Iyer–Szybalski mechanism, namely that the

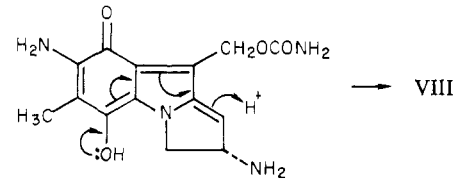
Scheme III



X

XI

Scheme IV



X

C_1 aziridine and C_{10} carbamate are eliminated spontaneously from IX, giving rise to intermediates X and XI, consecutively. Attack by nucleophiles then follows at C_1 and C_{10} of the quinone methide XI (Scheme III). The net result is the same as that of the direct attacks on IX. Our findings provide good evidence that the "first half" of this mechanism is correct: formation of VIII is best explained by protonation of the presumed intermediate X of Moore (Scheme IV). The striking increase of the yield of VIII with lowering of pH (Table III) supports this mechanism. At pH 5, VIII is almost the sole product of the reductive metabolism, while none is formed at pH 9. It is possible that intramolecular proton transfer is involved from the protonated 2-amino group of X to the 1 position of the double bond. (Note that the IX → X → VIII conversion represents an internal redox reaction.) In summary, VIII can be regarded as a "trapped form" of X, providing strong experimental support for X as an actual intermediate. Whether the 1-substituted mitosenes IV–VII are also formed via X by addition of the nucleophile to the double bond of X at the 1 position (Moore mechanism) or directly from IX is not indicated by our results. The ratio of 1,2-cis and -trans isomers (approximately 1:1) does not give an obvious clue to this question. The fact, however, that X is present during the reaction makes the Moore mechanism quite likely.

Our experiments prove that MC is activated to an alkylating agent by enzymatic reduction. The reactive site is the C_1 aziridine position, as previously postulated. The second postulated alkylating function, namely the C_{10} carbamate position, is inert in our systems. A consistent result was briefly reported recently by Hashimoto et al. (1980). They isolated the guanine analogue of Vb as the only identifiable product upon reaction of 5′-GMP with MC under H_2/palladium activating conditions. It should be remembered that the existence of *two* alkylating functions of MC was originally postulated (Iyer & Szybalski, 1964) simply because DNA was found cross-linked after exposure to MC, implying two points of attachments of the drug. The monofunctional binding mode greatly predominated over the extent of cross-linking (Szybalski & Iyer, 1964; Weissbach & Lisio, 1965). The postulate made no

prediction as to which of the two functions (C_1 or C_{10}) should be more reactive. It is clear from the present work that the aziridine (C_1) of activated MC is far more reactive than any second potential alkylating function, and consequently, it is most likely to be responsible for the observed monofunctional binding to DNA. We looked hard for evidence for any reaction at C_{10}. In order to provide an authentic reference for the predicted product of reductive hydrolysis at both C_1 and C_{10} (III; bifunctional activation product), III, a new compound, was synthesized. However, III was not detectable in any reduction mixture of MC or in alkaline phosphatase digests of phosphate alkylation products isolated from such mixtures. Attempts to activate the C_{10} carbamate by using more drastic conditions (10-fold increase of NADPH or more prolonged hydrogenation (30 min) resulted in a diffuse pattern of products with UV spectra unknown in the MC and mitosene series,[3] and no phosphate seemed to be attached to any of them (unpublished results). This indicated that even if the C_{10} carbamate was lost at this stage, the products were not the 7-aminomitosene type compounds as predicted by the hypothesis. These results contrast with those of a chemical model system recently reported by Hornemann et al. (1979). When $Na_2S_2O_4$ as reducing agent in the presence of potassium ethyl xanthate ($EtOC(S)S^-K^+$) as nucleophile is used, the 1,10-bis(ethylxanthyl)-7-aminomitosene was obtained as the main product (40% yield). This $Na_2S_2O_4$–induced model alkylation system of MC, however, may not be directly comparable to the bioreductive system used in the present work since the former is known to be complicated by side reactions: HSO_3^-, the oxidation product of $Na_2S_2O_4$, itself reacts rapidly with reduced MC, forming one known and three unknown bisulfite adducts (Hornemann et al., 1976). Thus, it is possible that some of these adducts mediate the course of reactions of MC with ethyl xanthate. When we substituted $Na_2S_2O_4$ for microsomes/NADPH or H_2 gas in our MC alkylation system, no phoshate alkylation, hydrolysis products, or VIII were observable (Figure 1d; Results). It appears that bisulfite competes for reduced MC with P_i, water, or the protonation reaction so effectively that only the bisulfite adducts are formed. In summary, the question of a second alkylating function of MC and, therefore, the nature of the cross-links in DNA remains open. In order to enhance the low reactivity (if any) of such a function under bioreductive conditions, "DNA-like" model nucleophiles should be used as probes in which the second, weaker alkylation step is facilitated by fixed proximities as in double-stranded DNA.

