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City University of New York, 1989

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ISOLATION AND IDENTIFICATION OF THE ADDUCTS OF MITOMYCIN C AND PORFIROMYCIN WITH DNA FORMED *IN VITRO* AND *IN VIVO*.

BY

DONDAPATI RENU CHOWDARY

A dissertation submitted to the Graduate Faculty in Biochemistry in partial fulfillment of the requirements for the degree of Doctor of Philosophy,

The City University of New York.

1989

This manuscript has been read and accepted for the Graduate Faculty in Biochemistry in satisfaction of the dissertation requirement for the degree of Doctor of Philosophy

5/19/89
Date

Chair of Examining Committee

5/16/89
Date

Executive Officer

Supervisory Committee

The City University of New York

ABSTRACT

ISOLATION AND IDENTIFICATION OF ADDUCTS OF MITOMYCIN C AND PORFIROMYCIN WITH DNA FORMED *IN VITRO* AND *IN VIVO*.

by

Dondapati Renu Chowdary

Adviser: Professor Maria Tomasz

The antitumor antibiotics, mitomycin C (MC) and porfiromycin (PM), are shown to form covalent complexes with DNA *in vitro*, under reductive activation conditions (both chemical and enzymatic). Three major covalent adducts have been isolated and identified as (i) N^2-guanine adduct with MC (structure **4a**), (ii) N^2-guanine adduct with 10-decarbamoyl mitomycin [(10-DMC); structure **16a**], and a bis-adduct of MC linked to two Gs at their N^2-positions (structure **6**). The adducts of PM with DNA formed *in vitro* are analogous (structures **19**, **20**, & **21**). Formation of adducts **6** and **16a** in CHO mammalian cells has been shown after exposing them to MC or 10-DMC, whereas formation of crosslink **6** *in vivo* has been demonstrated after injecting rats with MC. The experiments done in tissue cultures with [1a-^{3}H]-porfiromycin show [^{3}H]-label in the unmodified A, G, and T thus suggesting the demethylation of PM to MC in cells. The methyl group containing [^{3}H] label was incorporated into nucleosides *via de novo* purine and thymidylate biosynthesis.

A consolidated enzymatic scheme for the hydrolysis of MC-modified DNA has been established and the resistance of such DNA to cleavage by several nucleases has been shown. Thus, only DNase I/SVD/alkaline phosphatase or nuclease P_1/SVD/alkaline phosphatase combinations can degrade MC-modified DNA into nucleosides.

A modified version of ^{32}P-postlabeling has been developed with *in vitro* authentic standards and this can be conveniently used in the future to detect MC-modified lesions obtained *in vivo*. By utilizing the alkaline ethidium bromide fluorescence assay, the crosslinking effect of MC, PM, and 10-DMC has been shown to occur in cells.

ACKNOWLEDGEMENTS

With great pleasure, I wish to express my sincere thanks to Professor Maria Tomasz for being so kind, patient, and co-operative research adviser during the entire course of this study. Her continuous support and encouragement will always be remembered. I wish to extend my thanks to all my laboratory colleagues: Roselyn Lipman for introducing me to various techniques and for being a great friend; Dr. Anil K. Chawla (presently at the University of Massachusetts Medical Center, Worcester, Massachusetts), for being a wonderful friend and co-worker; and others.

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LIST OF ABBREVIATIONS

AEB	alkaline ethidium bromide assay
ATP	adenosine triphosphate
b.r.	binding ratio; mole of bound drug per mole of mononucleotide unit
CD	circular dichroism
CHO	Chinese hamster ovary
cpm	counts per minute
DHFR	dihydrofolate reductase
10-DMC	10-decarbamoyl mitomycin C (for structure, see page 11)
DNA	deoxyribonucleic acid
DNase I	deoxyribonuclease I
ε	molar extinction co-efficient (M^{-1} cm^{-1})
EGTA	ethylene glycol bis(β-aminoethyl ether) N,N,N',N'-tetraacetate
HAT	hypoxantine, aminopterin, and thymidine
HMW	high molecular weight
H_2/Pd-C	hydrogen/palladium-charcoal
HPLC	high performance liquid chromatography
H_2/PtO_2	hydrogen/platinum oxide
IR	infrared
LSC	liquid scintillation counter/counting
MC	mitomycin C (for structure, see page 2)
NADH	nicotinamide adenine dinucleotide (reduced)
NADPH	nicotinamide adenine dinucleotide phosphate (reduced)
NMR	nuclear magnetic resonance
$Na_2S_2O_4$	sodium dithionite

PBS	phosphate-buffered saline
PM	porfiromycin (for structure, see page 3)
RNA	ribonucleic acid
RNase A	ribonuclease A
SDS	sodium dodecyl sulfate
SVD	snake venom phosphodiesterase
THF	tetrahydrofolate
ts	temperature sensitive
UV	ultraviolet

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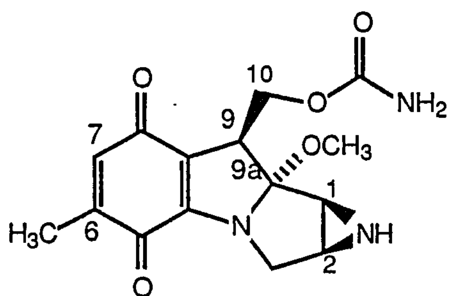
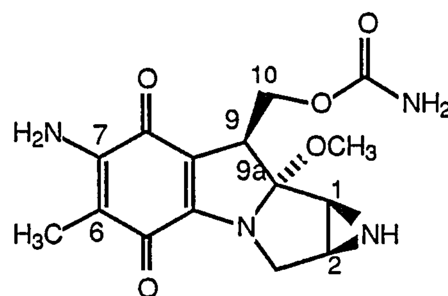
INTRODUCTION

Successful chemotherapy is a crucial event in the treatment of any disease. In case of neoplasia, curative therapy requires that all tumor cells capable of indefinite replication be eradicated by a careful administration of appropriate chemical agents. Chemical agents, both naturally occuring and synthetic, that are employed clinically in the treatment of cancer can be divided into six classes: (1) alkylating agents (nitrogen and sulfur mustards, chlorambucil, cyclophosphamide, melphalan etc.), (2) antimetabolites (methotrexate, 6-mercaptopurine, 5-fluorouracil etc.), (3) antibiotics (actinomycin D, bleomycin, doxorubicin, mitomycin C etc.), (4) specific mitotic inhibitors (vincristine, vinblastine), (5) steroidal hormones (prednisone, predisolone), and (6) other drugs (platinum coordination complexes). Of these, alkylating agents and antibiotics act primarily on DNA. The antitumor antibiotics can interact with DNA either by intercalation, groove binding or by covalent binding. Although there are several thousand intercalating compounds, only a small number have been studied for their mechanism of interaction with DNA. The same is true with the covalent modifying drugs. The use of these agents is very limited due to their toxic effects on several tissues. Despite the extensive search for more effective agents, only a few have been known to possess antitumor property with less cytotoxicity than the parent drug. However, the molecular mechanism of their action is not well understood. In order to arrive at more effective chemotherapeutic agents, it is necessary to understand the nature and/or mode of action of such drugs.

The mitomycins and porfiromycin produced by several strains of Streptomyces, were discovered in the 1950s and have been shown to be bactericidal and cytotoxic.[1] The

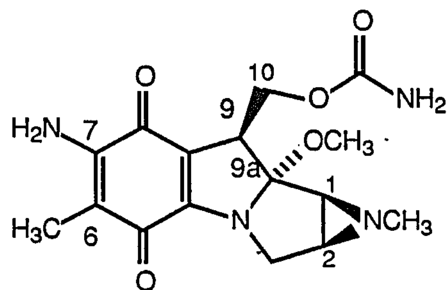
central structure, mitosane **1** is common to all of these drugs. Bactericidal and cytotoxic properties as well as the inhibitory properties of mitomycins on cell growth are the direct manifestations of their action on several cell components.[1] One of the members of this family, mitomycin C (MC, **2**), has been widely used in clinical chemotherapy of breast, cranial, and gastrointestinal solid tumor carcinomas.[2] The known ability of MC to alkylate DNA *in vitro* and *in vivo* is manifested by (i) the reversible melting behavior of MC-exposed DNA, attributed to formation of covalent crosslinks between the





complementary strands[1] and (ii) by covalent association of the ultraviolet chromophore of MC with DNA.[3] The crosslinks were suggested to be the direct cause of irreversible cidal effects[4] whereas the monofunctional DNA damage by the antibiotic (that is repairable) is thought to be related to the reversible growth inhibition and filament formation.[1] The cytotoxicity of MC is most likely a direct result of DNA alkylation as indicated by the parallels in biologial activity of MC with a number of known "DNA damaging agents": selective inhibition of DNA replication,[1] strong induction of the SOS response[5] and sister chromatid exchange,[6] and cross-resistance or cross-hypersensitivity of bacterial[1,7] and mammalian cells[8] to UV light and MC. The alkylation process requires low pH[9] or reduction[1] to unmask MC into a transiently active form, which is

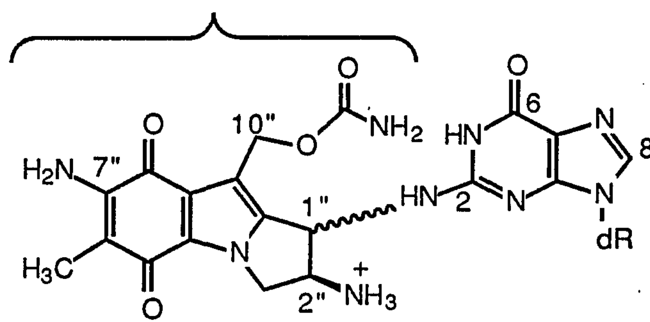
thought to occur in cells and can be mimicked easily *in vitro* chemically or enzymatically.[1] This property of MC and porfiromycin (PM, 3), requiring reductive activation prior to alkylation, led Sartorelli to coin the word for these drugs as prototype "bioreductive alkylating agents."[10]



3

In spite of the potent antitumor properties and clinical significance of MC, its DNA-alkylation products have remained unknown for over 20 years. From their original observations, Szybalski and Iyer[1] postulated a mechanism for the reactivity of MC: the C-1 aziridine and C-10 carbamate groups are the two masked alkylating functions involved in the crosslinking of two complementary strands of DNA. Several laboratories have initiated efforts in understanding the molecular nature of action of MC on DNA. Despite their intensive efforts, it had remained elusive mainly for the following reasons: (i) difficulty in isolating the low molecular weight adducts; (ii) resistance to degradation of MC-modified DNA to nucleases; and (iii) loss of bound drug moiety by chemical cleavage methods (alkali or acid).[3,11-13] On the other hand, with the exception of some cytotoxicity data, studies *in vivo* have especially been hindered until recently (see ref. 12, 14, & 15) due to the unavailability of radiolabeled MC.

More recently, a deoxyguanosine-MC adduct arising from the model reaction of reductively activated MC (microsomes/NADPH or H_2/PtO_2) with dinucleoside phosphate d(GpC) was isolated in our laboratory and the structure was elucidated in collaboration with Nakanishi's laboratory.[16] The structure was rigorously established as N^2-(2"β,7"-diaminomitosen-1" α-yl), 2'-deoxyguanosine **4** (The term mitosene refers to the bracketed structure in **4** without substituents at the 1", 2", and 7" positions; structure **5**).



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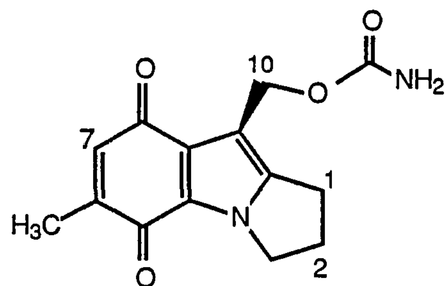
a : 1" = α-

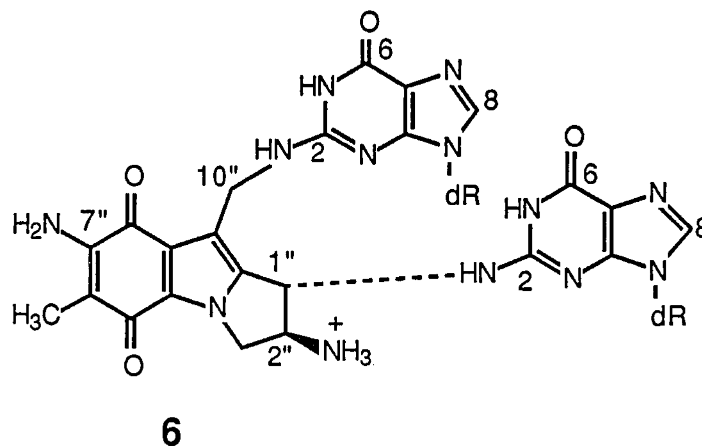
b : 1" = β-

With this model compound as a reference, the reaction products of chemically reduced MC with DNA have been isolated and characterized.[17,18,19] Thus, the C-1 position

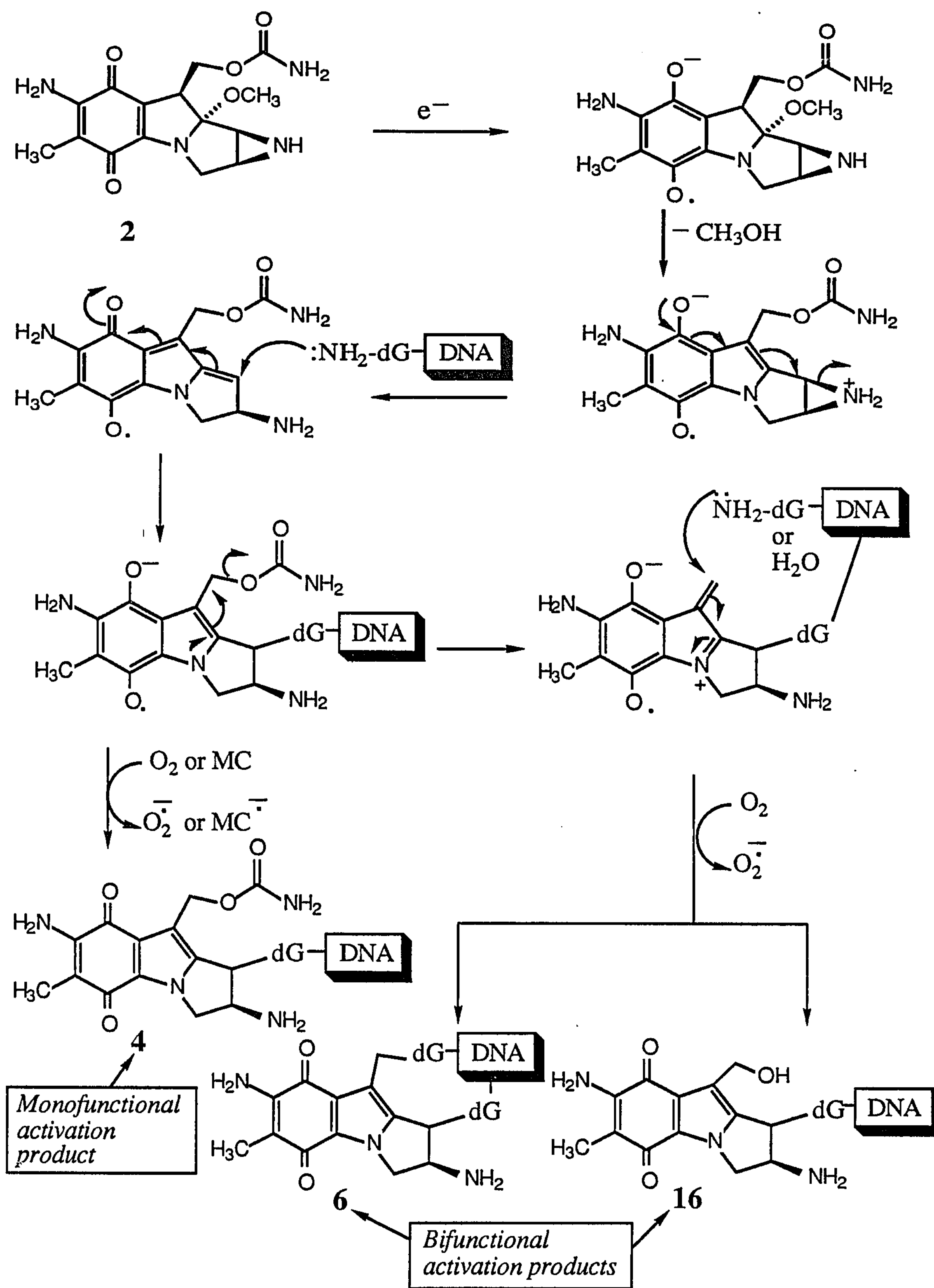


5

of MC had been confirmed as the reactive site involved in binding to DNA. The report also showed that three adducts of MC, obtained under identical conditions from DNA were incorrectly identified by another group.[12] By utilizing a different chemical reducing agent, $Na_2S_2O_4$, with high redox potential, the Tomasz-Nakanishi team also reported the isolation of a bis-adduct of MC with DNA **6**.[14] The bis-adduct was shown to contain two guanines linked at N^2- position to a mitosene moiety at its C-1 and C-10 positions as shown in **6**. Thus, for the first time, a long sought for candidate, the MC-crosslink **6**, had been isolated and fully characterized.



Although the MC-modified bases have been identified, the mechanism of reductive activation of MC and the fate of the reaction intermediates were unknown until very recently. An "auto-catalytic pathway" has been put forward by Peterson and Fisher[20] which was later confirmed in our laboratory using DNA itself as the nucleophile.[21] Several groups have identified and characterized the various reduction intermediates of this complex process,[13,20-30] named the "activation cascade" by Danishefsky and his collaborators.[25,28] This mechanism is summarized in *Scheme I*, page 6.



Scheme I. Mechanism of activation of MC.

Despite the considerable amount of progress made on the nature of *in vitro* adducts of MC; the cause of cytotoxicity *in vivo* has remained only speculative. Interstrand crosslinks are thought to be the lethal lesions resulting from the reduced species of MC,[4] but the production of DNA-protein crosslinks, DNA monoadducts, alkylated proteins,[4] and cytotoxic hydroxyradicals has also been reported.[31] In one of the most interesting developments with respect to their chemotherapeutic utility, Sartorelli and co-workers[32] showed that MC and PM are more toxic to hypoxic tumor cells than to aerobic ones and that this process can be utilized to target hypoxic core tissues of solid tumors selectively. The increased toxicity of these drugs to cell cultures under hypoxia was correlated with increased alkylating potential.[33,34] The same research group has shown PM to be a better antitumor agent than MC based on the following observations: PM was shown to exhibit similar toxicity as MC to hypoxic cells, whereas it was much less toxic to aerobic cells than MC.[34,35] Very recently, Marshall and Rauth[36] have shown that PM is not as effective as MC at oxygen concentrations greater than 0.02%. This suggests that the ability of PM in eliminating the cancerous tissue insensitive towards radiation therapy is limited, although it exhibits less toxicity towards well-oxygenated cells. The radiation resistant tumors have an intermediate oxygen concentrations i.e. 0.02% - 1.0%. Taken this into consideration, MC might be a better antitumor agent than PM in combination chemotherapy. Recent studies in our laboratory with [^{3}H]-PM in tissue cultures have shown that PM is demethylated into MC.[18,37] (also see Part III of this thesis). The latter finding now raises the question as to whether the *in vivo* effect is due to PM or MC.

In spite of the early recognition of their intrinsic significance for the mode of action of MC and PM,[1] the *in vivo* DNA-alkylation products have long remained unknown. The formation of interstrand crosslinks, both *in vivo* and *in vitro*, has been demonstrated by

utilizing physical studies like melting behavior of MC-exposed DNA,[38] alkaline elution studies,[34,39] and alkaline sucrose gradient centrifugation.[40] Genetic studies also show that the rate of removal of interstrand crosslinks by mammalian cells is important to the sensitivity of cells to MC.[8, 41] Unavailability of radioactive MC made it difficult to explore the underlying nature of cytotoxicity. Recently, Hashimoto and co-workers[12] were able to detect adducts from liver DNA digests of rats injected with a high dose of MC (10 mg/rat) by their UV absorbance on HPLC. However, their structural assignments were found to be erroneous.[17,18] After injecting rats with a high dose of MC (30 mg/rat), we have isolated and identified the MC-crosslink **6**.[14,18] By using their sensitive ^{32}P-postlabeling assay, Reddy and Randerath[42] have detected MC-DNA adducts formed in various tissues of MC-injected rats, of which >90% were guanine-derived but they were not characterized further.

Detection of full adduct patterns of DNA exposed to MC in the cell which could be compared to those obtained and unambiguously assigned *in vitro* [14,17] has been lacking. Detecting such patterns is of particular interest in view of the finding *in vitro* that the conditions of the reductive activation of MC can be manipulated to yield two different types of reductive alkylation products, viz., the monofunctional adduct **4a** and the bifunctional adducts **6** and **16a**, depending on the activation environment (*Scheme I*, page 6).[14,21] Such environmental modulation of MC reactivity may be expected to occur also in cells and tissues, and this, in turn, could have a modulating effect on the cytotoxicity, since monofunctional alkylation of DNA is thought to be generally less destructive than the bifunctionally induced crosslinks. Since the DNA-adduct pattern reflects the nature of MC activation, the *in vivo* adduct patterns should be useful as a tool in relating the DNA lesions caused by MC and the cytotoxic effects.

The specific objectives of the present thesis were the following:

1: Investigation of the structures of the three MC-DNA adducts, proposed earlier by Hashimoto and co-workers.[12] This was warranted by results previously observed in our laboratory[16,43] which conflicted with theirs.

2: Isolation and characterization of MC-DNA formed *in vivo* and in the tissue cultures.

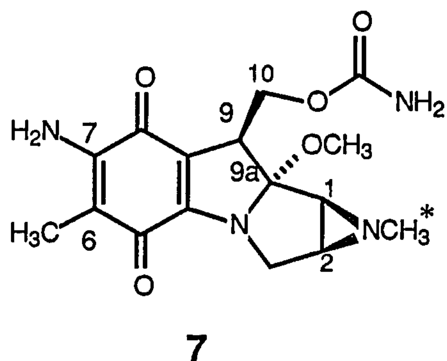
3: Development of a highly sensitive assay for detection and identification of MC-DNA adducts formed in cells.

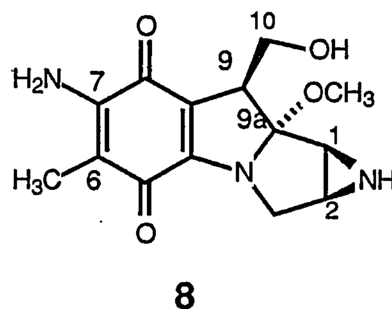
Accordingly, the results and discussion are organized into Parts I, II, and III, corresponding to these three objectives. One general "Materials and Methods" and one "Bibliography" section serve all three parts.

MATERIALS & METHODS

MATERIALS:

Materials and their sources were as follows: Calf thymus DNA (Type I, sonicated before use), potato apyrase, RNase A, bacterial alkaline phosphatase (Type III-R), and xanthine oxidase (grade III) were from Sigma; *Micrococcus luteus* DNA was from Miles laboratories, M13 single stranded DNA (strain BK8) was a gift of M.Z. Humayun of New Jersey Medical School, Newark; Poly(dG-dC), d(GpC), d(GpA), d(GpG), d(GpT), d(CpG), micrococcal nuclease and nuclease P_1 were from Pharmacia P-L Biochemicals; DNase I and snake venom phosphodiesterase, SVD, (Phosphodiesterase I) were from Cooper Biomedicals; proteinase K, spleen exonuclease (phosphodiesterase II; 0.002 unit/μg), and calf intestine alkaline phosphatase (Cat # 405 612, Lot # 10756422-14; the lyophilized powder has been dissolved in 30 mM triethanolamine/3 M NaCl/1 mM $MgCl_2$/0.1 mM $ZnCl_2$, pH 7.6) were from Boehringer Mannheim Biochemicals; [γ-^{32}P]-ATP and polynucleotide kinase were from New England Nuclear; Pic A reagent (tetrabutyl ammonium phosphate, HPLC grade) was from Millipore; liquid scintillation fluid, Ready flow III, was from Beckman Instruments; mitomycin C, porfiromycin and [1a-^{3}H]-porfiromycin **7** (19.7 mCi/mmol; >90% pure as checked by HPLC) were all gifts from Bristol-Myers Laboratories, and 10-decarbamoyl MC, 10-DMC, **8** was synthesized as described.[44] The hexamer, d(TACGTA), and its MC-crosslink were obtained from Roselyn Lipman of this laboratory. Each batch of alkaline phosphatase was checked for any contaminating 3'-phosphodiesterase activity prior to use.





METHODS:

Preparation of MC-DNA, PM-DNA or MC-Poly(dG-dC) complexes using chemical or enzymatic reducing agents for activation of the antibiotic.

(i) H_2/PtO_2 activation. DNA (0.67 μmole/ml), MC or PM (0.67 μmole/ml), and PtO_2 catalyst (100 μg per μmole of MC) were mixed in 15 mM Tris.HCl, pH 7.4 or 17 mM NaH_2PO_4, pH 7.4 and helium gas was bubbled for 10 min followed by hydrogen gas until the blue color of MC was changed to purple (7-10 min). The reaction mixture was exposed to air after bubbling helium for 5 minutes. Isolation of the complex and determination of binding ratio (b.r.) will be described later.

(ii) NADH-xanthine oxidase activation. DNA (1.0 μmole/ml), MC (1.0 μmole/ml), and NADH (2 μmoles/ml) in 20 mM NaH_2PO_4, pH 7.4 were incubated with xanthine oxidase (0.4 unit/ml) for 20 min at 37°C under a helium atmosphere.

(iii) NADPH-cytochrome *C* reductase activation. DNA (1.0 μmole/ml), MC (1.0 μmole/ml), and NADPH (2 μmoles/ml) in 20 mM NaH_2PO_4, pH 7.4 buffer were

incubated with cytochrome *C* reductase (0.4 unit/ml) for 20 min at 37°C under a helium atmosphere. The formation of complex was evident when the color changed from blue to purple.

(iv) Na$_2$S$_2$O$_4$ activation. A complex between CHO cell DNA and MC was made by using Na$_2$S$_2$O$_4$ as reducing agent according to the published procedure of Tomasz et al.[3]

(v) Reaction of MC with DNA according to Hashimoto et al.[12] A stock solution of MC (1 mg/ml, 50 ml total) was mixed with an aqueous solution of calf thymus DNA (2 mg/ml) and the solution mixture was stirred with 25 mg of 5% Pd on charcoal in presence of helium gas for 30 minutes. Later, hydrogen gas was bubbled through until the color changed from blue to reddish-purple. The resulting reaction mixture was filtered and the MC-modified DNA was either ethanol precipitated or isolated as described for other complexes.

(vi) Preparation of 10-DMC-CHO DNA complexes. Same as the above conditions except that 10-DMC was used instead of MC and the pH of the reaction mixture was 8.0.

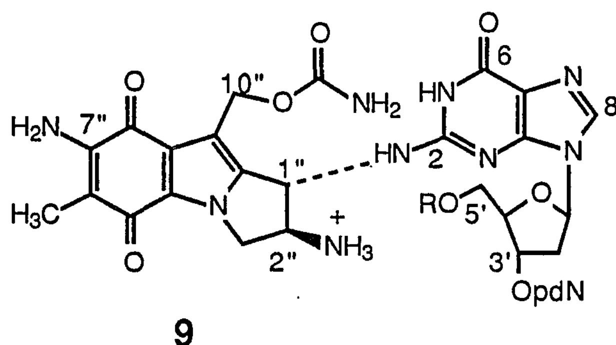
Isolation of MC-modified DNA and determination of binding ratio of the complexes.

All the above reaction mixtures were filtered through Whatman #1 filter paper and the complexes were separated from the other reaction products by Sephadex G-100 column chromatography (2.5 x 28 cm column for analytical purposes and 5.0 x 56 cm column for preparative scales), using 20 mM NH$_4$HCO$_3$ as eluent. Binding ratios (b.r.; mole of

bound drug/mole of mononucleotide unit) of the complexes were determined by the ultraviolet absorbance of mitosene at 310 nm using ε_{310} of 11,500 M^{-1} cm^{-1} and 11,200 M^{-1} cm^{-1} for denatured and native DNA complexes, respectively. A detailed method of determination of b.r. is described elsewhere.[3]

Synthesis of the MC-dinucleoside phosphate adducts 9 and 10.

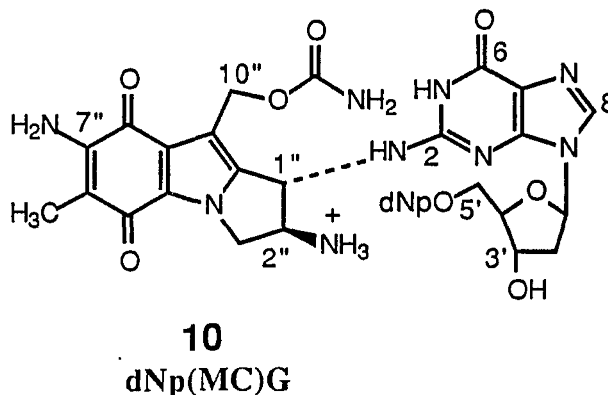
Reductively activated MC (H_2/PtO_2) was reacted with d(GpC), d(GpA), d(GpG), d(GpT), or d(CpG) as described earlier for the reaction of MC with d(GpC).[43] The



9

a : R = H; **dG(MC)pN**

b: R = p; **dpG(MC)pN**



10

dNp(MC)G

modified dinucleoside phosphates were purified to homogeneity by Sephadex G-25 chromatography[43] followed by HPLC [HPLC conditions (i)]. Each one was characterized by digestion with SVD to **4a** and d(pN) in a 1:1 molar ratio.

Digestion of MC-DNA, PM-DNA or 10-DMC-DNA complexes by various nuclease combinations.

(i) DNase I/SVD/alkaline phosphatase. Drug-DNA complex in 5 mM Tris.HCl, 1 mM $MgCl_2$, pH 7.0 (3.0 A_{260} units/ml), was digested at 37°C according to the following protocol: DNase I (16 units/A_{260} unit) at 0 h and 1 h; SVD (1.25 units/A_{260} unit; pH increased to 8.2) at 2 h and 5 h; alkaline phosphatase (0.5 unit/A_{260} unit) at 7 h, incubation continued until 24 hours.

(ii) Nuclease P_1. MC-DNA complex (3.0 A_{260} units/ml) in dilute acetic acid, pH 5.5 was incubated with nuclease P_1 (0.5 unit/A_{260} unit) at 55°C for 2 hours. In another experiment designed to duplicate the previously reported conditions,[12] 8 mg of MC-modified DNA per ml and 6 mg of nuclease P_1 per mg of DNA were used, corresponding to a 50-fold higher concentration of DNA and 16-fold higher concentration of nuclease P_1 than above.

(iii) Nuclease P_1 and alkaline phosphatase. The nuclease P_1 digest solutions were brought to 10 mM Tris.HCl, pH 8.2 by the addition of 0.01 vol of 1.0 M Tris.HCl, pH 8.2. Alkaline phosphatase (1.6 units/A_{260} unit) was added and the digestion was

allowed to proceed for 2 h at 37°C.

(iv) Micrococcal nuclease, spleen exonuclease and alkaline phosphatase. MC-DNA complex in 20 mM Na succinate/10 mM $CaCl_2$, pH 6.0 (3.0 A_{260} units/ml), was digested with micrococcal nuclease (9.0 units/A_{260} unit) and spleen exonuclease (0.45 unit/A_{260} unit; the enzyme was dialyzed against H_2O at 4°C for 15 h prior to use as described.[42]) at 37°C according to the method described by Reddy and Randerath.[42] The pH of the digest was brought to 8.2; alkaline phosphatase (1.0 unit/A_{260} unit) was added and incubation was continued overnight at 37°C.

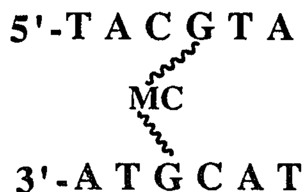
Digestion of MC-dinucleoside phosphate adducts by various nuclease systems and the hydrolytic stability of d[G(MC)pC] and dG(MC).

(i) The dinucleoside phosphate adducts, d[G(MC)pC] (**9a**, dN = dC) or d[C(MC)pG] (**10**; dN = dC) (0.5 - 3.0 units/ml), SVD (2.5 units/A_{260} unit), and alkaline phosphatase (1.3 units/A_{260} unit) were incubated in 10 mM Tris.HCl, pH 8.2 at 37°C for 2 hours.

(ii) Nuclease P_1 and alkaline phosphatase digestion was carried out as described above for MC-DNA complexes.

(iii) The hydrolytic stability of d[G(MC)pC] (**9a**, dN = dC) and monofunctional MC-adduct and dG(MC) **4a** was checked by boiling the samples in water at 100°C for 25 minutes.

Digestion of d[G(MC)pC] (9a, dN = dC), d[C(MC)pG] (10, dN = dC), or MC-hexamer crosslink 11 by spleen exonuclease and alkaline phosphatase.

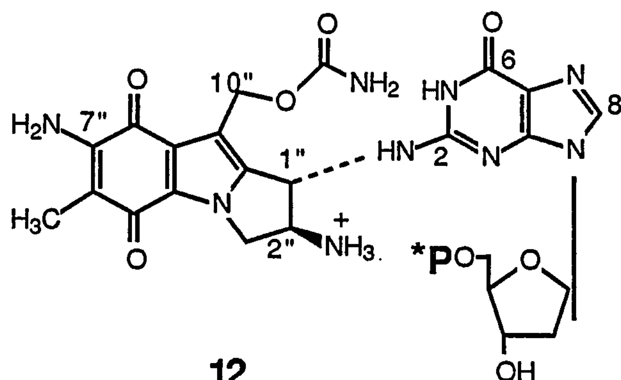


11

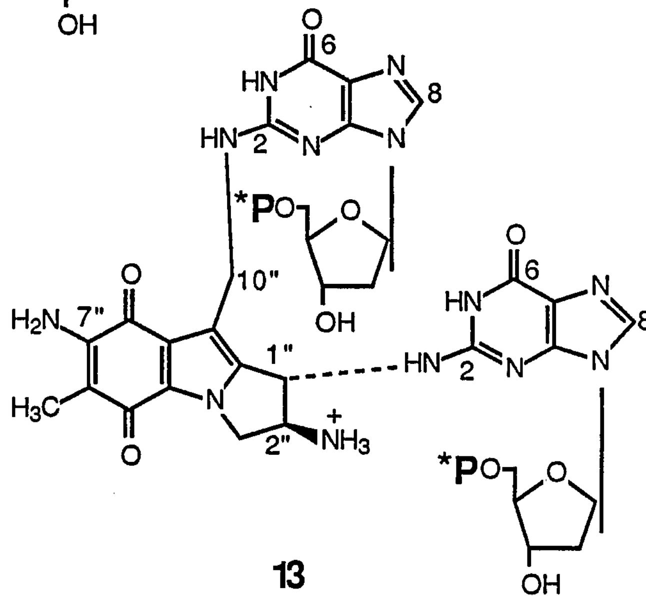
(i) The sample (1.0 - 3.0 A_{260} units/ml) in 15 mM Tris.HCl, pH 6.5 was incubated at 37°C with spleen exonuclease (0.2 - 20 units/A_{260} unit) for 2 hours. The pH of the solution was brought to 7.6 and alkaline phosphatase (1.0 unit/A_{260} unit) was added followed by incubation for 1 more hour at 37°C.

Modification of ^{32}P-postlabeling assay for MC-DNA complexes.

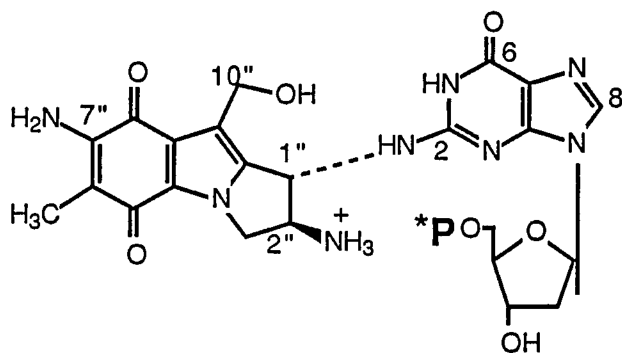
(i) Authentic standards of MC-modified 5'-nucleotides(*in vitro*). Non-labeled monofunctional authentic standard of MC-modified 5'-nucleotide, p[dG(MC)] **12**, was made from MC-DNA complex (H_2/PtO_2 activated) by digesting with nuclease P_1 (0.5 unit/A_{260} unit) and SVD, at 37°C, (0.5 unit/A_{260} unit) as described earlier for other complexes and purified by Sephadex G-25 chromatography and HPLC (not shown). For a crosslink standard, p_2[dG(MC)dG] **13**, an MC-hexamer crosslink ($Na_2S_2O_4$ activation) was digested with SVD (1.0 unit/A_{260} unit) and analyzed directly on



12
*P[dG(MC)]



13
*P_2[dG(MC)dG]



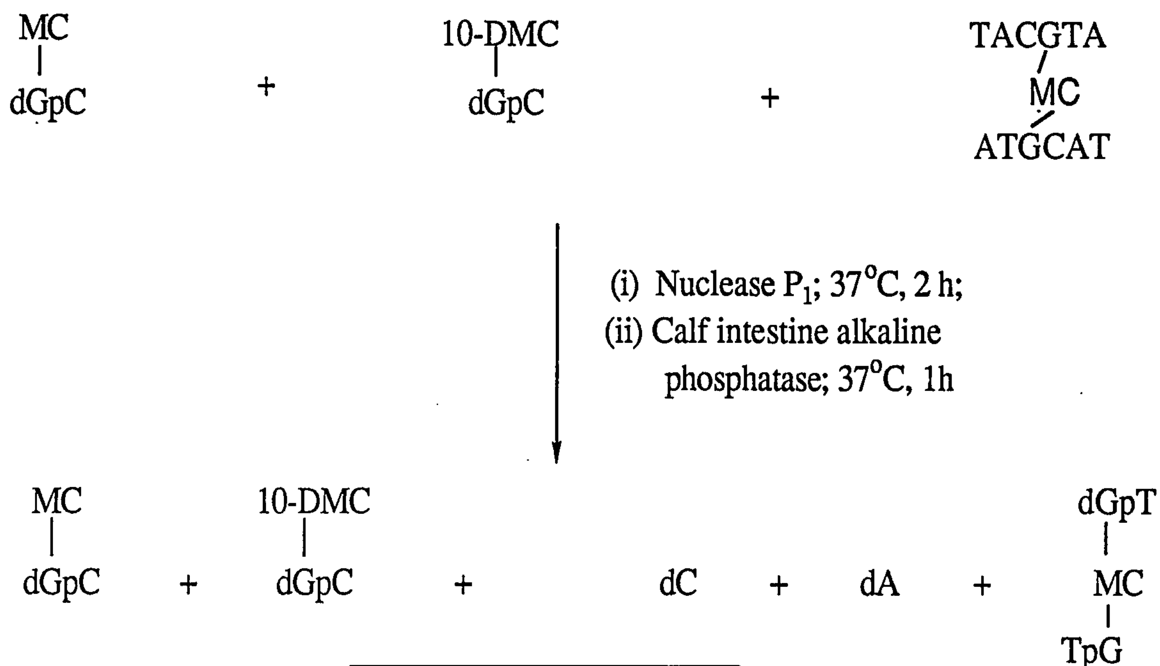
14
*P[dG(10-DMC)]

HPLC. The DMC-nucleotide adduct standard, p[dG(10-DMC)] **14**, was obtained by boiling p[dG(MC)] **12** in H_2O at 100°C for 30 minutes.

(ii) ^{32}P-postlabeling of MC-dinucleoside adduct **9a** (dN = dC) and MC-hexamer crosslink **11**. This method is outlined in *Scheme II*, page 19. d[G(MC)pC] **9a** (dN = dC), pre-boiled d[G(MC)pC], or an MC-hexamer crosslink, d[$(TACGTA)_2$MC] **11** was dissolved in dilute acetic acid, pH 5.5 (0.8 A_{310} unit/ml) and incubated at 37°C for 2 h with nuclease P_1(1.0 unit/ A_{260} unit). The pH of the digest was changed to 7.6 by adding 0.1 vol of 150 mM Tris.HCl, pH 7.6, calf intestine alkaline phosphatase (1.0 unit/A_{260} unit) was added and incubation was continued at 37°C for an extra hour. At the end of this step, the reaction mixture will contain the "1st step products" of *Scheme II* (page 19). The controls contained only the unmodified samples.

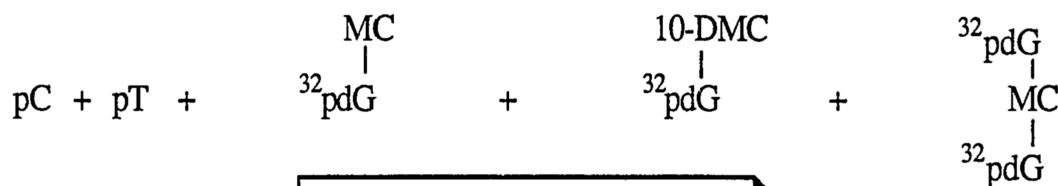
In order to inactivate calf intestine alkaline phosphatase, the above digestion mixture was brought to 5 mM EGTA [ethylene glycol bis(β-aminoethyl ether) N,N,N',N'-tetraacetate] with a 500 mM stock solution and incubated at 70°C for 1 h followed by immediate quenching on ice. The samples were centrifuged on a Microfuge for 3 min and the contents were gently transferred to new tubes.

For labeling experiment, 50 picomoles (based on the ultraviolet absorbance readings, using ε_{310} of 11,500 $M^{-1}cm^{-1}$) of the "1st step products" were taken and 1 μl each of 100 mM $MgCl_2$ and 100 mM 2-mercaptoethanol, 10 units of polynucleotide kinase, and [γ-^{32}P]ATP (1.0 Ci/mmol; 1.0 picomole/picomole of adduct) were added and incubated



1st STEP PRODUCTS

(i) Heat inactivate alkaline phosphatase ; 70°C, 1 h
(ii) Polynucleotide kinase, [γ-32P]ATP; 37°C, 3 h
(iii) SVD, 37°C, 1 h



FINAL STEP PRODUCTS

Scheme II. ^{32}P-postlabeling of the MC-modified dinucleoside phosphates and hexamer.

at 37°C for 3 hours. After the kinasing step, SVD (1.0 unit) was added and incubated at 37°C for 1 h followed by the addition of potato apyrase (0.1 unit) and further incubation for 1/2 more hour. The resultant mixture containing the "final products" (*Scheme II*, page 19) was analyzed by HPLC [HPLC conditions (iv)] after centrifuging for 3 min on a microfuge. The fractions were collected and counted as described below.

The substrate mixture for ^{32}P-postlabeling method described above, contained the following samples:

(a) contained 50 picomoles of "1st step products" resulting from d[G(MC)pC] (**9a**, dN = dC) digestion;

(b) contained 50 picomoles of "1st step products" resulting from pre-boiled d[G(MC)pC] (**9a**, dN = dC) digestion; and

(c) 50 picomoles of "1st step products" resulting from d[(TACGTA)$_2$MC] **11** digestion.

HPLC separations.

(i) Semi-preparative runs.

A reverse-phase column (Beckman Ultrasphere ODS; 1.0 x 25 cm), assembled with a Beckman model 110A pump, model 165 variable wavelength detector, 427 integrator and a 2.0 ml sample loop, was used; flow rate was 2.0 ml/minute; eluent was 8:92 CH_3CN/30 mM KH_2PO_4, pH 5.0. In order to separate the nucleosides with larger retention times, the same eluent was used but 4:96 CH_3CN/30 mM KH_2PO_4, pH 5.0. Samples of digests (usually 1.5 ml) were injected directly, after adjusting the pH to 6.0 -

7.0. The model 165 detector is capable of fast scanning ultraviolet absorbance spectra of eluates (20 nm/second; "on the flight" scanning).

(ii) Analytical runs. A reverse-phase column (Beckman Ultrasphere ODS; 0.46 x 25 cm) column was used with sample volumes of up to 100 µl and a flow rate of 0.5 ml/minute with 8:92 CH_3CN/30 mM KH_2PO_4, pH 5.0 or 1.0 ml/minute with 4:96 CH_3CN/30 mM KH_2PO_4, pH 5.0.

(iii) Separation of [^{3}H]-PM-DNA digests. This was same as in step (i) but the eluent was 4:96 CH_3CN/30 mM KH_2PO_4, pH 5.0 and the eluate was collected into scintillation vial fractions (0.5 minute/fraction; 1.0 ml) by using a fraction collector. To each of the collected fractions, 4 ml of the liquid scintillation fluid (Ready Flow III) was added and the counting was done by using Beckman LS 6800 scintillation counter.

(iv) Separation of MC-modified or ^{32}P-postlabeled adduct 5'-nucleotides. Separation of MC-modified 5'-nucleotides (labeled or non-labeled) was accomplished by using a Beckman Ultrasphere C18 reverse-phase column (1.0 x 25 cm). A gradient elution system was utilized, consisting of two eluents. Eluent A was 30 mM KH_2PO_4, pH 5.5 containing 5 mM Pic A reagent (tetrabutyl ammonium phosphate) and eluent B was an equal mixture of eluent A and CH_3CN. A linear gradient was started from initial 100% A to a final elution mixture of 50%A and 50%B over a period of 40 minutes. The flow rate was 2.0 ml/minute. The column was re-equilibrated with 100% eluent A prior to next injection. The collection of fractions and their counting was performed as described in (iii).

Cell lines.

A subline of Chinese hamster ovary (CHO) cells, CHO-K1; a temperature sensitive mutant of Balb/c-3T3 mouse cell line, *ts*20; and an African green monkey kidney cell line, CV_1 were all obtained from the laboratory of Harvey L. Ozer (New Jersey Medical School, Newark). $P388D_1$ murine leukemia cells were obtained from American Type Culture Collection (Rockville, Maryland). All cells were grown in monolayer cultures.

Cell cultures.

CHO cells. CHO cells were grown at 33°C in an equal mixture of Dulbecco's modified Eagle's medium and F-10 Ham's medium (DF-10) supplemented with 10% newborn calf serum (M.A. Bioproducts, Maryland), 50 μg/ml proline and maintained in humidified atmosphere of 92.5% air and 7.5% CO_2, as described previously.[45] The doubling time of this cell line at 33°C was 14 - 16 hours. For cells grown in "HAT" medium, the following concentrations of hypoxanthine (H), aminopterin (A), and thymidine (T) were used: $[H] = 5\times10^{-5}$ M, $[A] = 2.5\times10^{-6}$ M, and $[T] = 1\times10^{-5}$ M.

Balb/c-3T3 cells. This cell line was grown at 33°C in the Dulbecco-Vogt modification of Eagle's medium (DME) supplemented with 10% newborn calf serum in a humidified chamber with 7.5% CO_2 and the typical doubling time of this cell line was about 30 - 34 hours. The growth characteristics of this cell line are described elsewhere.[46]

CV_1 cells. CV_1, the African green monkey kidney cells were grown in DF-10 medium

containing 10% newborn calf serum, at 37^oC with 7.5% CO_2 and the doubling time of this cell line was not determined. The growth characteristics and culture conditions of this cell line are detailed elsewhere.[47]

<u>$P388D_1$ cells</u>. These cells were grown in RPMI 1640 medium supplemented with 15% fetal bovine serum (HyClone laboratories, Logan, Utah), penicillin (50 units/ml), and streptomycin (50 μg/ml) and grown at 37^oC, 92.5% humidity and 7.5% CO_2. The cells were always passaged by 1:4 split of a 80% confluent dish and were never grown to a high density. The cells were scraped off the dish using a cell scraper (Costar, Cambridge, Massachusetts) without trypsin as recommended by the supplier.

Treatment of the cells with drugs.

Drug treatment was carried out in monolayer cultures. Cells, which were grown to a density of $5x10^6$ to $1x10^7$ cells/100 mm dish or $1x10^7$ to $2x10^7$ cells/150 mm dish were treated with 10, 100, or 250 μM of MC , 10-DMC, PM, or [^{3}H]-PM. Control cells were treated with water or methanol. After exposure to either drug or vehicle for 2 h, cells were washed twice with 10 ml of cold, sterile phosphate-buffered saline (0.2 g KCl, 0.2 g KH_2PO_4, 8 g NaCl, and 1.15 g Na_2HPO_4/liter, pH 7.4) and trypsinized into the same, except for $P388D_1$ cells which were scraped off. The cells were pelleted by centrifugation and the cell pellet was stored at -70^oC until DNA extraction.

Survival curve determinations (CHO cells).