Elucidation of the anaerobic metabolism of MC receives added importance in view of findings of Sartorelli and his group (Kennedy et al., 1980a,b) that at low doses MC is selectively toxic to *hypoxic* tumor cells in vitro.[5] It is most likely that the anaerobic metabolites and/or their reactive precursors identified in the present work play a direct role in this cytotoxicity.

Acknowledgments

We thank David Tucker for skillful assistance in parts of this work.

[5] This selectivity disappears at higher doses of MC. The killing of well-oxygenated cells at higher levels of drug is suggested by the above authors to be due to the repeated one-electron reduction/autoxidation cycles of MC, generating hydrogen peroxide (Tomasz, 1976) and its precursor, the superoxide radical (Bachur et al., 1978; Lown et al., 1976; Handa & Sato, 1975).

References

Bachur, N. R., Gordon, S. L., & Gee, M. V. (1978) *Cancer Res. 38*, 1745.
Cederbaum, A. I., Becker, F. F., & Rubin, E. (1976) *J. Biol. Chem. 251*, 5366.
Handa, K., & Sato, S. (1976) *Gann 67*, 523.
Hashimoto, Y., Shudo, K., & Okamoto, T. (1980) *Chem. Pharm. Bull. 28*, 1961.
Hornemann, U., Ho, Y. K., Mackey, J. K., & Srivastava, S. C. (1976) *J. Am. Chem. Soc. 98*, 7069.
Hornemann, U., Keller, P. J., & Kozlowski, J. F. (1979) *J. Am. Chem. Soc. 101*, 7121.
Iyer, V. N., & Szybalski, W. (1963) *Proc. Natl. Acad. Sci. U.S.A. 50*, 355.
Iyer, V. N., & Szybalski, W. (1964) *Science (Washington, D.C.) 145*, 55.
Kennedy, K. A., & Sartorelli, A. C. (1979) *Fed. Proc. Fed. Am. Soc. Exp. Biol. 38*, 443.
Kennedy, K. A., Rockwell, S., & Sartorelli, A. C. (1980a) *Cancer Res. 40*, 2356.
Kennedy, K. A., Teicher, B. A., Rockwell, S., & Sartorelli, A. C. (1980b) *Biochem. Pharmacol. 29*, 1.
Kinoshita, S., Uzu, K., Nakano, K., & Takahashi, T. (1971) *J. Med. Chem. 14*, 109.
Lin, A. J., Cosby, L. A., & Sartorelli, A. C. (1976) *ACS Symp. Ser. No. 30*, 71.
Lown, J. W., Begleiter, A., Johnson, D., & Morgan, A. R. (1976) *Can. J. Biochem. 54*, 110.
Matsumoto, I., & Lark, K. G. (1963) *Exp. Cell Res. 32*, 192.
Mercado, C. M., & Tomasz, M. (1972) *Antimicrob. Agents Chemother. 1*, 73.
Miller, J. A., & Miller, E. C. (1979) in *Chemical Carcinogens* (Searle, C. E., Ed.) ACS Monogr. No. 173, p 737, American Chemical Society, Washington, D.C.
Moore, H. W. (1977) *Science (Washington, D.C.) 197*, 527.
Patrick, J. B., Williams, R. P., Meyer, W. E., Fulmor, W., Cosulich, D. B., Broschard, R. W., & Webb, J. S. (1964) *J. Am. Chem. Soc. 86*, 1889.
Schwartz, H. S., Sodergren, J. E., & Philips, F. S. (1963) *Science (Washington, D.C.) 142*, 1181.
Small, G., Setlow, J. I., Kooistra, J., & Shapanka, R. (1976) *J. Bacteriol. 125*, 643.
Stevens, C. L., Taylor, K. G., Mink, M. E., Marshall, W. S., Noll, K., Shah, G. D., & Uzu, K. (1964) *J. Med. Chem. 8*, 1.
Szybalski, W., & Iyer, V. N. (1964) *Microb. Genet. Bull. 21*, 16.
Taylor, W. G., & Remers, W. A. (1974) *Tetrahedron Lett.* 3483.
Taylor, W. G., & Remers, W. A. (1975) *J. Med. Chem. 18*, 307.
Tomasz, M. (1976) *Chem. Biol. Interact. 13*, 89.
Tomasz, M., & Lipman, R. (1979) *J. Am. Chem. Soc. 101*, 6063.
Webb, J. S., Cosulich, D. B., Mowat, J. H., Patrick, J. B., Broschard, R. W., Meyer, W. E., Williams, R. P., Wolf, C. F., Fulmor, W., Pidacks, C., & Lancaster, J. E. (1962) *J. Am. Chem. Soc. 84*, 3185.
Weiss, M. J., Redin, G. S., Allen, G. K., Jr., Dornbush, A. C., Lindsay, H. S., Poletto, J. F., Remers, W. A., Roth, R. H., & Sloboda, A. E. (1968) *J. Med. Chem. 11*, 742.
Weissbach, A., & Lisio, A. (1965) *Biochemistry 4*, 196.