For cell survival studies, drugs (MC or 10-DMC) were added to exponentially growing

cultures at a density of 1 - $1.5x10^5$ cells/ml (10 ml total; 100 mm petri dishes). The cells were exposed to various concentrations of drugs for 2 h at 33°C. At this time the medium was removed, cells were washed twice with fresh medium without serum and the centrifuged cells were seeded in triplicates in 60 mm dishes and allowed to form colonies for 10 - 14 days. Colonies were counted after Geimsa staining. The percent of variation between two determinations at 0 to 2 μM doses was within 10%, whereas it was about 24% at 4 μM dose. At the 100 μM or 250 μM doses, none of the cells formed new colonies. However, no evidence was found for the loss of cell integrity, as assayed quantitatively by cell counts in a hemocytometer of tryphan blue-stained cells.

Isolation of DNA from cells.

DNA was isolated from cells by the standard methodology[48] with some modifications. Briefly, 10^8 cells were suspended at a concentration of 5 $x10^6$ cells/ml in 10 mM Tris.HCl/100 mM NaCl/1 mM EDTA, pH 8.0, containing 0.5% sodium dodecyl sulfate and proteinase K (100 μg/ml) was added and incubated at 37°C overnight. The solution was extracted twice with phenol [pre-equilibrated with Tris.HCl, pH 8.0)/"chloroform"; ("chloroform" = $CHCl_3$:isoamyl alcohol ; 24:1; v/v)] followed by "chloroform" and the nucleic acids were precipitated by ethanol. RNA was removed by treatment with RNase A (100 μg/ml) at 37°C for 1 h and the DNA was ethanol precipitated after extracting with phenol/"chloroform" as described above. The yields of DNA ranged from 0.8 - 1.0 mg, as determined by spectrophotometry.

Animals. Male Sprague-Dawley rats were used and the experiments were carried out in the laboratory of Arthur Cederbaum of the Mt. Sinai School of Medicine.

Injection of rats with MC.

In a typical experiment, a fasted male Sprague-Dawley rat (185 g) was injected intraperitoneally with 15 mg of MC in 1.5 ml of 15 mM KCl twice, at a 2 h interval, and killed 1 h after the second injection. The liver was quickly perfused with 50 mM Tris.HCl, pH 7.5 and excised. The liver was frozen immediately and stored at -70°C until DNA extraction. The control rat received only the vehicle.

Isolation of nuclei from rat liver.

The nuclei and the nucleic acids from the liver were isolated essentially as described by Croy et al.[49] The liver was suspended in 24 ml of homogenizing buffer (250 mM sucrose/2 mM $CaCl_2$/10 mM Tris. HCl, pH 7.5) and cut into small pieces with a pair of scissors and homogenized for 5 min using a motor driven Potter-Elvehjem type homogenizer fitted with a teflon pestle. The homogenate was filtered through a double layered cheese cloth to remove any large clumps. The filtrate was centrifuged at 1000xg for 10 min at 4°C. The sediment was resuspended in the same buffer containing a final concentration of 5% Triton X-100, by repeated expulsions from a pipet and centrifuged at 1000xg for 10 min at 4°C. The supernatant was discarded and the nuclear pellet was re-extracted twice as above. After the third extraction, the crude nuclear pellet was suspended in homogenizing buffer and stored at -70°C until DNA extraction.

Isolation of DNA from the nuclei.

The homogenizing buffer was aspirated off the nuclear pellet and the pellet was

suspended in 50 mM Tris.HCl, pH 6.5. Appropriate volumes of 5% SDS and 4 M NaCl were added to result in a final concentration of 1% SDS and 1M NaCl (final volume, 20 ml). An equal volume of CH_3Cl/isoamyl alcohol (24:1; v/v) was added and the two phases were shaken vigrously for 20 min at room temperature. The aqueous and organic phases were separated by centrifugation at 3000 rpm for 20 min in a Sorvall GLC-3 table-top centrifuge. The aqueous phase was re-extracted for second time in the same way and the nucleic acids from the aqueous phase were precipitated by adding 2 volumes of cold ethanol (-20°C). The precipitated nucleic acids were spun on a glass rod, washed with 70% ethanol, air dried and dissolved in 20 ml of 100 mM NaCl/50 mM Tris.HCl, pH 7.0 (12.5 mg). RNase A (100 μg/ml) was added and incubated at 37°C for 1 hour. The solution was cooled and the DNA was ethanol precipitated (11 mg) and dissolved in dilute acetic acid, pH 5.5 (157 μg/ml).

Hydrolysis of DNA into nucleosides.

The DNA thus obtained was hydrolyzed into nucleosides by using nuclease P_1/SVD/alkaline phosphatase method as described above.

Isolation of modified nucleosides.

The hydrolyzed DNA was loaded on to a Sephadex G-25 (5.0 x 56 cm) column and eluted with 20 mM NH_4HCO_3. The "adduct region" corresponding to the elution volumes of the authentic standard adducts[14,43] was pooled, concentrated, and lyophilized. The resultant lyophilized modified nucleosides were dissolved in 1.5 ml of

30 mM KH_2PO_4, pH 7.0 and analyzed by HPLC [HPLC conditions (i)] as described above.

Spectrophotometry.

The UV spectra of the samples was carried out by using Cary 219 spectrophotometer (Varian Instruments). The fluorescence measurements were determined by using Perkin Elmer model LS 5 fluorimeter.

Alkaline ethidium bromide fluorescence assay for crosslinking of DNA.

The alkaline ethidium bromide (AEB) fluorescence assay was carried out using the method of Matsuo and Ross[50] and was done at Rockefeller University in the laboratory of Peter Ross, under his guidance.

DNA samples. High molecular weight (HMW) DNA (> 50 kb as checked by agarose gel electrophoresis) was isolated as described above and dissolved in TE buffer (10 mM Tris.HCl/1 mM EDTA, pH 8.0) at a concentration of 50 μg/ml. All samples used in this assay had an A_{260}/A_{280} ratio in the range of 1.8 - 1.9. The pipet tips used for this experiment were cut at the ends to avoid shearing of DNA.

Alkaline denaturation. Twenty microliters of sample containing ~1 μg of DNA was denatured by adding 2.5 μl of 1.0 M NaOH and incubating for 30 min at 37°C. This solution was neutralized by the addition of 2.0 μl of 1.7 M acetic acid. The samples not to be denatured (non-denatured samples) were incubated with 0.225 vol of a mixture of

1.7 M acetic acid and 1.0 M NaOH (1:1.25) in order to keep identical ionic concentrations among all samples.

Alkaline fluorescence assay. All measurements were carried out in siliconized, borosilicate glass tubes (Kimble, 10x75 mm). Two milliliters of AEB fluorescence buffer (50 mM KCl/6 mM KOH, pH 11.8 containing 0.5 μg/ml ethidium bromide) was added to each sample. The final pH was checked just before the fluorescence measurements and was 11.8. This pH of 11.8 prevents the formation of regions of short self-complementarity so that the potential ethidium bromide intercalation sites in the control native DNA are destroyed. On the other hand, if the drug has introduced an interstrand crosslink, this will serve as a locked site for rapid "zipping-up" of both the strands when the samples are renatured following denaturation.[51] Fluorescence was measured at 525 nm excitation and 600 nm emission. The background fluorescence of the buffer (blank) was set to zero.

Measurement of DNA crosslinks. The percent of DNA crosslinks was determined by measuring the difference in fluorescence of denatured control DNA and denatured drug treated DNA sample as described by Garcia et al.[52] using the formula,

$$C_t = \frac{f_t - f_c}{1 - f_c} \times 100$$

where,

C_t = percent of DNA crosslinks in the drug-treated sample.

$$f_t = \frac{\text{fluorescence of the denatured drug-treated sample}}{\text{fluorescence of the non-denatured drug-treated sample}}.$$

$$f_c = \frac{\text{fluorescence of the denatured control}}{\text{fluorescence of the non-denatured control}}.$$

RESULTS

Formation of MC-DNA complexes under chemical or enzymatic reductive activation conditions.

Brief treatment of a mixture of MC and calf thymus or Chinese hamster ovary (CHO) DNA in neutral buffer with either H_2/PtO_2, NADPH-cytochrome cytochrome *C* reductase, or xanthine oxidase/NADH resulted in the formation of a covalent complex between MC and DNA. Such variation of the reductive activating agent did not significantly affect the b.r., which was typically in the range of 0.04 - 0.07. M13 DNA and heat or alkali denatured calf thymus DNA gave similar results. Poly(dG-dC), however, formed complexes that exhibited a higher b.r. (0.10 - 0.12).

Adduct HPLC patterns from various DNA-MC complexes digested with DNase I/SVD/alkaline phosphatase.

The digest of various DNA-MC complexes was analyzed by HPLC (Fig. 1a). With the exception of the early-eluting unmodified nucleosides, only one major peak was evident in the pattern, at 45 minutes. The two minor peaks, at 39 and ~120 min, each represent <5% of the peak area of the 45 min major peak.

MC-calf thymus DNA complexes formed under enzymatic activation (NADPH-cytochrome *C* reductase or xanthine oxidase/NADH) yielded patterns (Fig. 1b, c) essentially identical to those obtained using H_2/PtO_2 (Fig. 1a). M13 DNA and denatured calf thymus DNA also gave similar patterns (not shown). MC-poly(dG-dC)

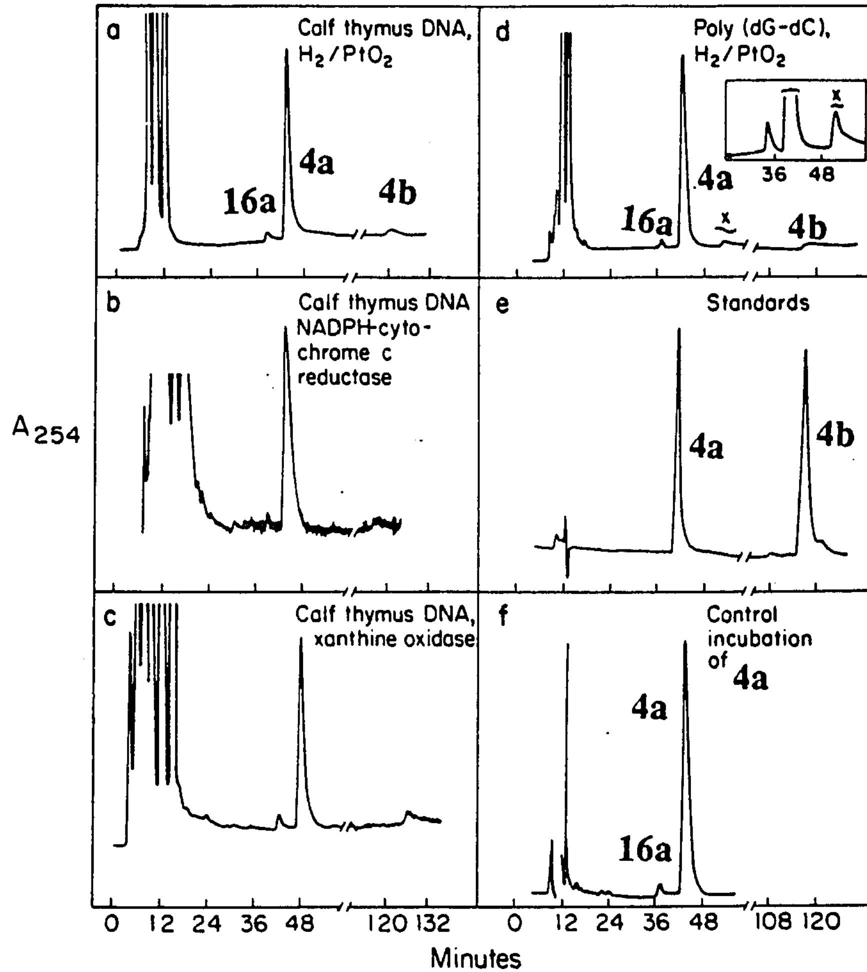
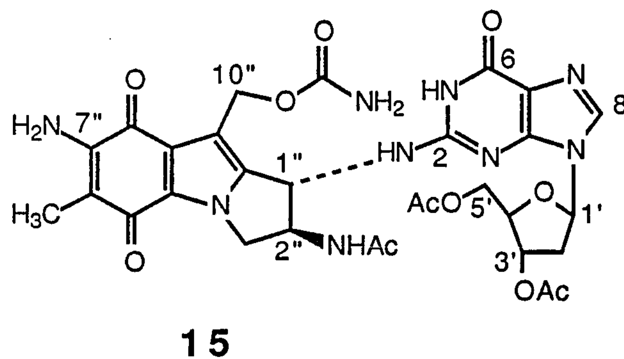


Fig. 1. HPLC patterns from DNase I/SVD/alkaline phosphatase digests of various MC-DNA and MC-poly(dG-dC) complexes. Digests of MC-calf thymus DNA complexes formed under reductive activation by (a). H_2/PtO_2 (b). NADPH-cytochrome cytochrome *C* reductase. (c) Xanthine oxidase/NADH. (d) Digest of MC-poly(dG-dC) complex (H_2/PtO_2 activation). (e) Authentic standards **4a** and **4b**. (f) Adduct **4a** incubated with DNase I/SVD/alkaline phosphatase.

complexes (H_2/PtO_2 or NADPH-cytochrome *C* reductase) also yielded the same pattern of adducts (Fig. 1d), except for an additional minor adduct peak, the crosslink **6,** marked **X**.

Identification of the major MC-DNA adduct as 4a.

The major component of each of the adduct patterns described above (45 min peak; Fig. 1a-d) was isolated by either HPLC or Sephadex G-25 chromatography as described.[43] Direct comparisons of this material with the authentic adduct **4a**[16,43] were made with respect to the following criteria: (i) HPLC elution time: identical (Fig. 1e); single homogenous peak when mixed with the authentic adduct **4a.** (ii) Sephadex G-25 elution volume[43]: identical. (iii) UV spectra (Fig. 2) obtained at three pH values: identical. (iv) Conversion to adduct triacetate **15** showed a very good comparison between the ^{1}H NMR spectra of **4a** obtained in this work and an authentic sample.[16]



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Furthermore, fast atom bombardment mass spectra, and Fourier transform IR spectra of

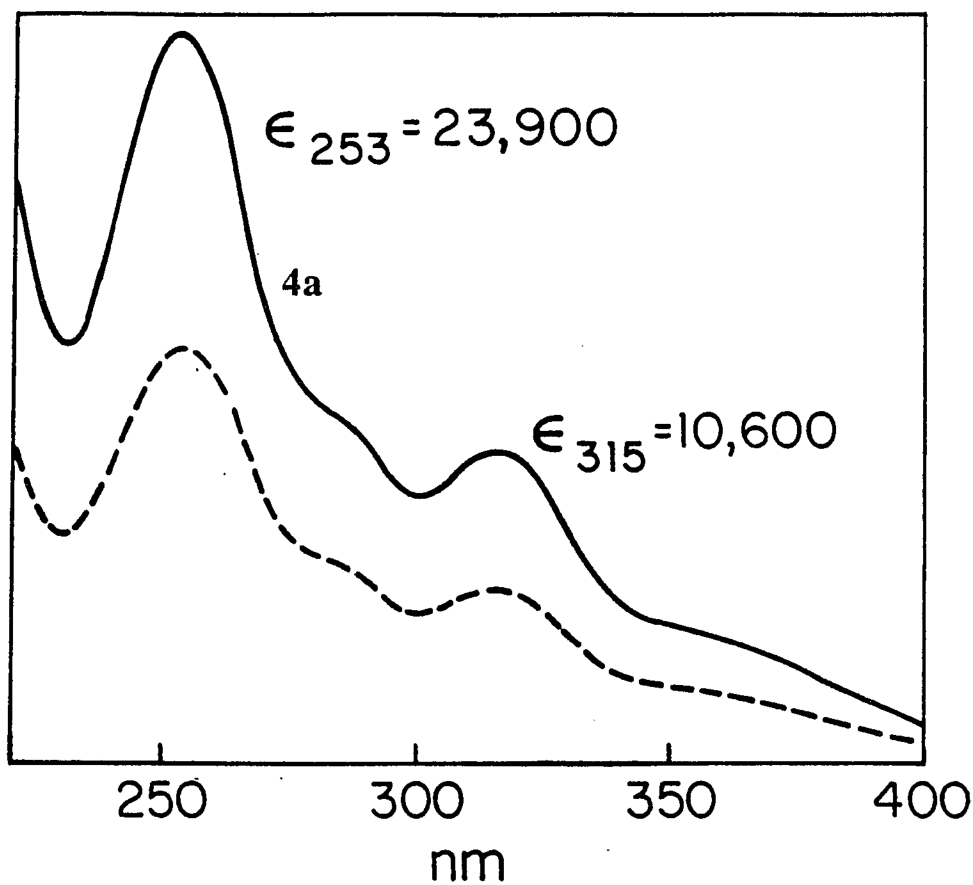


Fig. 2. Comparison of the UV spectra of synthetic **4a** with that obtained from enzymatic digestion of MC-calf thymus DNA. Buffer: 10 mM potassium phosphate, pH 7.0.

the authentic[43] (all of these were done by G. Verdine; Columbia University) and DNA-derived **4a** were virtually identical.

Reinvestigation of Hashimoto's MC-DNA adducts.[12]

a) Nuclease P_1 digestion products of the MC-DNA complex.

The MC-DNA complex made according to the published protocol of Hashimoto et al.[12] (see ref. and methods) (b.r. 0.02) was digested with nuclease P_1 strictly according to the published procedure.[12] An aliquot of the digest was analyzed by HPLC (10:90 CH_3CN/0.3% aqueous NH_4Cl[12]), giving rise to the pattern in Fig. 3a. The three peaks under the bracket reproduce Hashimoto's pattern;[12] those were the "three adducts" they investigated and proposed structures for. With a more polar eluent, however, (6:94 CH_3CN/0.3% aqueous NH_4Cl) to distribute the products over a larger elution time, the apparent triplet at >11 min (Fig. 3a) resolved into a complex series of products with various peak intensities and widths (Fig. 3b); thus, it is clear that the "triplet" shown under the bracket in Fig. 3a is actually composed of larger number of overlapping components.

b) Dephosphorylation of nuclease P_1 digestion products with alkaline phosphatase.

Another aliquot of the nuclease P_1 digest was treated with alkaline phosphatase. HPLC of this digest resulted in the trace shown in Fig. 4b. Four adduct peaks were evident; none

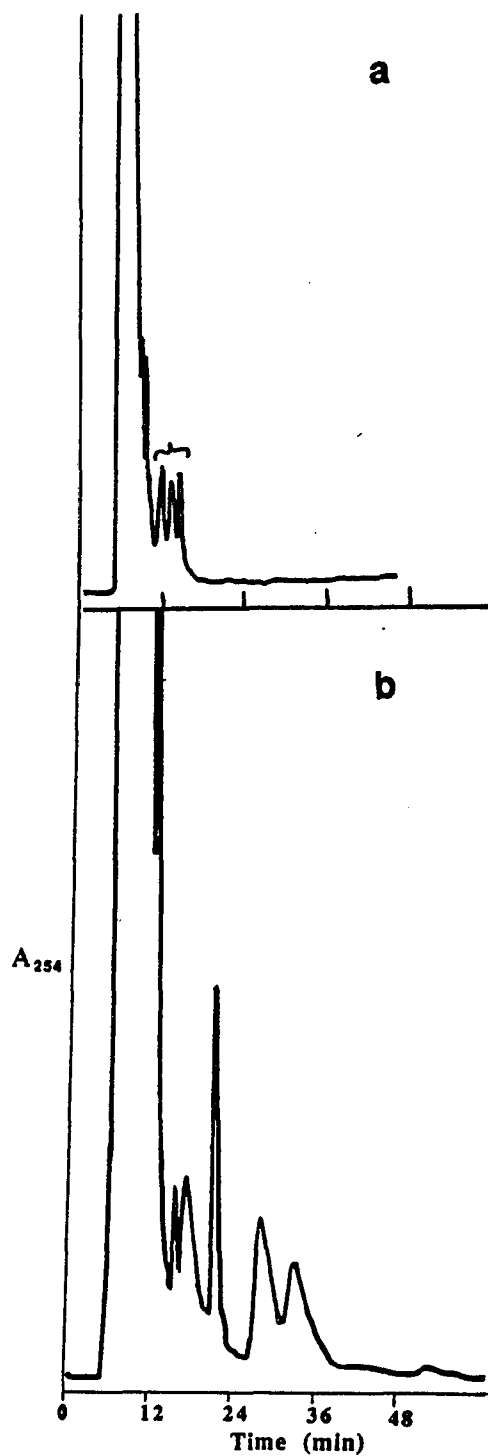


Fig. 3. HPLC patterns of nuclease P_1 digest of MC-calf thymus DNA complex (b.r. = 0.02; H_2/Pd-C activation). Eluent (a) 10:90 CH_3CN/0.3% aqueous NH_4Cl; (b) 6:94 CH_3CN/0.3% aqueous NH_4Cl.

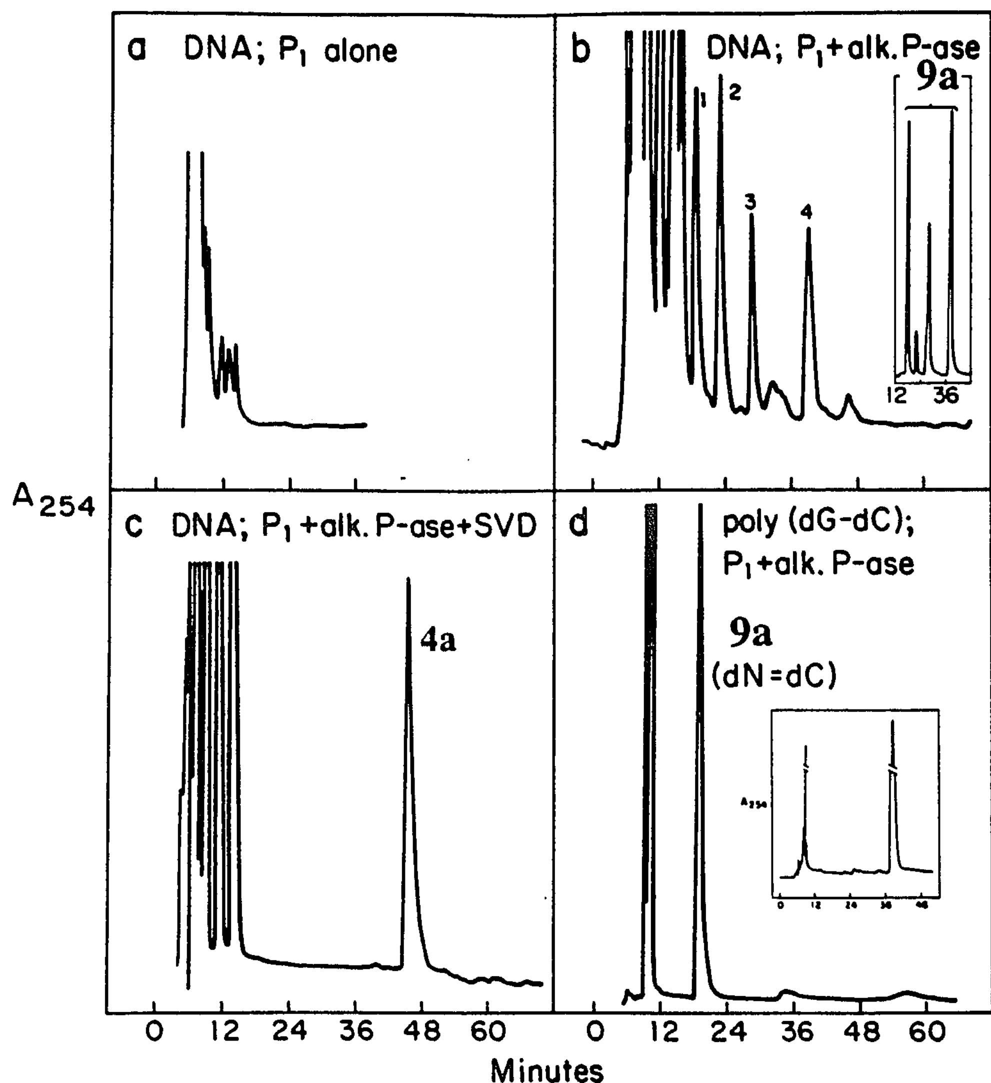


Fig. 4. HPLC patterns of digests of MC-calf thymus DNA and MC-poly(dG-dC) using several nuclease treatment combinations. (a) MC-calf thymus DNA complex (b.r. = 0.02; H2/Pd-C activation) digested with only nuclease P_1. (b) Complex in *a* digested further with alkaline phosphatase. (*Inset*) The four synthetic adducts, **9a** (dN = dC, dG, dT, and dA, in respective order of increasing elution time). (c) Same complex as in *a* and *b* digested further with SVD/alkaline phosphatase. (d) MC-poly(dG-dC) complex (b.r. = 0.12; H_2/PtO_2 activation) digested with nuclease P_1/alkaline phosphatase. (*Inset*) Further digestion of purified **9a** peak (dN = dC) with SVD/alkaline phosphatase; the early eluting peak (11') is deoxycytidine and the latter eluting peak (38') is **4a**.

of them corresponded to the major, well-characterized adduct **4a** (see above and Fig. 4). Nuclease P_1/ alkaline phosphatase digestion of an MC-poly(dG-dC) complex (b.r. 0.12) gave rise to a single detectable MC-linked adduct, which was identical to peak 1 in Fig. 4b.

c) Further digestion of nuclease P_1 products with SVD and alkaline phosphatase.

An aliquot of the above nuclease P_1 digest was adjusted to 10 mM Tris.HCl, pH 8.2 by adding 0.01 vol of 1 M Tris.HCl, pH 8.2, and was incubated with alkaline phosphatase (0.5 unit/A_{260} unit) and SVD (2.5 units/A_{260} unit) for 2 h at 37°C. Direct HPLC analysis indicated essentially a single adduct **4a** (Fig. 4c) as judged by its spectral and chromatographic properties. Application of the same digestion conditions *to any of the four isolated nuclease P_1 / alkaline phosphatase products* (Fig. 4b; peaks 1 - 4) *resulted in each case in the quantitative release of **4a** and the corresponding nucleoside in a 1:1 ratio*; for example, treatment of pure peak 1 (**9a**; dN = dC) (Fig. 4d) gave rise to the early eluting deoxycytidine peak and a later eluting peak, **4a** (*inset*).

d) Identification of the nuclease P_1 digestion products of MC-DNA complex: Hashimoto's compounds[12] are MC-dinucleotide adducts.

The nuclease P_1 digestion products [see (a) above] designated by Hashimoto et al.[12] as three MC-mononucleotide adducts were dephosphorylated and analyzed by HPLC, as

described in (b) above. The HPLC peaks (Fig. 4b) were compared to HPLC peaks of the authentic synthetic standards **9a**, i.e. the MC-dinucleoside phosphates, resulting in identifying peaks 1 - 4 as d[G(MC)pC], d[G(MC)pG], d[G(MC)pT], and d[G(MC)pA], respectively (see general formula **9a**).

e) Digestion of dinucleoside phosphate adduct of MC, d[C(MC)pG (<u>10</u>, dN = dC) with nuclease P_1 or spleen exonuclease and alkaline phosphatase.

Dinucleoside phosphate adduct of MC, d[C(MC)pG] (**10**, dN = dC), was digested with nuclease P_1 or spleen exonuclease followed by alkaline phosphatase. In both the cases, it was hydrolyzed into adduct **4a** and dC in 1:1 molar ratio (Fig. 5).

Summary: <u>*Scheme III*</u> (page 40). The adducts from nuclease P_1/alkaline phosphatase digestion are MC-dinucleoside phosphates, **9a**. This is concluded from their identity with the authentic synthetic standards with respect to the following properties; (i) HPLC retention times; (ii) degradation by SVD/alkaline phosphatase to **4a** and deoxynucleoside (molar ratio, 1:1) (e.g., see Fig. 4d); and (iii) resistance to nuclease P_1.

Micrococcal, spleen exonuclease and alkaline phosphatase digestion products of MC-DNA complex.

The HPLC patterns of the micrococcal nuclease, spleen exonuclease and alkaline phosphatase digests of complexes of DNA with MC, activated by either H_2/PtO_2 or

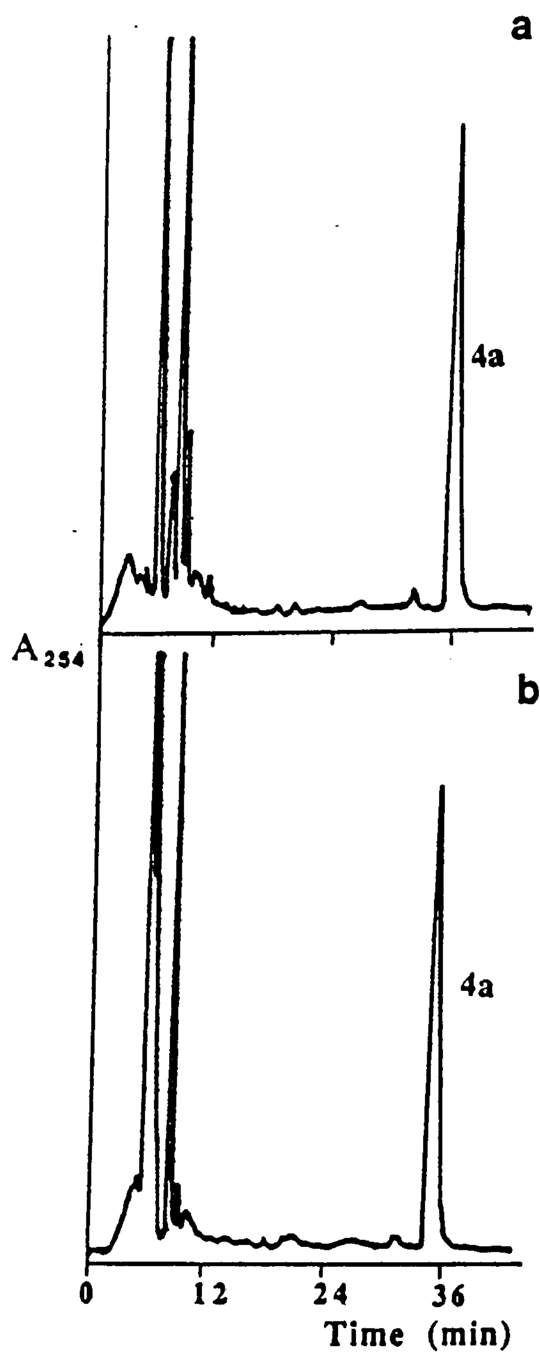
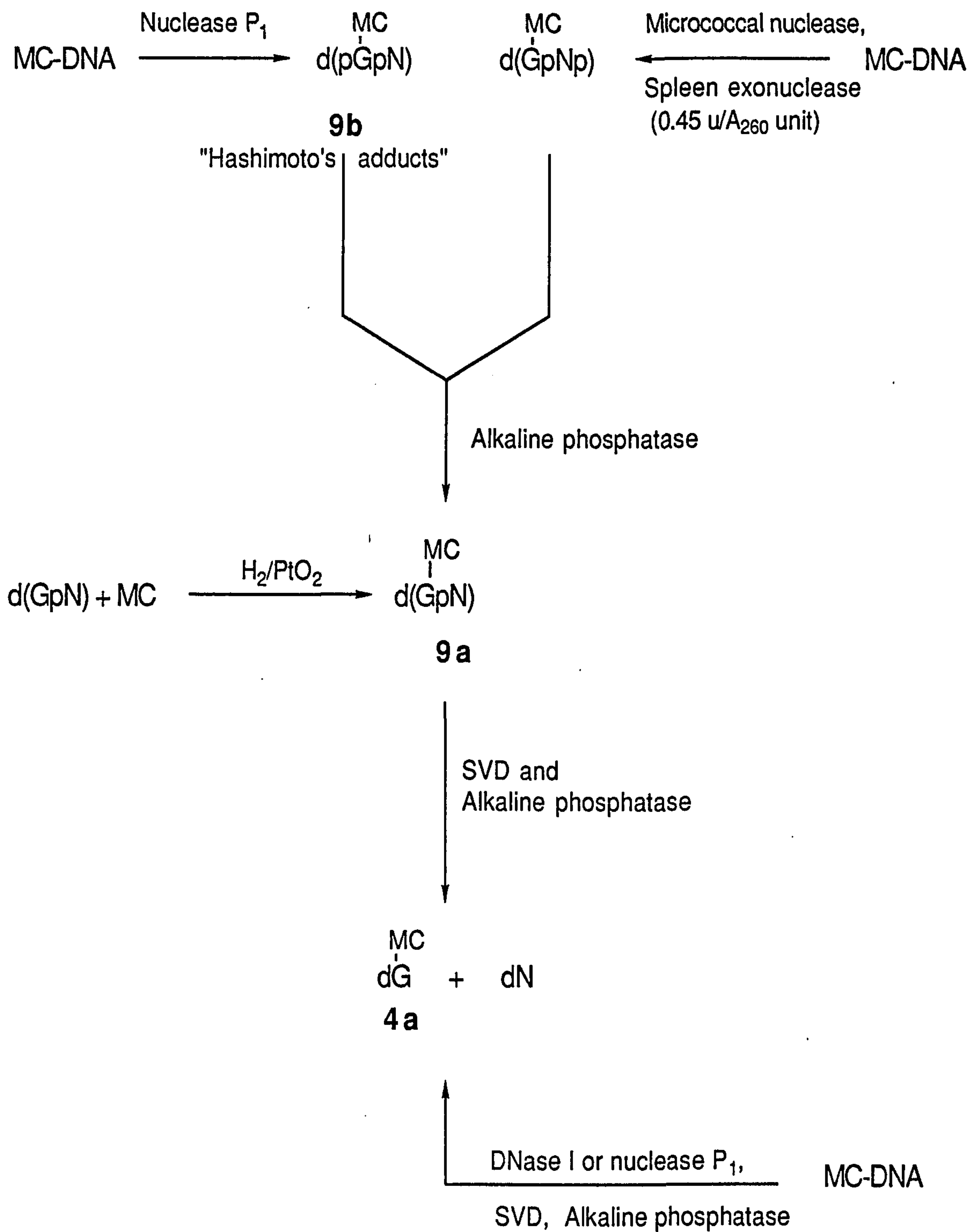


Figure 5. HPLC patterns of the digests of d[C(MC)pG] (**10**, dN = dC) with

a) nuclease P_1 and alkaline phosphatase,

b) spleen exonuclease and alkaline phosphatase.



Scheme III. Products of MC-DNA digested with various nuclease combinations.

$Na_2S_2O_4$ method are shown in Fig. 6 b & d, respectively. These patterns were compared with those obtained by hydrolyzing the same complex using our standard enzymatic system i.e. DNase I/SVD/alkaline phosphatase (Fig. 6a & c). No adduct was seen corresponding to the authentic standards. Once again, it is quite clear that micrococcal nuclease and spleen exonuclease combination cannot hydrolyze MC-modified DNA into mononucleotides. In order to arrive at a conclusive evidence, we used the same enzymatic system with the model compounds, MC-dinucleoside phosphate adduct **9a** (dN = dC) or MC-hexamer crosslink **11** obtaining results as follows.

Spleen exonuclease and alkaline phosphatase digestion products of d[G(MC)pC] (9a, dN = dC) and d[(TACGTA)$_2$MC] 11.

The HPLC patterns resulting from spleen exonuclease and alkaline phosphatase digestion of MC-modified dinucleoside phosphate, d[G(MC)pC] (**9a**, dN = dC) and MC-hexamer crosslink, d[(TACGTA)$_2$MC] **11** are shown in Figs. 7 and 8. The pattern indicated that these adducts were resistant to cleavage by this enzymatic system (Fig. 7b and Fig. 8b) although the controls were cleaved into mononucleosides (not shown). For comparison, the HPLC patterns of the same samples digested with SVD/alkaline phosphatase are shown in Figs. 7a and 8a. Interestingly, when a very high concentration of spleen exonuclease (20 units/A_{260}) corresponding to 100-fold excess of the amount used in the published report[42] was used, only the monofunctionally modified species was cleaved but not the crosslinked one (results not shown). Therefore, it can be concluded that MC-modified DNA is resistant to cleavage by spleen exonuclease.

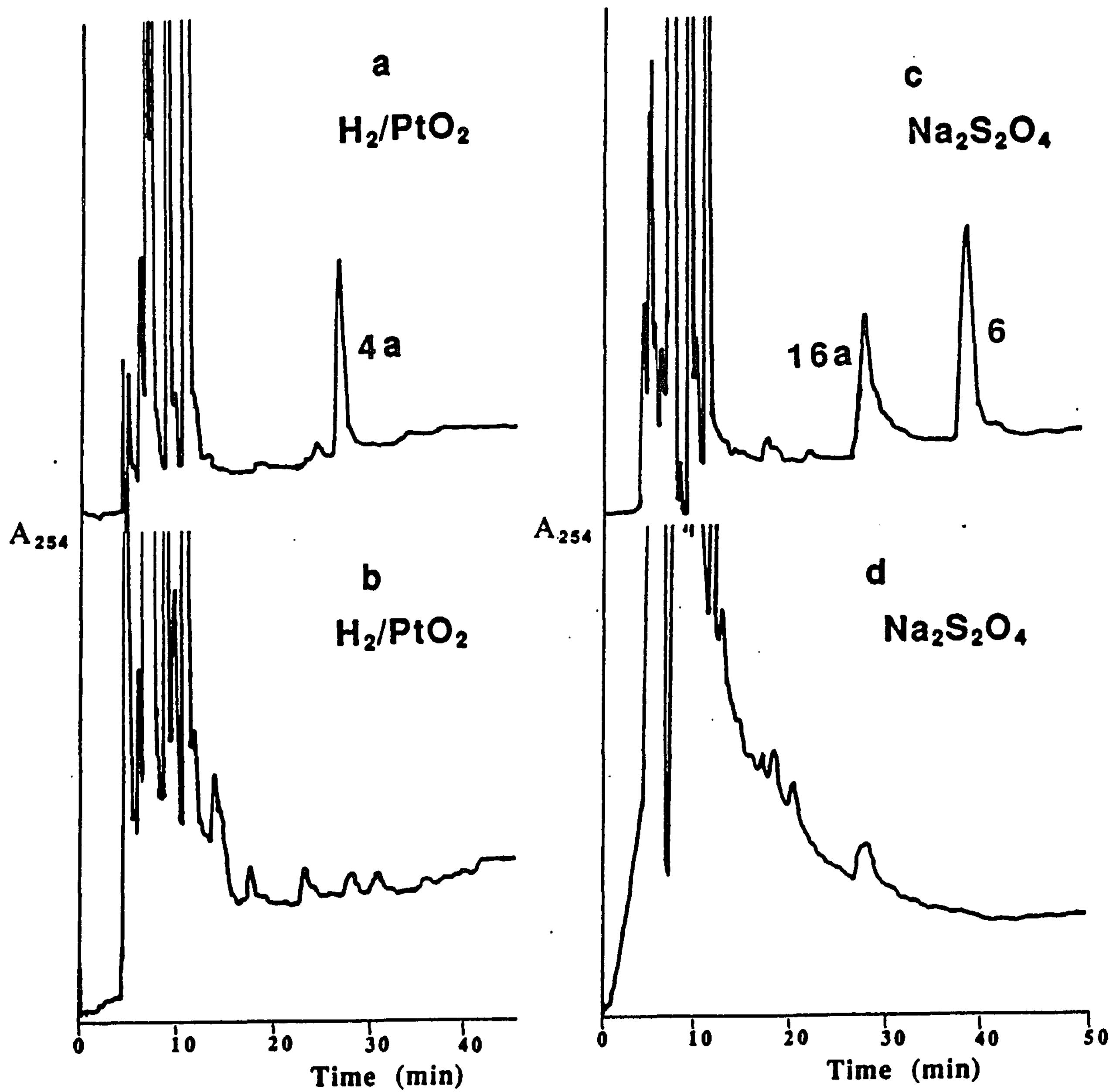


Fig. 6. HPLC pattern of digests of MC-DNA complexes with DNase I, SVD, and alkaline phosphatase (a & c) or micrococcal nuclease, spleen exonuclease, and alkaline phosphatase (b & d).

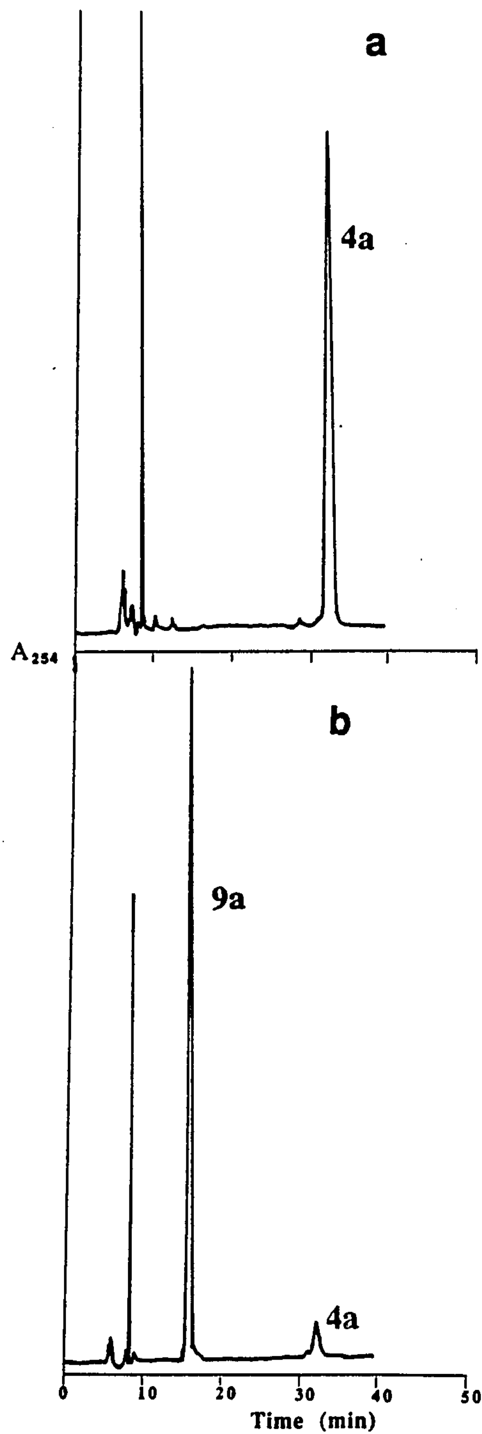


Fig. 7. HPLC pattern of the digests of dG(MC)pC with

(a) SVD and alkaline phosphatase or

(b) spleen exonuclease and alkaline phosphatase.

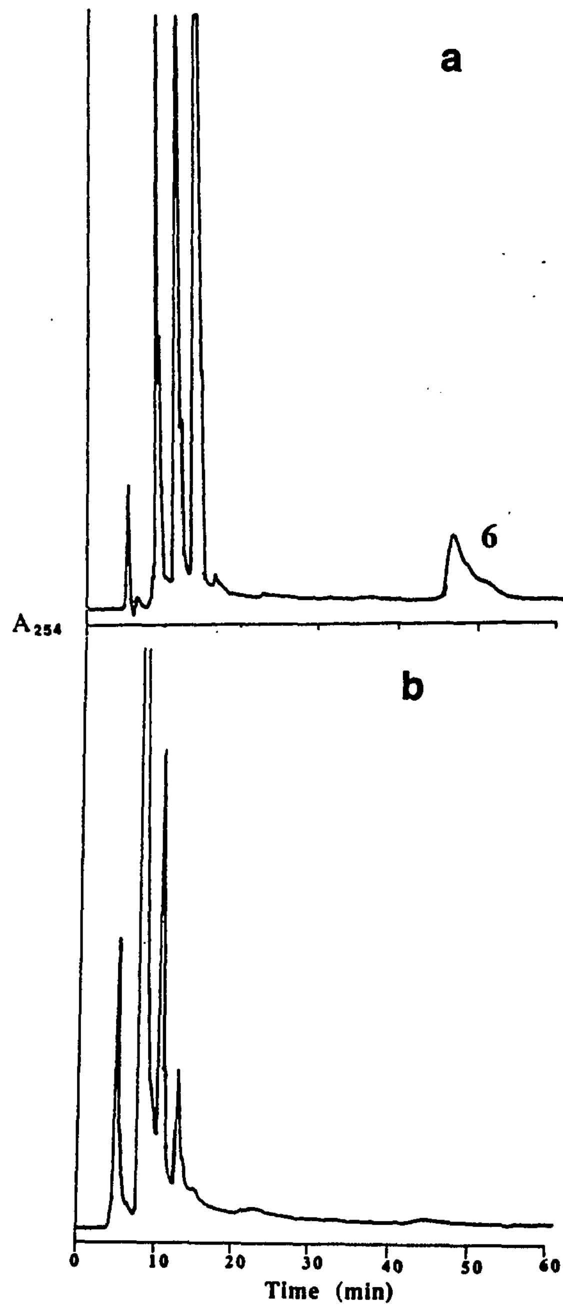


Fig. 8. HPLC pattern of the digests of d[(TACGTA)$_2$MC] with

(a) SVD and alkaline phosphatase or

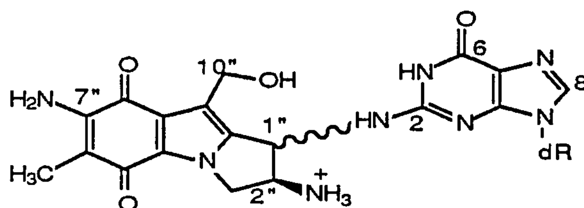
(b) spleen exonuclease and alkaline phosphatase.

Differential susceptibility of dinucleoside phosphate adducts d[G(MC)pN] 9a and d[NpG(MC)] 10 to nuclease P_1 but not to SVD.

Both **9a** (dN = dC) and **10** (dN = dC) were incubated with nuclease P_1, followed by alkaline phosphatase, and analyzed directly by HPLC (Fig. 4d, *inset*). Although **9a** remained unchanged even in the presence of a 100-fold increase of nuclease P_1 concentration, **10** was degraded readily to **4a** and deoxycytidine (Fig. 5a). On the other hand, SVD and alkaline phosphatase degraded both **9a** (dN =dC) and **10** (dN = dC) to **4a** and deoxycytidine.

The thermal stability of d[G(MC)pC] and dG(MC) was tested by boiling at 100°C in H_2O. The adducts slowly converted into d[G(10-DMC)pC] and dG(10-DMC), respectively, with the amounts increasing with the duration of incubation (Figs. 9 & 10).

Adducts formed with 10-DMC. 10-DMC readily formed a complex with DNA under all reductive activation conditions (H_2/PtO_2, $Na_2S_2O_4$ or xanthine oxidase/NADH). Only single major adduct was formed which was identified as **16a** (Fig. 11a).[19] The only other minor adduct formed was its 1"-β isomer **16b**.[19]



16
a: 1" = α-
b: 1" = β-

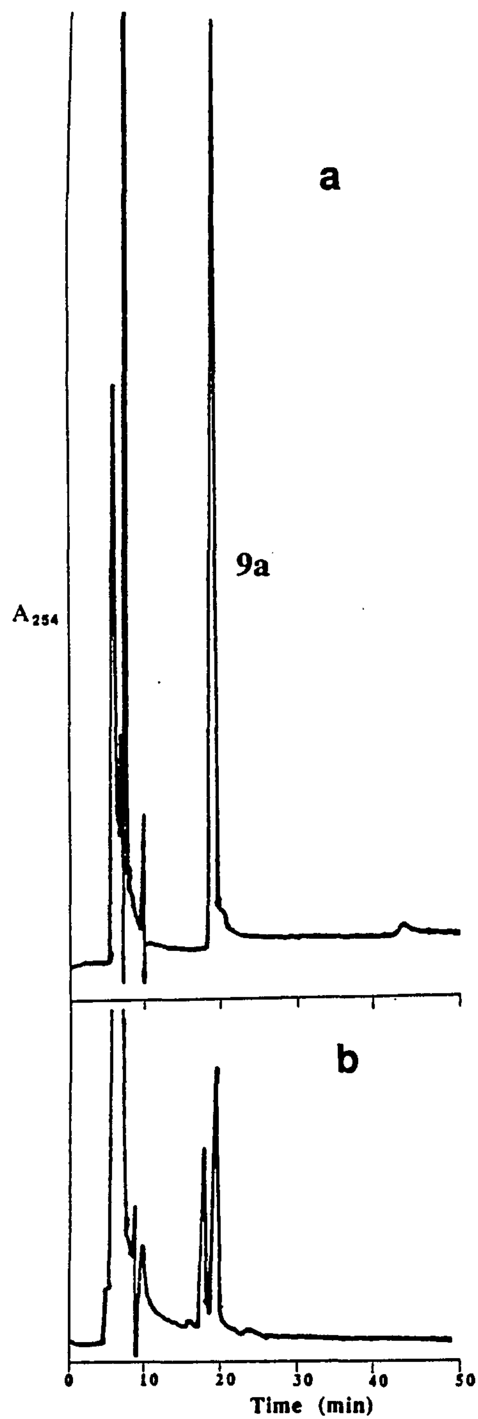


Fig. 9. HPLC pattern of dG(MC)pC

(a) control or

(b) boiled at 100°C for 30 minutes.

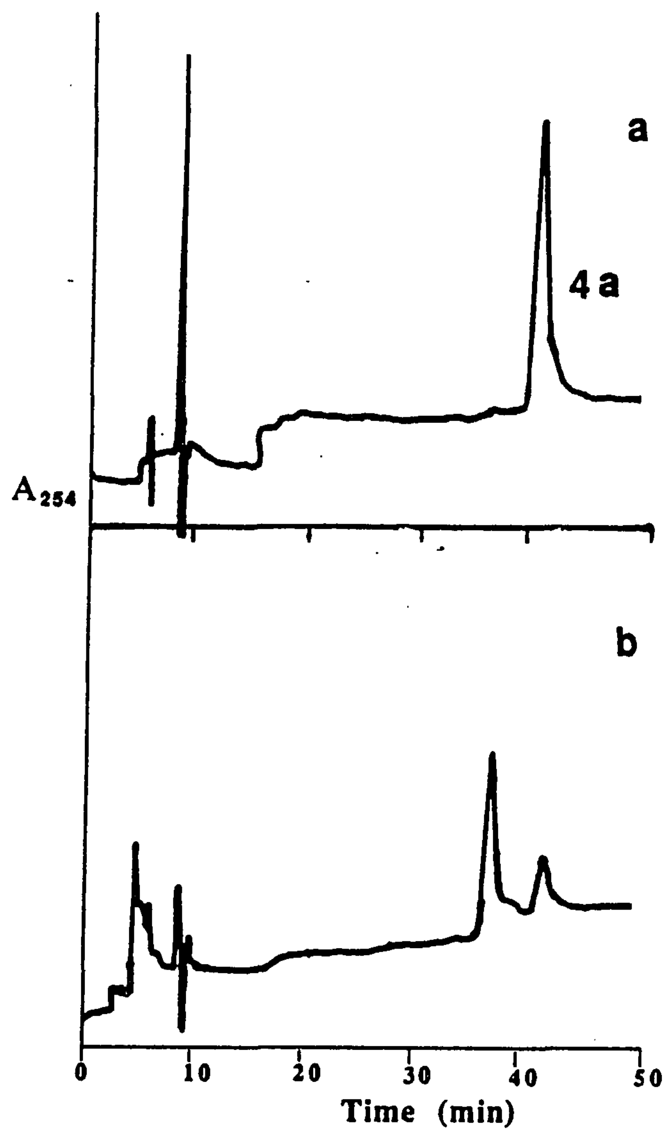


Fig. 10. HPLC pattern of dG(MC)

(a) control or

(b) boiled at 100°C for 30 minutes.

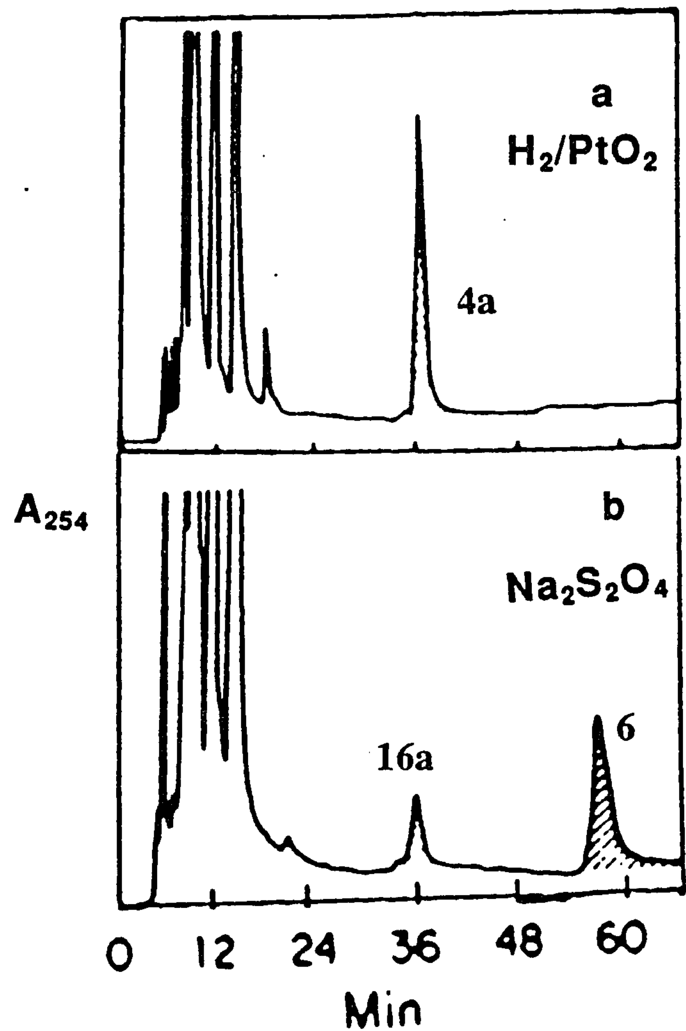
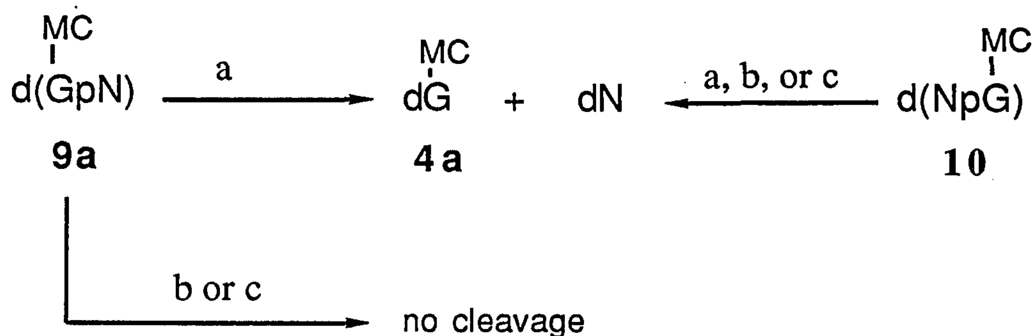


Fig. 11. HPLC patterns from DNase I/SVD/alkaline phosphatase digests of drug-CHO DNA complexes: (a) 10-DMC-DNA complex (H_2/PtO_2). (b) MC-DNA complex ($Na_2S_2O_4$).

Adducts formed with MC under $Na_2S_2O_4$ activation. The HPLC pattern of the digest of DNA-MC complex resulting from the $Na_2S_2O_4$ activation is shown in Fig. 11b. With the exception of the early eluting peaks, two major adducts were seen. The first one with the elution time of 36 min was identified as **16a,** a monofunctional 10-DMC-adduct, and the other with elution time of 50 min was identified as **6**, an MC-crosslink containing two Gs.

DISCUSSION

The finding in our laboratory[17] that N^{2-} position of guanine was the only detectable site of binding of reductively activated MC in DNA was in conflict with a previous report by Hashimoto and coworkers.[12] Reinvestigation of the reported work as Part I of this thesis revealed a likely source of error as follows: according to the authors,[12] nuclease P_1 digestion of MC-modified calf thymus DNA gave a three-peak pattern on HPLC, the components of which were used directly for structural studies and were concluded to be mononucleotides substituted by MC at N^{2-} and O^{6-} of guanine and N^{6-} of adenine, respectively. Similar HPLC pattern was obtained with such nuclease P_1 digest in our laboratory (Fig. 4a). Upon further analysis, however, we have shown that these three peaks actually consist of *four* MC dinucleotide adducts, rigorously identified by several lines of evidence as **9b** (dN= dG, dA, dC and dT; all four are formed in native DNA; Fig. 4b, *Scheme III,* page 40). No conditions could be found for hydrolysis of the 3'-phosphodiester bond in **9a** (isolated or synthetic) or in **9b** by nuclease P_1. Apparently, the presence of an N^2-MC-Gua residue totally inhibits the cleavage of its 3'-phosphodiester linkage by nuclease P_1. Thus, the previous structure determination[12] must have inadvertently used MC dinucleotides **9b** rather than what were believed to be MC mononucleotides, leading to erroneous interpretations of the resulting data. On the contrary, *5'-phosphodiester bond* of N^2-MC-Gua was susceptible to cleavage as evidenced by incubating d[Np(MC)G] **10** with nuclease P_1 and alkaline phosphatase resulting in the release of adduct **4a** and dN in 1:1 molar ratio (Fig. 5 and *Scheme IV*, page 51).[17]



a = SVD and alkaline phosphatase

b = Nuclease P_1 and alkaline phosphatase

c = Spleen exonuclease and alkaline phosphatase

Scheme IV. Digestion of MC-modified dinucleoside phosphate adducts with different enzymatic combinations.

These results completed the picture of the monofunctional alkylation products of DNA with reduced MC, and this may be summarized as follows: The major covalent adduct formed between MC and DNA *in vitro* in a variety of systems is the guanine N^2-linked mitosene **4a**. The other two minor adducts also bear a guanine N^{2-} linkage;[16] **16a** is a secondary degradation product of **4a**, whereas **4b** is the 1"-β stereoisomer , arising from attack of the guanine NH_2 group from the β rather than the α side of activated MC. Model-building studies (Fig. 12) indicate severe steric hindrance to this mode of binding to native B-DNA structure. Therefore, **4b** may actually be formed preferentially in

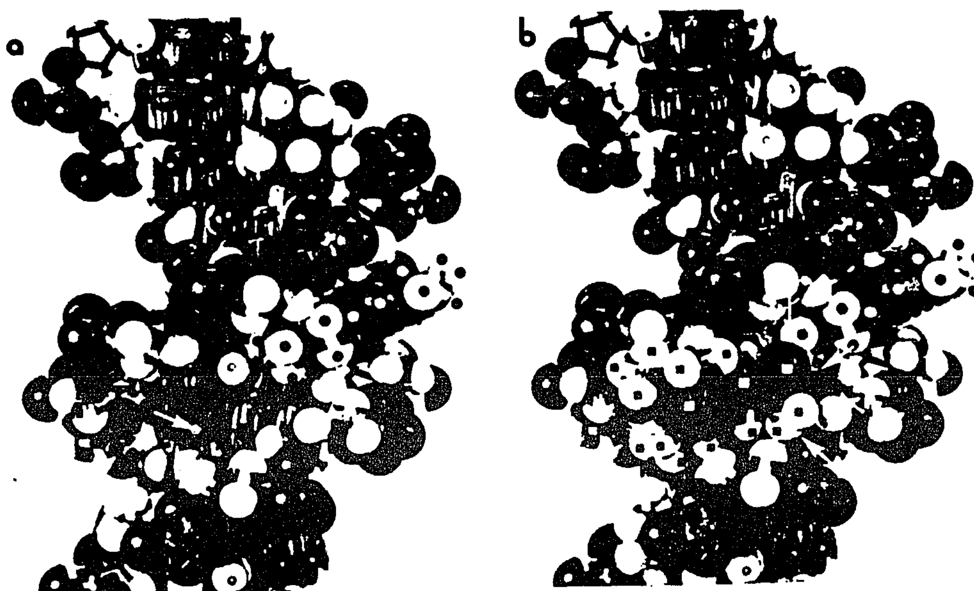


Fig. 12. Proposed model of the MC-DNA complex using space-filling models (Molecular Design Inc., Academic Press). (a) View of adduct **4a** incorporated into DNA; the bound MC moiety is indicated by dots; the 2"-NH_3+ group is located on the left, the 10"-$CONH_2$ group is on the right. (b) Two molecules of adduct **4a** incorporated into DNA at a $C_1G_1C_2G_2$-sequence. Dots and squares indicate the atoms of MC_1 and MC_2, respectively. Arrows (clockwise from top): H bond between 10a"-carbonyl oxygen of MC_2 and 2"-NH_3+ of M_1; two H bonds between 2"-NH_3+ of MC_1 and O^2 atoms of C_2 and the C opposite G_1; H bond between 10a"-NH_2 of MC_2 and 3'-O of G_2. Also apparent in the model but occluded in the photo is an H bond between 10"-O of MC_2 and 2-NH_2 of the G opposite C_1. The M molecules are shown in the probable "head-to-tail" orientation; each MC occupies exactly the minor groove space associated with 2 base pairs.

transiently denatured segments of DNA. Accordingly, twice as much **4b** was obtained from denatured as from native DNA. The reaction between reductively activated MC and deoxyguanosine itself yielded **4a** and **4b** in almost equal amounts.[16] The three-dimentional features of adduct **4a** in double-stranded DNA were studied by space-filling molecular models, leading to the remarkable result that in one unique conformation the N^2-Gua-bound MC residue fits snugly into the minor groove without appreciable perturbation of DNA structure (Fig. 12). In the MC model, the 2" NH_3^+ group lies inside the minor groove and the four H bonds are seen between functional groups of the MC residue and the DNA. By using molecular mechanics simulation of MC bound to N^{2-} of guanine in $d(GC)_5$, Rao et al.[53] observed a similar orientation, but only two of the four H bonds. This model is consistent with a number of previous experimental observations about MC-modified DNA as follows: (i) Intercalation by covalently bound MC residues was ruled out by linear flow dichroism studies.[54] (ii) Binding of MC was uninhibited to T2 DNA,[55] even though the major groove is severely blocked by glucosylated 5-hydroxymethyl cytosines. (iii) MC-modified DNA is more stable than native DNA, as indicated by increased melting temperature;[3,56] the favorable secondary interactions of covalently anchored MC units with the surrounding minor groove readily account for this fact. The location of the 2"NH_3^+ group inside the groove-i.e. the site of minimum electrostatic potential is especially favorable.[57] The stabilizing effect of bound MC on DNA duplex structure is analogous to that of anthramycin, a molecule of similar size and binding site.[58] (iv) The experimental saturation binding ratio of MC to poly(dG-dC) is 0.25 (i.e., one MC for every 2 base pairs).[59] According to the proposed model, at a $(CG)_n$ sequence the guanine of every second base pair may be substituted by MC; this way, the drug molecules fill the minor groove completely, with

head-to-tail H bonds to one another, in agreement with the experimental saturation b.r. Results with synthetic polyribonucleotide analogs are also consistent with guanine-N^{2-} as the binding site: binding was uninhibited to poly(O^6-methylguanine) or poly(G, 7-methyl-guanine) but inhibited to poly(I).[60]

Consolidated enzymatic degradation scheme of MC-DNA complexes.

The results obtained from the digestion of MC-DNA complexes with various nuclease combinations (*Scheme III,* page 40) show that these complexes are resistant to degradation by a variety of enzymes. The results obtained with MC-modified dinucleoside phosphates and oligonucleotides indicated that nuclease P_1 could not cleave a 3'-phosphodiester bond of a MC-modified G (for e.g. see Fig. 4d; *Scheme IV*, page 51); spleen exonuclease, on the other hand, failed to hydrolyze the 5'-phosphodiester bond of a modified G (for e.g. see Fig. 7). Very high concentrations of spleen exonuclease hydrolyzed the 5'-phosphodiester bond of monofunctionally modified compounds but not the crosslinked ones. However, digestion of such samples with SVD yielded mononucleotides. It might be due to the fact that both nuclease P_1 and spleen exonuclease recognize the d(G-) and d(Gp-) portion, respectively, whereas SVD recognizes the d(-pC) part of the MC-modified dinucleoside. This type of selective resistance of MC-modified species to nuclease P_1 and spleen exonuclease indicates an alteration in the structure of d(Gp-) moiety. This is in agreement with the observed circular dichroism (CD) spectral differences between these MC-modified model compounds and their controls.[61] A combination of micrococcal nuclease and spleen exonuclease did not hydrolyze the MC-modified DNA either. Thus, the general enzymatic

system (micrococcal nuclease and spleen exonuclease) used in the ^{32}P-postlabeling assay[42] is not suitable for MC-modified DNA samples (see Results and Part III of this thesis). Results observed with various enzymatic combinations tested thus far showed that the inclusion of SVD is necessary in order to obtain mononucleotides. Thus, the following combinations of enzymes hydrolyze MC-DNA complexes into mononucleosides: *DNase I, SVD, and alklaine phosphatase or nuclease* P_1*, SVD, and alkaline phosphatase*.

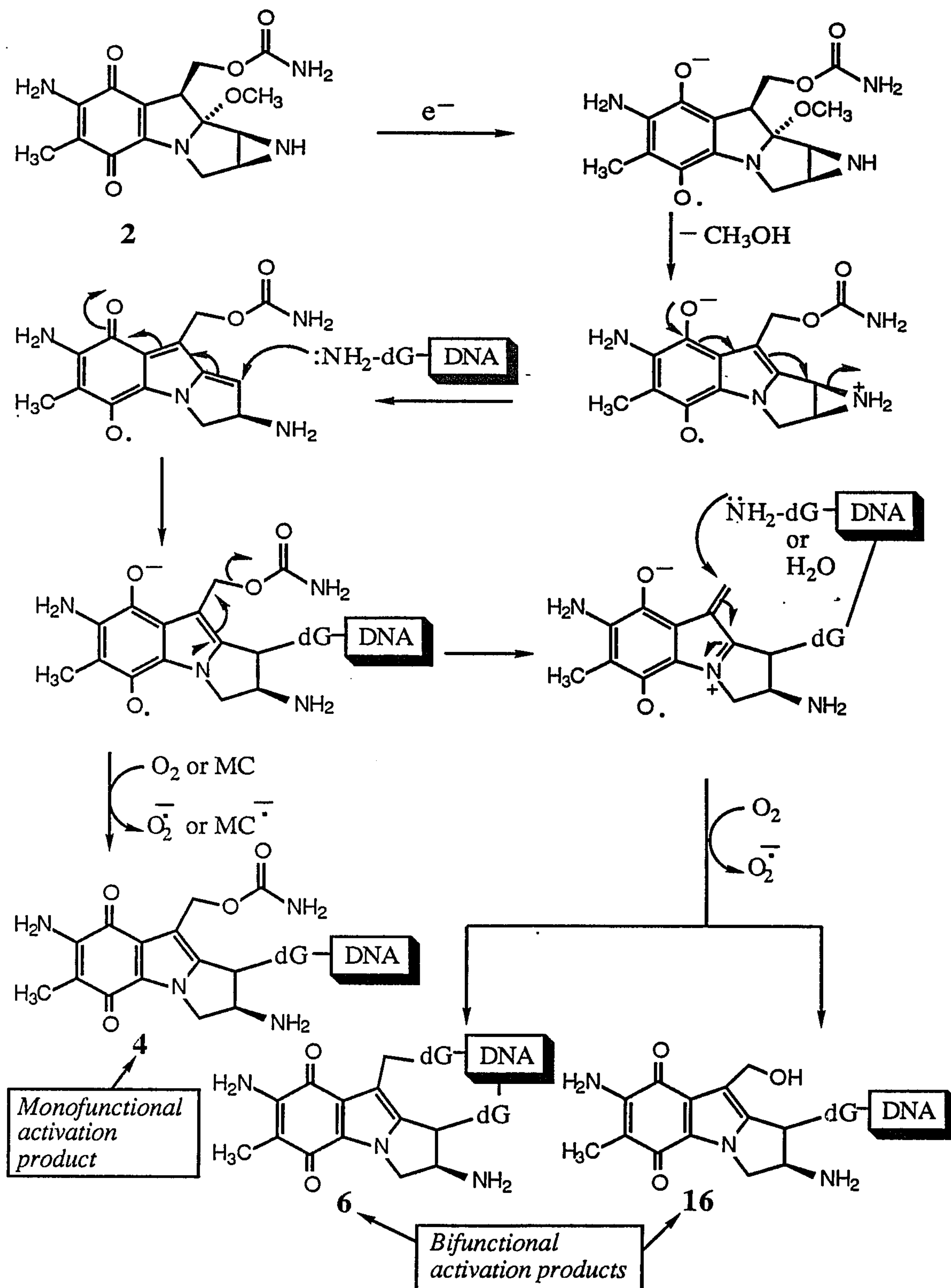
The hydrolytic stability (thermal stability) experiments showed that in case of monofunctional MC-adduct **4a**, the 10"-carbamate group is hydrolyzed upon heating (Fig. 10). This finding should be helpful in synthesizing 10-DMC-modified adducts from MC-modified DNA since 10-DMC does not react as efficiently as MC with DNA.

On the Origin of the MC-Induced Crosslinks in DNA.

This question was solved in 1987 by the joint efforts of the Tomasz and Nakanishi groups,[14] soon after the elucidation of the monofunctional chemistry described above. The monofunctional adducts **4a, 4b, 16a**, and **16b** resulting from the monofunctional activation of MC account essentially quantitatively for the observed amount of drug bound to DNA in the above experiments. What is then the origin of the interstrand crosslinks of MC, which are detectable by several physical methods?[33,34,36,38-40] Presumably, the resolution of this conflict lies in the very low incidence of crosslinking of DNA by MC: typically, < 1 crosslink per 10^6 Da DNA;[1] thus, a "crosslink"-type adduct could escape detection by ultraviolet absorbance methods. In the MC-poly(dG-dC) digests, however, a promising crosslink candidate, **X** (Fig. 4), is

clearly discernible (= 5% of monofunctional adducts) [based on the ultraviolet absorbance and base analysis data (see Results)]. A peak corresponding to **X** also becomes evident in native DNA-MC complexes, which have higher b.r. values.

In a very dramatic development, as reported by Tomasz et al.[14,21] the reaction products of MC and DNA were different when the reducing agent, $Na_2S_2O_4$ was used. When $Na_2S_2O_4$ was used as reducing agent, no detectable trace of **4a** was observed. But the reaction products were adducts **6** and **16a** along with a minor peak at 22 minutes (Fig. 11b). The structure of **6** was determined. It accounts fully for the long-sought-for MC-DNA crosslink, the *bifunctional* alkylation product of MC, linking *two guanines of the complementary strands* together. The differential binding of MC to DNA under $Na_2S_2O_4$ reduction, as opposed to H_2/PtO_2 or flavoenzymatic reduction, can be rationalized. Subsequent to DNA alkylation, the activated monoadduct species (*Scheme I*, page 57) can react further by two pathways . In their reports,[14,21] the authors have shown that O_2 inhibits the bifunctional activation pathway (crosslinking of DNA) due to inactivation of the active intermediate by O_2 (*Scheme I*, page 57). This may be relavent to the greater toxicity of MC under hypoxic conditions, for example, to cells in solid tumors.[62] Since crosslinking of DNA is more lethal than monofunctional alkylation, this explains the differential cytotoxic effects of MC towards aerobic and hypoxic cells. In the latter case, there is increased bifunctional activation of MC, hence, more crosslinking of DNA than in their aerobic counter parts where O_2 may inhibit bifunctional pathway.



Scheme I. Mechanism of activation of MC (also shown on page 6).

RESULTS

Formation of adducts 6 and 16a with CHO DNA and MC or 10-DMC *in vitro*.

DNA isolated from CHO cells was treated with MC *in vitro* using $Na_2S_2O_4$ as a reducing agent, resulting in the formation of MC-DNA complex with a b.r. of 0.05. The complex was digested to nucleosides by using DNase I/SVD/alkaline phosphatase and analyzed by HPLC. The modified nucleosides were again the same ones as seen with calf thymus DNA,[14] viz., the decarbamoyl adduct **16a** and the crosslink **6** as seen in HPLC pattern (Fig. 13a).

Isolation of adducts 6 and 16 from CHO cells.

Treatment of CHO cells with 250 μM MC, followed by isolation and digestion of their DNA to mononucleosides resulted in the HPLC pattern shown in Fig. 13b. Although the b.r. of MC to DNA was too low to detect binding of the drug spectrophotometrically[3] before digestion, the HPLC pattern of the digest clearly showed the presence of the same two adducts **6** and **16a**, as those seen *in vitro* (Fig. 13a). Digests of the same amount of DNA from control (untreated) CHO cells showed no reproducible peaks above noise level in the adduct eluting region (Fig. 13c). Treatment of cells with lower doses of MC (50 or 100 μM) resulted in no detectable adduct patterns.

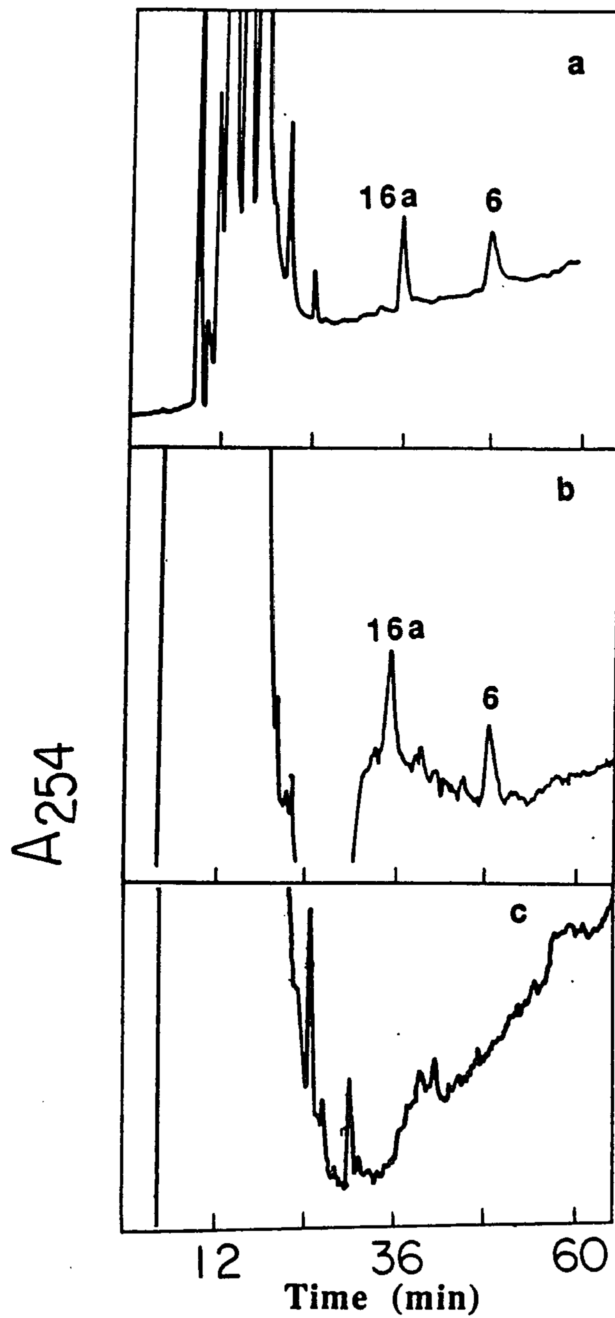


Fig. 13. HPLC patterns of digests of CHO DNA exposed to MC *in vitro* or in CHO cells.

(a) *in vitro* ($Na_2S_2O_4$ activation),

(b) in CHO cells (250 μM MC), and

(c) CHO cells (control).

Adducts with 10-DMC.

The same series of experiments were also carried out using 10-DMC instead of MC. The HPLC pattern of the digest from the reaction of 10-DMC with CHO cell DNA *in vitro* (Fig. 14c) indicated the formation of adduct **16a** , but none of the crosslink **6**. This result is identical with that obtained previously using *M. luteus* DNA[19] instead of CHO DNA. DNA from CHO cells treated with 250 μM 10-DMC showed the presence of adduct **16a** in the HPLC pattern (Fig. 14a). Besides having identical HPLC elution time with that of adduct **16a** originating from the reaction with DNA *in vitro* (Fig. 14c, also "mixed run", not shown), its ultraviolet absorbance spectrum, obtained "on-the-flight" during HPLC elution (Fig. 14a, *inset*), was also identical with the ultraviolet absorbance spectrum of the latter, obtained similarly (Fig. 14c, *inset*). A peak corresponding to the elution time of crosslink **6** was seen in some runs of the 10-DMC treated cell digests but it was not reproducible consistently. The results utilizing alkaline ethidium bromide fluorescence assay showed 18 and 23 percent crosslinking in DNA samples from cells treated with 10 and 100 μM of 10-DMC, respectively (Table I, page 62). A digest of the control CHO cell DNA showed a noisy baseline on HPLC in the adduct region (Fig. 14b), indicating clearly that in Fig. 14a only the peak corresponding to adduct **16a** is a significant component above noise level from the 10-DMC treated cells. The "on-the-flight" ultraviolet absorbance spectrum from the 36 min region of the HPLC of control digest (Fig. 14b, *inset*) indicated the absence of a UV-absorbing artifact which could have been mistaken for an adduct spectrum. Treatment of cells with 100 μM dose, but not with 50 μM dose, of 10-DMC also gave a clearly detectable peak of adduct **16a.**

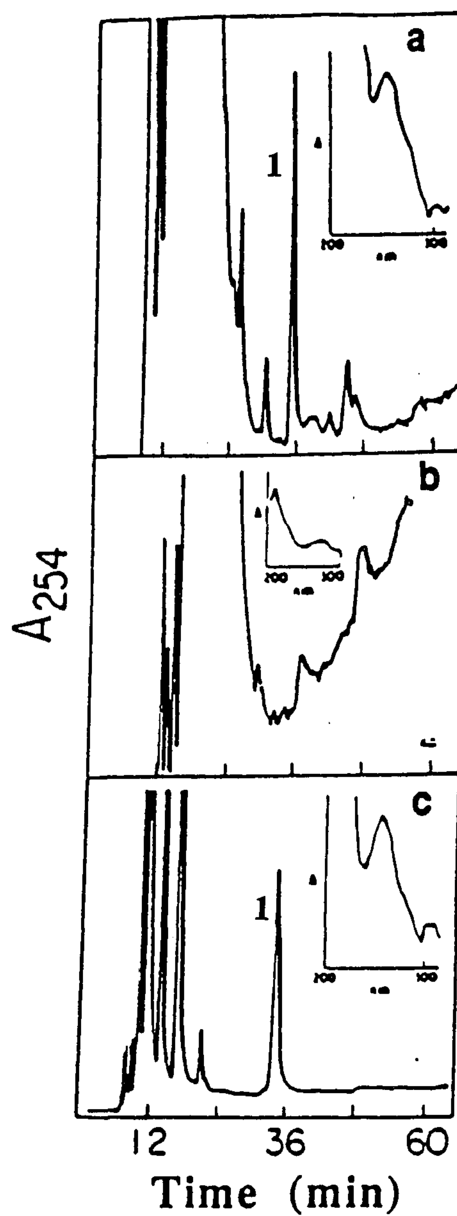


Fig. 14. HPLC patterns of digests of DNA from CHO cells exposed to 10-DMC.

(a) 250 µM (*Inset*: "on-the-flight" UV spectrum of peak **1** recorded during its elution),

(b) Control, and

(c) *in vitro* ($Na_2S_2O_4$) activation (*Inset*: "on-the-flight" UV spectrum of peak **1** recorded during its elution).

Table I. Percent crosslinking in DNA from cells treated with drugs, as detected by alkaline ethidium bromide (AEB) fluorescence assay.

	10-DMC	MC	PM
10 μM	18	4	–
100 μM	23	13	5

Isolation of the crosslink 6 from rat liver.

The DNA isolated from the liver of rats injected with MC was digested to nucleosides by nucleases. The modified nucleosides were separated from the unmodified ones by Sephadex G-25 chromatography.[14,43] The "*adduct region*" (Fig. 15) corresponding to the elution volumes of the authentic samples was further analyzed by HPLC. The HPLC chromatogram is shown in Fig. 16a, in which two major peaks are seen. The earlier one with the elution time of 42 min corresponding to the authentic crosslink **6** was collected (0.55 A_{260} units). Its identity with the authentic crosslink **6** was established with respect to the following criteria: (i) HPLC elution time: identical (Fig. 16a & b); single homogenous peak when mixed with the authentic **6** (Fig. 16c). (ii) ultraviolet absorbance spectrum: identical (Fig. 17). The other peak with elution time of 51 min was an artifact, a possible contamination of enzyme(s) from Sephadex chromatography. Its non-adduct property was evident from the UV spectrum where there was no peak with λ_{max} at around 315 nm, a diagnostic property of the mitosene skeleton. A 254 nm absorbing material has been shown to be associated with alkaline phosphatase which has a similar spectrum.[53]

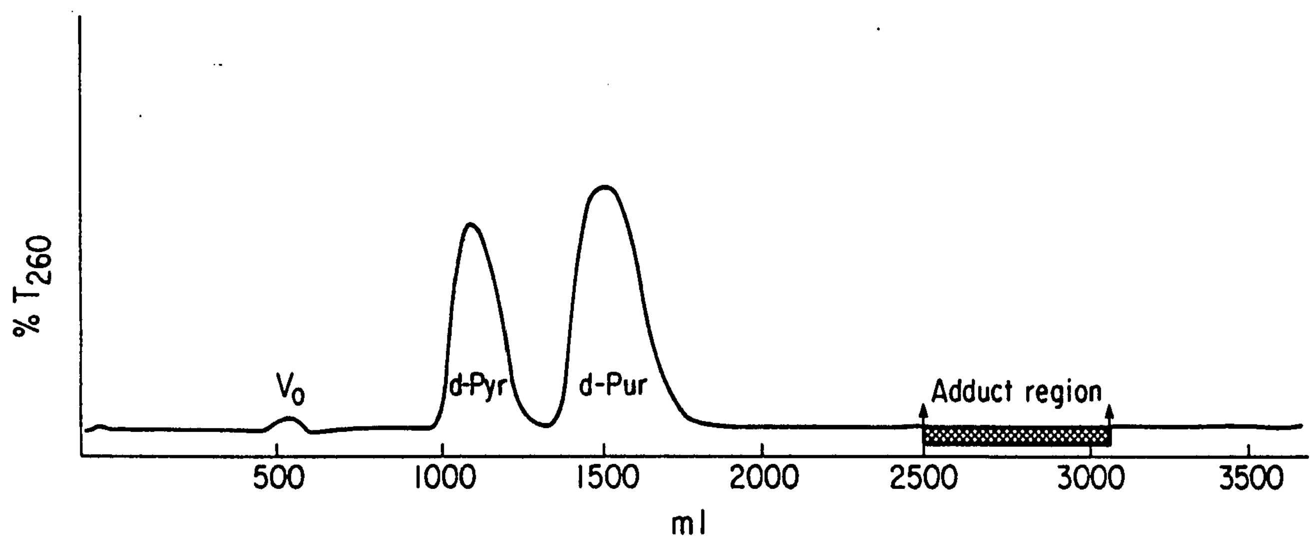


Fig. 15. Sephadex G-25 chromatography of rat liver DNA digested with nuclease P_1/SVD/alkaline phosphatase.

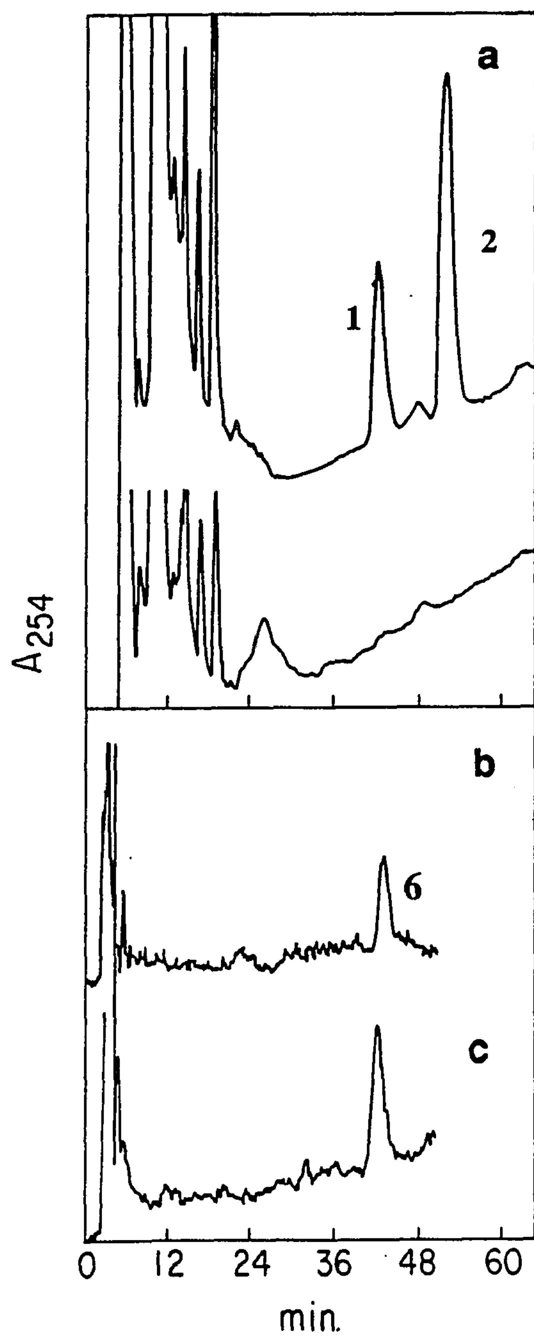


Fig. 16. HPLC patterns of

(a) "Adduct region" from Sephadex G-25 chromatography,

(b) Authentic crosslink **6**, and

(c) "Mixed run" of peak **1** with the same amount of the authentic crosslink **6** as shown in (b).

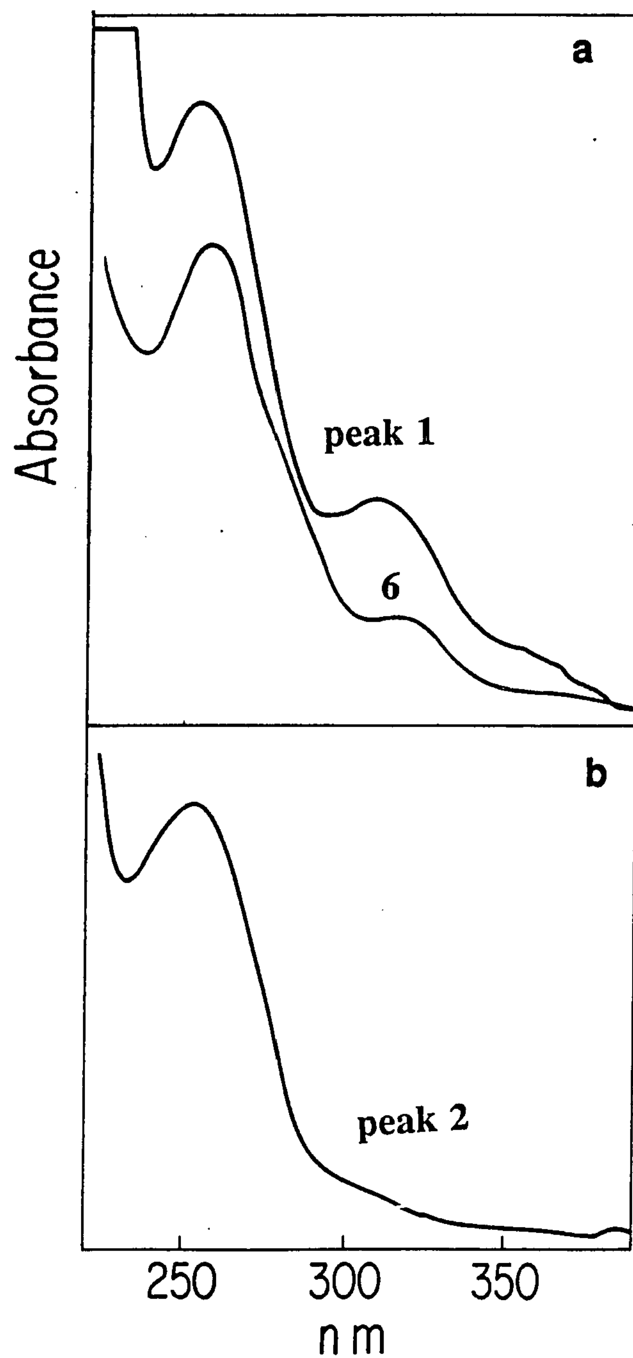


Fig. 17. Comparison of the UV spectra of the synthetic authentic crosslink **6** with those obtained from HPLC of MC-injected rat liver DNA digest.

(a) Peak **1** from Fig. 16 (a) and the authentic crosslink **6**,

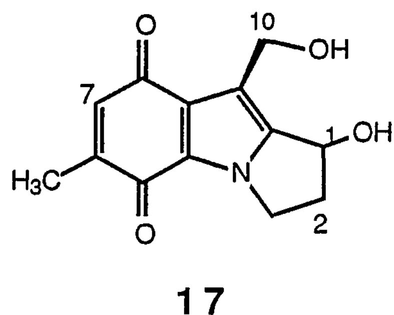
(b) peak **2** from Fig. 16 (a).

DISCUSSION

The detection of covalent adducts of MC formed with DNA in CHO cells and rats confirms recent reports that MC alkylates DNA in the cell.[14,42,64,65] The structures of the adducts were identified, for the first time, indicating that a monofunctional and a bifunctional, crosslink-type adduct (**16a** and **6**) were formed *in vivo*. In case of CHO cells both **6** and **16a** were formed as the two major DNA modifications, whereas in case of rats only the crosslink **6** was isolated. It is possible in the latter case that the monofunctional adducts are fewer in number and are therefore, below the level of detection as their repair kinetics could be different from that of the crosslink adduct. Although no differential rate of excision repair has been reported for MC adducts, such a differential repair of closely related adducts has been observed previously for adducts of *cis*- and *trans*-diaminedichloroplatinum(II),[66,67] the N^2- and C-8 adducts of 2-acetylaminofluorene with guanine,[68] and various adducts of aflatoxin *B*,[69] among others. The appearence of crosslink **6** accounts for the crosslinked behavior of CHO cell DNA observed after treatment of the cells with MC[15,33,34,36] and its formation in rats injected with MC[14] shows that it is probably a general effect of this drug *in vivo*. The identity of the adduct pattern to that obtained upon reductive activation of MC *in vitro* by $Na_2S_2O_4$ indicates that the intracellular activation of the drug is also reductive. An alternative non-reductive, low-pH-activated alkylation of DNA, observed *in vitro*, results in entirely different adducts i.e. N7-alkylated adducts.[70] Direct comparison of reductive adduct patterns of *in vitro* studies with those of *in vivo* from the rats, although unassigned in their specific components, allowed workers to draw the same conclusion previously.[42] The present results indicate, furthermore, that the activation of

MC is bifunctional in the CHO cells: adduct **4a**, the product of monofunctional activation is absent; only **6** and **16a**, the pair resulting from bifunctional activation[14,15,21] (*Scheme I*, page 69) are detectable.

The results obtained with 10-DMC are very interesting as it has been shown for the first time that it crosslinks DNA *in vivo* but not *in vitro*. The AEB fluorescence assay results clearly indicate such crosslinks in the DNA samples isolated from the cells treated with 10-DMC (Table I, page 62). It is known that 10-DMC cannot form crosslinks *in vitro*,[19] but no one tested this *in vivo* before. It has been previously reported that 10-DMC is as mutagenic as MC[6] and even the results obtained in our laboratory indicated that 10-DMC is as lethal to CHO cells as MC when grown in aerobic cultures (Fig. 18). Very recently, Reinhoudt and co-workers[71] have reported that mitosene diol **17** and mitosene diacetate **18** are very active against the gastric tumor xenograft GXF 97. It is possible that the 10-hydroxyl group is converted *in vivo* into a better leaving group by acylation or phosphorylation, thus facilitating crosslinking of DNA. In order to arrive at a definitive conclusion, further investigation utilizing a more sensitive *in vivo* assay is needed. Therefore, studies using alkaline elution method,[72] a sensitive assay for the detection of interstrand crosslinks, are planned which would confirm the crosslinking activity of 10-DMC.



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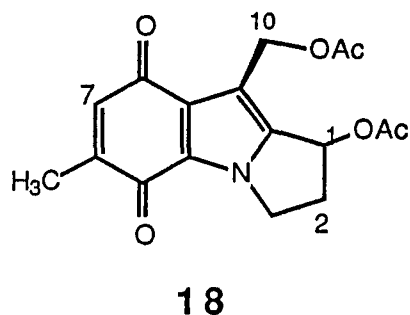

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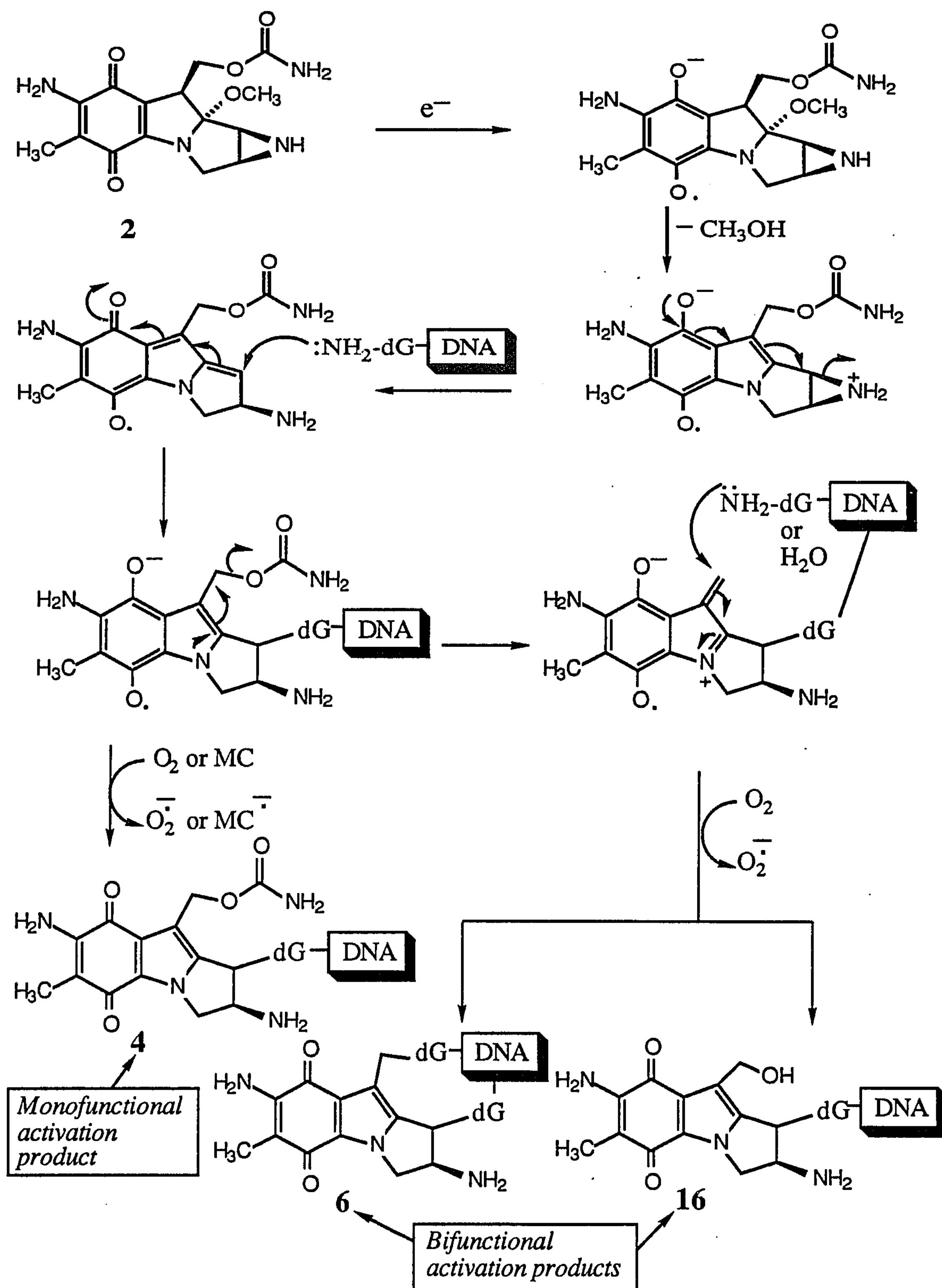


Scheme I. Mechanism of activation of MC.

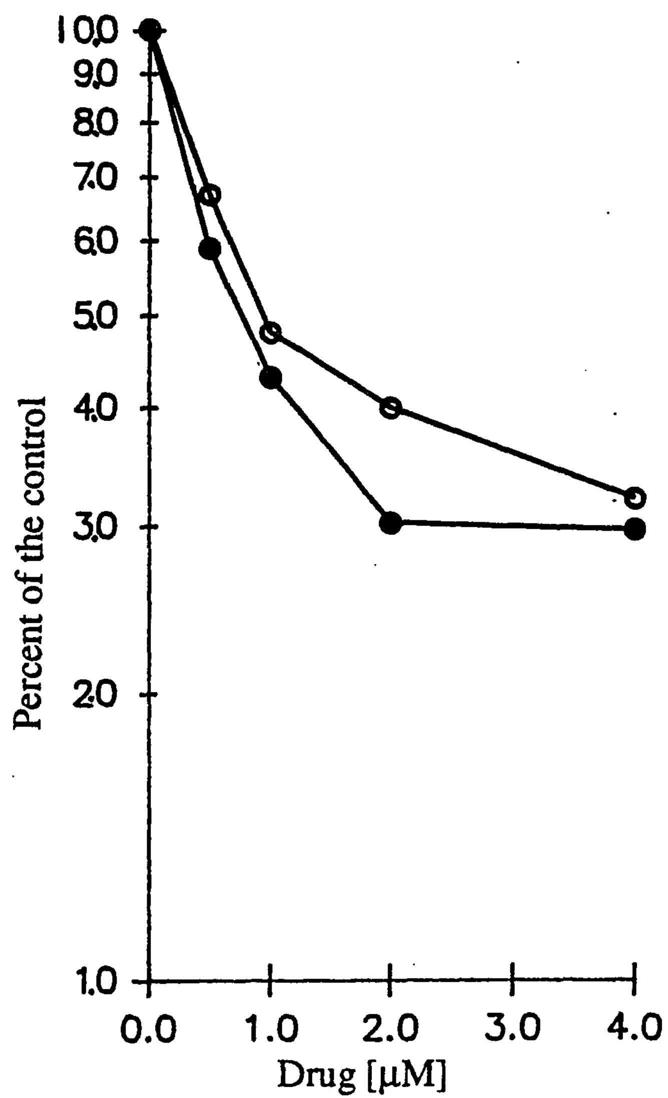


Fig. 18. Percent survival of CHO (K1) cells treated with various concentrations of MC (•) or 10-DMC (o).

It is noteworthy that reductive DNA-alkylation products of MC in CHO cells are formed under aerobic conditions. Isolation of adduct **6** from rat liver represents another instance.[14] The ^{32}P-postlabeled adducts from the rats formed under much lower dose conditions than used here also indicate aerobic DNA-alkylating activity of MC.[42] Very recently, utilizing the same technique, Kato et al.[65] have reported similar kind of results from liver autopsy samples of cancer patients treated with MC. Detection of crosslinked DNA in aerobic tumor cells[34,39] and CHO cells[33] has been interpreted similarly. Thus, DNA-alkylation clearly occurs under aerobic conditions although it may be diminished[33,34] and/or modulated in adduct distribution as compared to that in hypoxic cells.

The cytotoxic effect seen in cells which accompanies the antitumor activity could be from a variety of lesions caused by MC such as production of superoxide or hydroxy radicals, or hydrogen peroxide which are able to damage DNA,[73,74] DNA-DNA crosslinks,[4,33,34,39] DNA-protein crosslinks and other kind of lesions with the cellular macromolecules.[4,21] To date, there is no direct evidence in support of the toxicity due to superoxide or hydroxy radicals nor there is any showing that it is not. Using the alkaline elution technique, Dorr et al.[39] did not observe any DNA single strand breaks in murine L1210 leukemia cells with doses of up to 60 μM MC nor any double strand breaks up to doses of 300 μM. But there are several reports showing a good correlation between the degree of crosslinking and cell toxicity.[33,34] Differential toxicity of MC towards hypoxic and well oxygenated cells is known where it is more toxic to hypoxic cells than to their aerobic counterparts and the antitumor activity of MC against hypoxic solid tumors has been attributed to this selectivity.[34] The increased

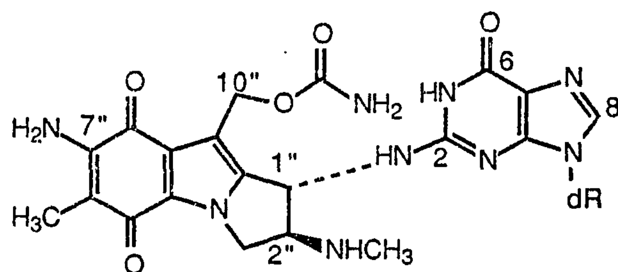
cytotoxicity of these agents in case of hypoxic cells is also shown to be due to the production of more crosslinks.[33,34] Such a property of MC can only be observed at very low drug doses in cell cultures. Taken these findings[33,34] and the mechanism[21] of activation of MC *in vitro* together (*Scheme I*, page 69), it is reasonable to speculate that the differential effect of MC is due to increased bifunctional activation that results in more crosslinking of DNA in hypoxia than in the aerobic cells, since in the latter, O_2 may selectively inhibit the bifunctional pathway. The existence of such inhibition in chemical systems is reported[21] but the *in vivo* systems tested so far showed fully bifunctional activation under aerobic conditions.[12,14,15] Although the present study and work by others[12] clearly showed the formation of adducts in cells, the amounts of drug used by us are larger than biologically customary doses. These findings do not invalidate the above theory, however, since the experiments were conducted under such high MC dose conditions that the O_2 effect would have been "swamped out" i.e. MC was in excess over O_2. Development of highly sensitive assays will be needed to explore further correlations between modes of activation,[21] adduct chemistry and physiological activities of MC. Such techniques, when developed, will allow the use of lower, physiologically relevant doses of MC in order to probe the proposed correlation among cellular O_2 tension, adduct distribution, and MC toxicity. Part III of this thesis addresses to this problem.

RESULTS

Adducts formed with porfiromycin (PM).

When PM or [^{3}H]-PM was treated with calf thymus DNA under reductive activating conditions, using either H_2/PtO_2 or $Na_2S_2O_4$ as reducing agents, complexes of PM-DNA were formed with b.r. of 0.05 - 0.1. The complexes were digested to nucleosides and analyzed by HPLC with the following results:

i) H_2/PtO_2 activation of PM. The only major peak seen in this case was the one with elution time of 36 min which was identified as **19**, a monofunctional PM adduct (Fig. 19a). Its identity was established by the following observations: (i) Similar HPLC elution time corresponding to **4a** arising from MC reaction[17] (44 min) and (ii) ultraviolet absorbance spectral characterstics are similar to **4a** (Fig. 19b).



ii) $Na_2S_2O_4$ activation. The complex of PM with DNA formed under $Na_2S_2O_4$ activation condition is shown in Fig. 20a. This pattern shows two modified nucleosides

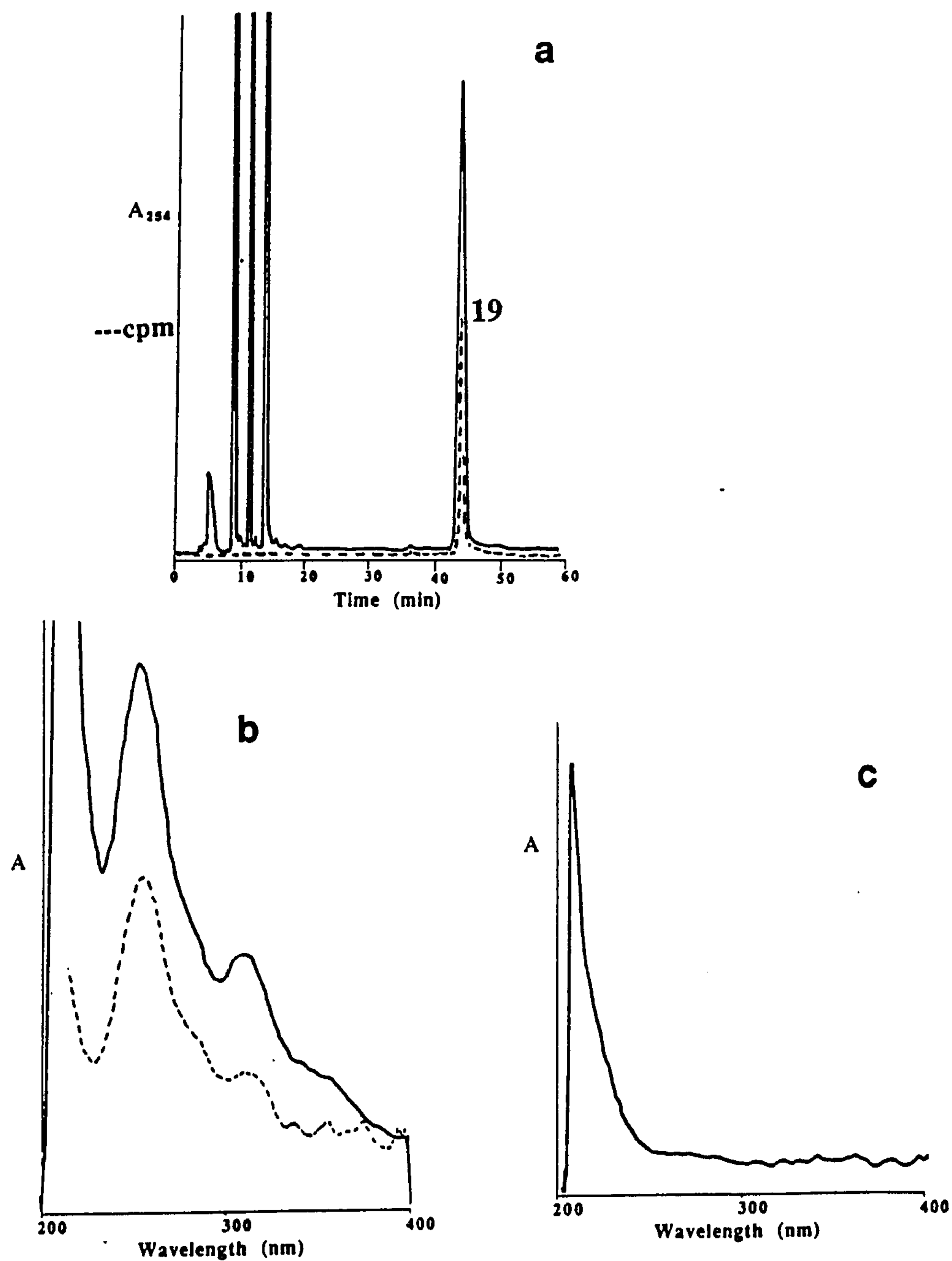


Fig.19. HPLC pattern and the UV spectra from DNase I/SVD/alkaline phosphatase digest of PM-DNA complex. (a) H_2/PtO_2 activation conditions (— A_{254}; --- cpm). (b) "On-the-flight" UV spectrum of peak **1** recorded during its elution (— PM; --- MC). (c) UV spectrum of blank recorded in the same way.

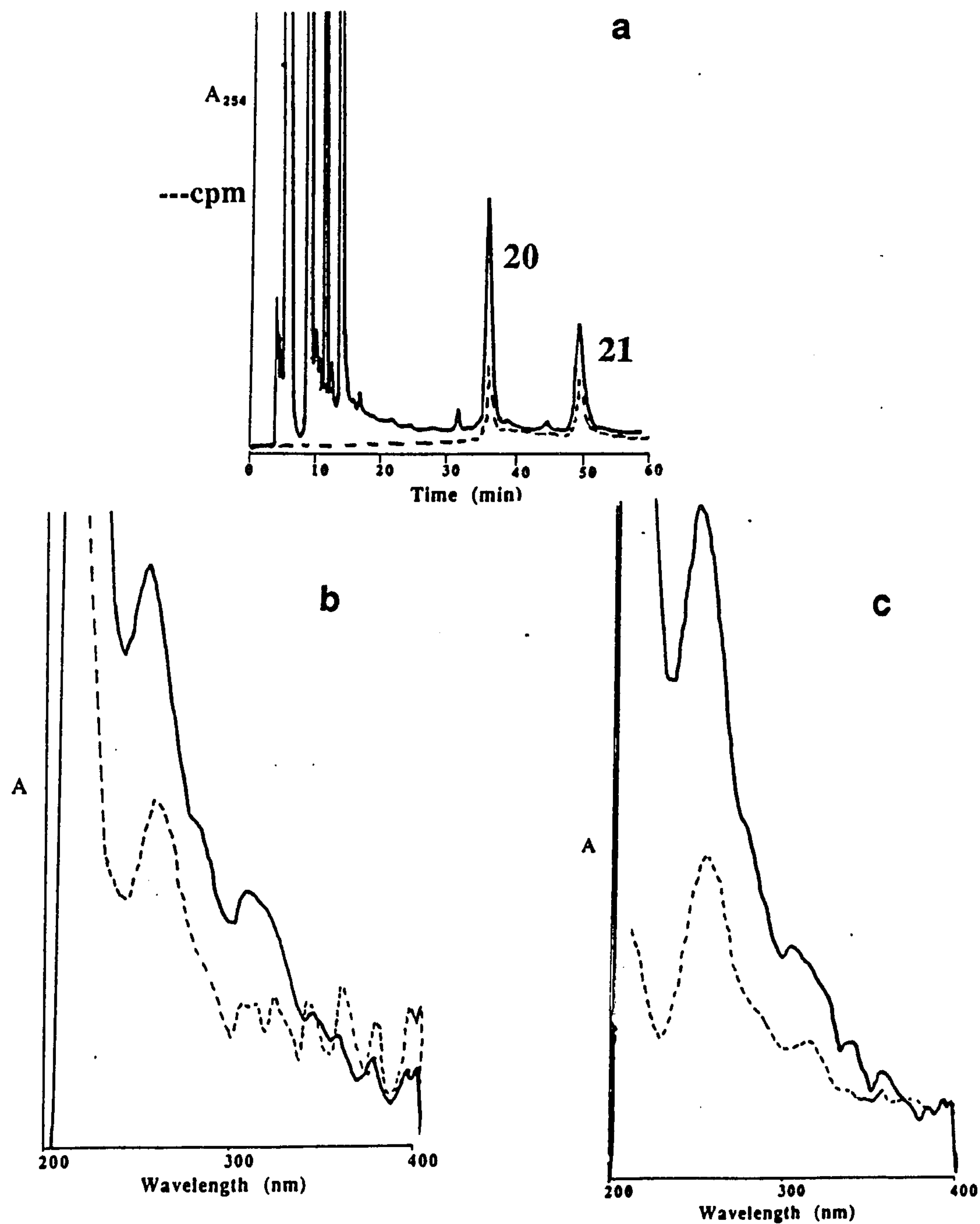
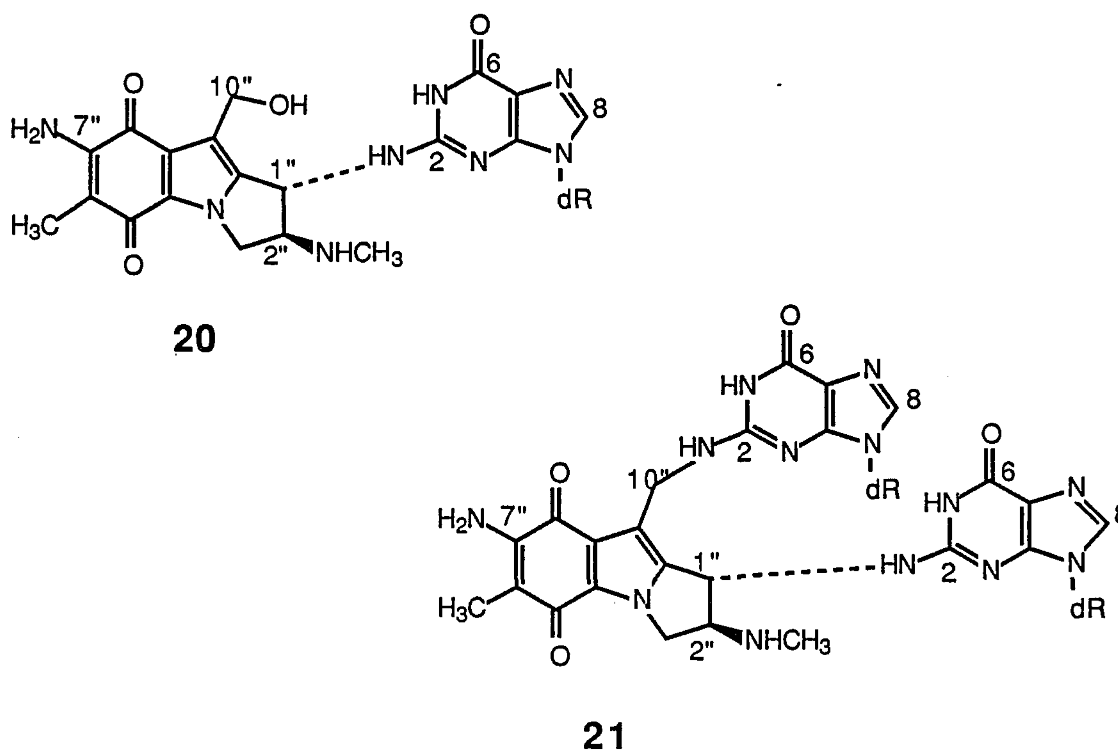


Fig. 20. HPLC pattern and the UV spectra from DNase I/SVD/alkaline phosphatase digest of PM-DNA complex. (a) $Na_2S_2O_4$ activation conditions (— A_{254}; --- cpm). (b) and (c) "On-the-flight" UV spectrum of peak **2** and **3**, respectively, during their elution (— PM; --- MC) .

which have similar elution times as the ones formed with MC under identical conditions[20] (36 min and 49 min, respectively; for e.g. see Fig. 13a) and the ultraviolet absorbance spectra of these compounds was very much similar to the MC-modified Gs, **16a** and **6**, respectively (Fig. 20b-c). The former peak was assigned as **20**, a monofunctional 10-decarbamoyl porfiromycin adduct whereas the latter as **21**, a crosslink of PM with two Gs.



HPLC and liquid scintillation counting of [^{3}H]-PM treated CHO cell DNA digests in search of adducts.

The HPLC patterns of the digest of DNA from CHO cells treated with 10 μM [^{3}H]-PM or vehicle are shown in Fig. 21. The radioactivity of [^{3}H] are plotted in the same

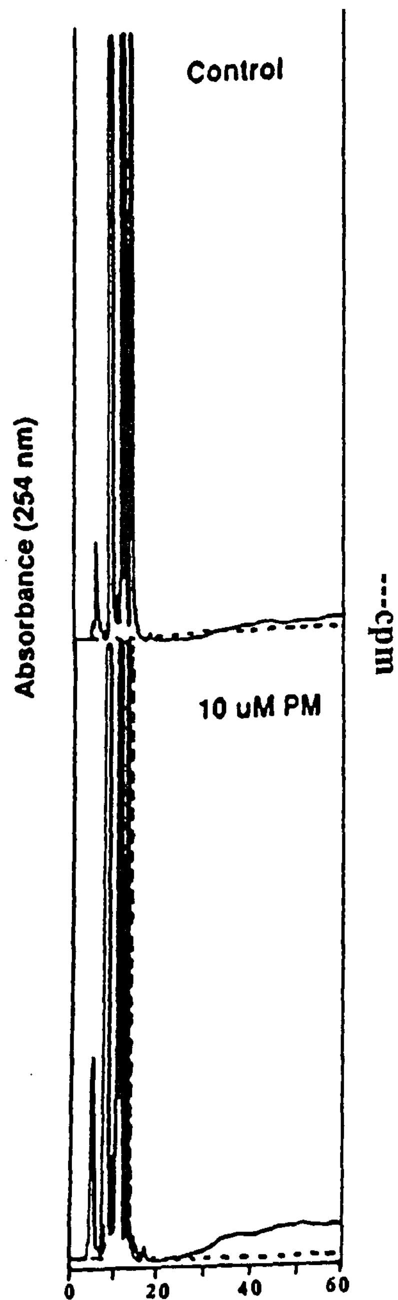


Fig. 21. HPLC patterns and liquid scintillation counting plots of DNA digests from CHO cells treated with 10 μM [^{3}H]-PM.

(a) control,

(b) 10 μM [^{3}H]-PM.

figure. It is evident from the pattern and the [^3H] plot that the label is seen only in the early eluting unmodified nucleosides but not in the drug-modified ones. In order to locate the [^3H] label more specifically, the digest was eluted with a more polar eluent i.e. 4/96 CH_3CN/30 mM KH_2PO_4, pH 5.0 [HPLC coditions (i)] and the HPLC pattern of such a run is shown in Fig. 22. When the cells grown in regular medium (DF-10) were treated with [^3H]-PM, [^3H] label was seen only in purines and thymidine but none in cytidine (Fig. 22b-c and Table II, page 80). On the other hand, the radioactivity disappeared from the nucleosides of digests of DNA obtained from cells grown in HAT medium with the same dose of [^3H]-PM (Fig. 22a).

Detection of crosslinks of DNA by alkaline ethidium bromide fluorescence assay.

The AEB fluorescence assay measurement readings were used in calculating the percentage of DNA crosslinking (Table I, page 62). No crosslinking was detectable in the DNA of CHO cells treated with 10 μM dose of PM although MC exhibited 4% crosslinking at the same dose. The DNA samples from 100 μM MC or PM treated cells showed 13% and 5% of crosslinking, respectively. These results also showed that 10-DMC caused 18 and 23% crosslinking of DNA when used in cells at 10 and 100 μM concentrations, respectively.

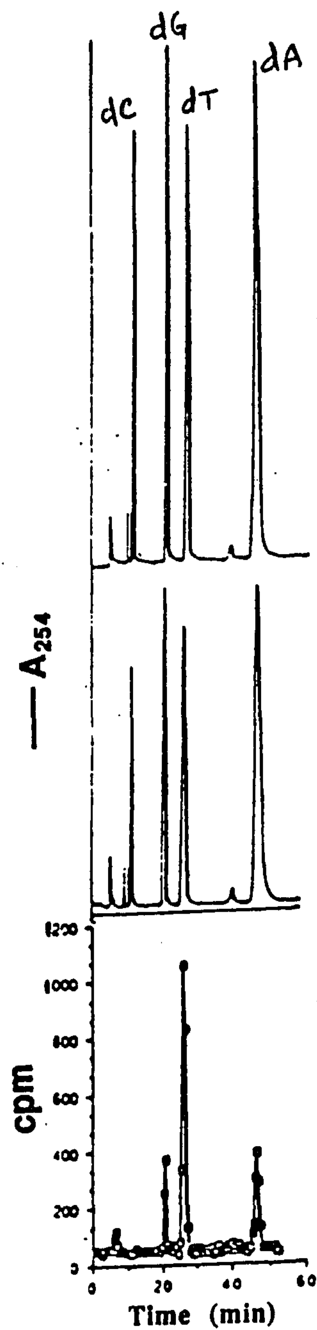


Fig. 22. HPLC patterns and [^{3}H] plot of the digests of DNA from CHO cells treated with 10 μM [^{3}H]-PM.

(a) "HAT" medium,

(b) DF-10 medium,

(c) [^{3}H] plot of the fractions collected from (b).

Table II. Percent distribution of [^{3}H]-label in the nucleosides of DNA from CHO cells treated with 10 μM [^{3}H]-PM.

Cell line	dC	dG	dA	dT
CHO (K1)	-	14.4	27.6	58.1
Balb/c-3T3 (*ts 20*)	-	(52.6)*		47.4
$P388D_1$	-	17.0	35.0	48.0
CV1	-	14.7	25.0	60.3

* indicates the total percent of [^{3}H]-label in the purines.

Authentic standards of MC-modified 5'-nucleotides for ^{32}P-postlabeling assay.

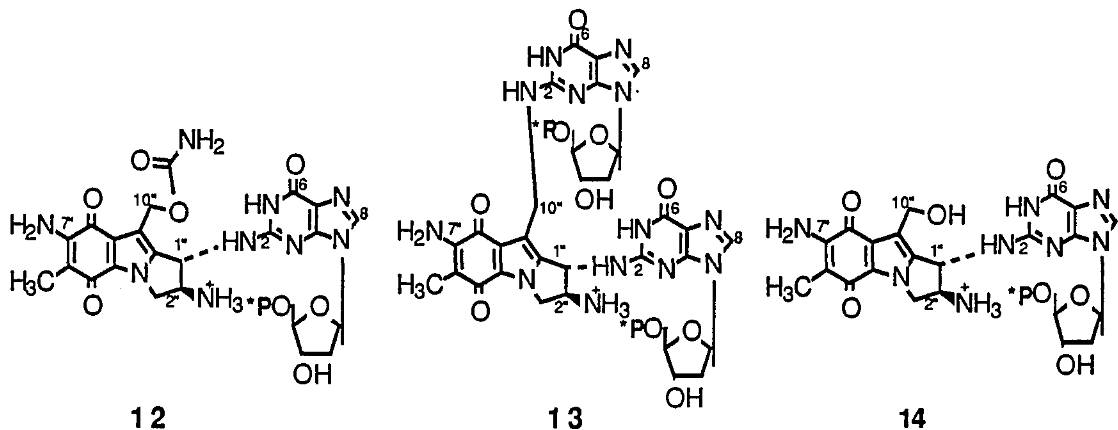
(a) non-radiolabeled standards

The authentic standards of MC-modified 5'-nucleotides, p[dG(MC)] **12**, p[dG(10-DMC)] **14**, and p_2[dG(MC)dG] **13** were made from MC-modified DNA or MC-modified hexamer as described in Materials and Methods (page16) and separated on HPLC using an ion-pairing chromatography [HPLC conditions (iv)] and the absorbance of the eluate was monitored at 310 nm. The patterns are shown in Fig. 23. and the peaks were identified. The HPLC elution times of the standards were as follows:

(i) 5'-p[dG(10-DMC)] **14** was 37 minutes;

(ii) 5'-p[dG(MC)] **12** was 38 minutes; and

(iii) 5'- p_2[dG(MC)dG] **13** was 48 minutes.



(b) ^{32}P-postlabeled standards

The same standards as above but with ^{32}P-label were made utilizing the synthetically modified MC-adducts, d[G(MC)pC] **9a** and d[MC(TACGTA)$_2$] **11**, [γ-^{32}P]ATP and polynucleotide kinase as described in Materials and Methods (page 18). The resulting mixture was separated on HPLC and the ^{32}P counts of the eluate were determined by liquid scintillation counting. The plots of the ^{32}P radioactivity are shown in Fig. 23. There is a good correlation between these plots and the HPLC patterns of the non-labeled standards (Fig.23 & 24).

In order to quantitate the yields of ^{32}P-postlabeled standards, ^{32}P-counts from the fractions corresponding to each peak were added and the number of moles of adduct was calculated using the following formula,

$$\text{\# of moles of adduct labeled with 32P} = \frac{\text{total cpm in the adduct}}{\text{specific activity of [}\gamma\text{-32P]ATP}}.$$

The percent yield of these adducts was calculated as follows

$$\text{\% yield of 32P-labeled adduct} = \frac{\text{\# of moles of adduct labeled with 32P}}{\text{\# of moles of substrate (based on the uv absorbance at 310 nm)}}.$$

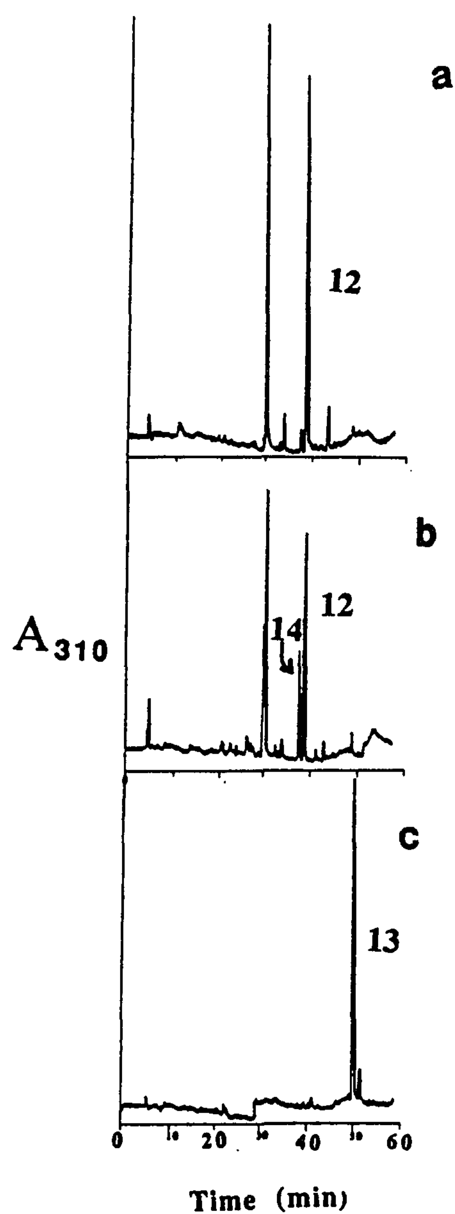


Fig. 23. HPLC patterns of the MC-modified 5'-nucleotide standards (unlabeled)*.

(a) p[dG(MC)] **12**,

(b) a mixture of p[dG(MC)] **12** and p[dG(10-DMC)] **14** resulting from the boiling at 100°C for 20 min of the former compound **12**, and

(c) p_2[dG(MC)dG] **13**.

*The non-numbered peaks in *a* and *b*, are dephosphorylated products of the respective standards.

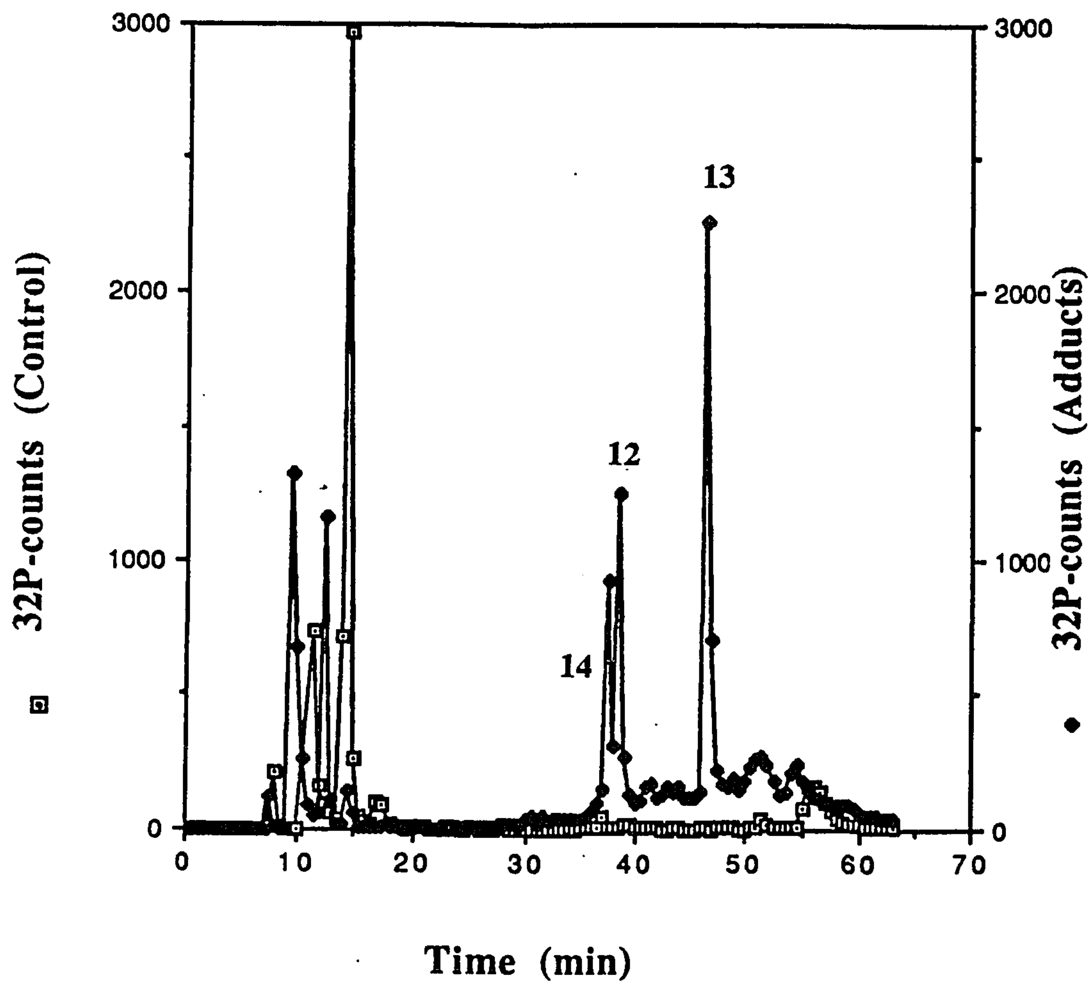


Fig. 24. LS counting plot of the ^{32}P-postlabeled MC-modified 5'-nucleotide standards (*in vitro*). Peaks a, b, and c were identified as p[dG(10-DMC)] **14**; p[dG(MC)] **12**; and p_2[dG(MC)dG] **13**; respectively. The early eluting peaks correspond to $^{32}P_i$ and ^{32}P-AMP.

DISCUSSION

In order to understand the molecular mechanisms of action of carcinogens, mutagens, drugs, or for that matter, any DNA damaging agent, it is very crucial to identify and quantitate the causative lesions that lead to the cell killing. In case of anticancer drugs, if such a study is established then new agents with less toxicity and higher therapuetic index could be conveniently designed for more effective and better treatment of cancer. In the early days, the primary pharmacologic action of nitrogen mustards was thought to be due to crosslinks.[74] Later, the interstrand crosslinking was suggested as a possible crucial effect of many anticancer agents.[4,76,77] Since then, several methods have been developed to measure DNA interstrand crosslinking with high sensitivity. Some of these techniques include alkaline sedimentation, reversible melting, and ethidium bromide fluorescence assay etc. A drawback of these techniques is that they require the isolation of DNA which is fragile. Several other difficulties are faced in tracking down the lethal lesions in intact cells/nuclei. In recent years, with the invention of new techniques like alkaline elution assay[72] and ^{32}P-postlabeling assay,[78] or improvement of the old techniques like alkaline ethidium bromide assay,[79] quite detailed information has been accumulated on the type of lesions caused by numerous agents. Some of these modified techniques don't even need purified DNAs (e.g. alkaline elution) thus avoiding any artifacts resulting from the isolation procedures.

Alkaline elution assay, developed by Kohn and his colleagues[72] is widely used to measure the macromolecular damage of DNA, viz., DNA-protein crosslinks, DNA-DNA crosslinks, single strand breaks and alkali labile sites caused by various DNA damaging agents. In this technique, the elution rate of DNA through the filters

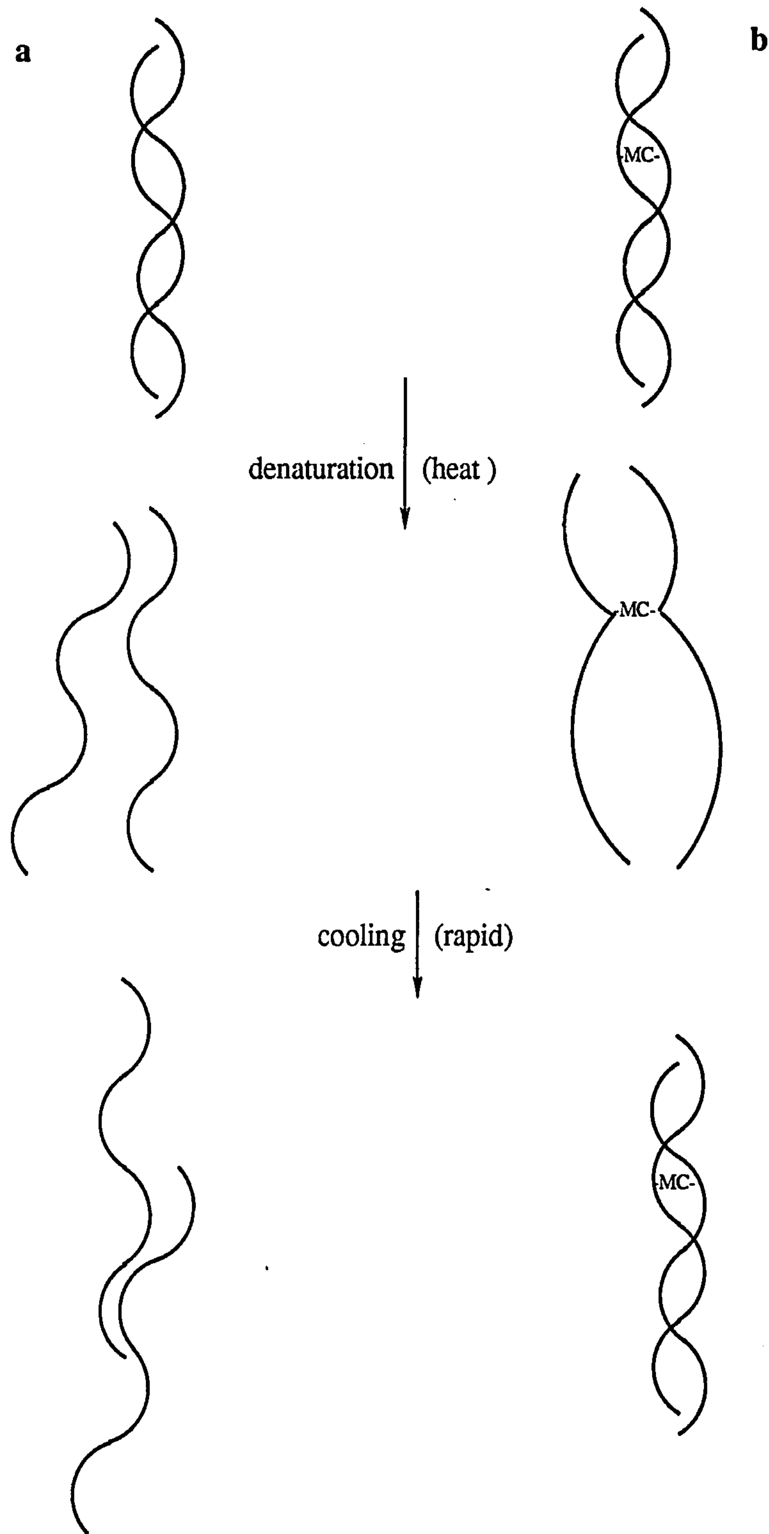
under alkaline conditions is measured. The cells with their DNA labeled (control or treated) are deposited on a membrane filter, lysed with a detergent solution and the lysate is allowed to flow through the filter which removes most of the non-DNA material of the cell. The high molecular weight DNA retarded on the filter is slowly eluted by means of an alkaline solution (pH ~12) which disrupts the hydrogen bonding between paired strands. Interstrand crosslinks reduce the elution rate due to the effective increase in their DNA size. Such a technique had been used in determining the interstrand crosslinks of DNA, introduced by mutagens and carcinogens.[34,36,39,72] The sensitivity of detection is 1 DNA lesion in 10^7 nucleotides.

Alkaline ethidium bromide assay is another type of technique used to determine the inter-strand crosslinks of DNA. This is based on the fact that the fluorescence of ethidium bromide, an intercalator, increases 20-25 fold on binding to double stranded DNA as compared to single stranded DNA. This property of ethidium bromide is employed to detect the double stranded DNA.[79] Interstrand crosslinks are detected by denaturing the DNA by heat or alkali followed by spontaneous renaturation. The high pH (>11.8) values used here prevent the formation of regions of short self-complementarity so that the intercalation sites are destroyed in control DNA and the fluorescence falls to zero. On the contrary, the interstrand crosslink introduced by a drug or carcinogen will serve as a "locked site" for rapid renaturation following the denaturing and renaturing steps. Using this technique, Matsuo and Ross[50] have detected the psoralen crosslinks with a sensitivity of 1.6 crosslinks per 10^7 basepairs.

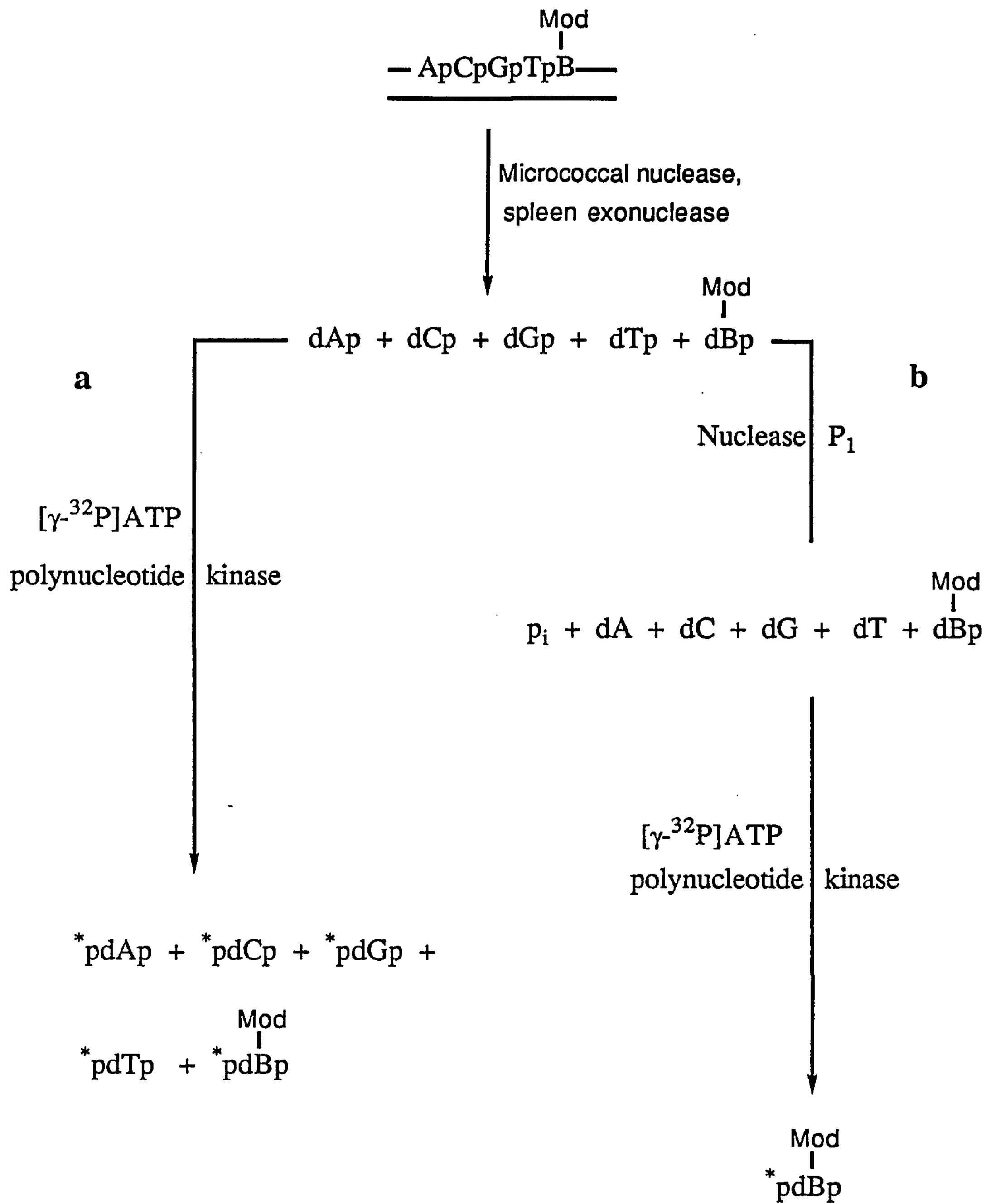
In the early 1960s, Iyer and Szybalski[4] showed the formation of interstrand crosslinks of MC with DNA by utilizing reversible melting technique which is illustrated in

Scheme V (page 88). In this technique, control DNA and MC-crosslinked DNA samples are denatured at high temperature and allowed to renature by rapid cooling. The denaturation of control DNA under these conditions is irreversible, since the alignment of the complementary strands is a slow and temperature-dependent process. On the other hand, introduction of an inter-strand crosslink by MC inhibits the complete separation of the complementary strands, hence, they remain in original alignment. MC-crosslink acts as a nucleation site for "zipping-up" of the complementary strands upon rapid cooling. Therefore the ultraviolet melting curve is reversible.

The crosslinks are not the only lesions of DNA caused by MC, however. Monofunctionally linked covalent adducts of MC and DNA[31] may also be biologically active. In correlating chemical and biological effects of MC, a sensitive assay for analysis of these adducts in living cells is also required. The general HPLC/UV absorbance assay developed by Tomasz et al.[3] is better suited for *in vitro* work only. Although we have demonstrated conclusively the formation and nature of such adducts by using this assay *in vivo*,[14,15,18] the large doses of MC used in those studies are not suitable for serial testing. Unfortunately, radiolabeled MC is not available. As a potential alternative, the ^{32}P-postlabeling assay method of Reddy and Randerath[42] is outlined in Scheme VI (page 89). This method is widely used to detect and quantitate the modified bases of DNA without the use of radioactive mutagens and carcinogens or radiolabeled DNA precursors. The DNA sample (control or treated) is digested with micrococcal nuclease and spleen exonuclease into 3'-mononucleotides. These 3'-nucleotides are then 5'-labeled with [γ-^{32}P]ATP and polynucleotide kinase resulting in the formation of 3',5'-deoxyribonucleoside diphosphates. Thus, the modified and



Scheme IV. Denaturation and renaturation kinetics of (a) control and (b) MC-crosslinked DNA.

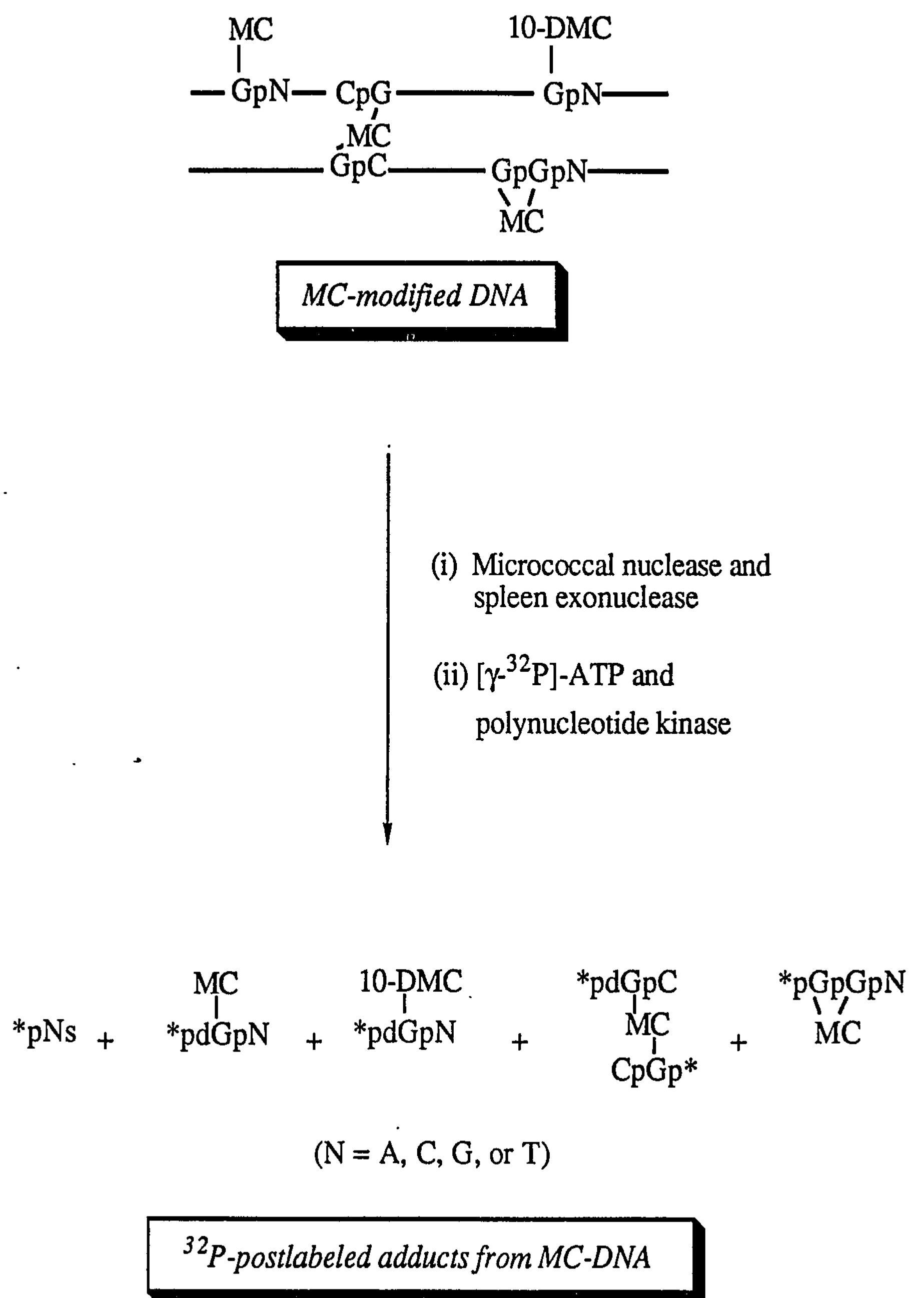


Scheme V. Outline of Reddy and Randerath's ^{32}P-postlabeling.

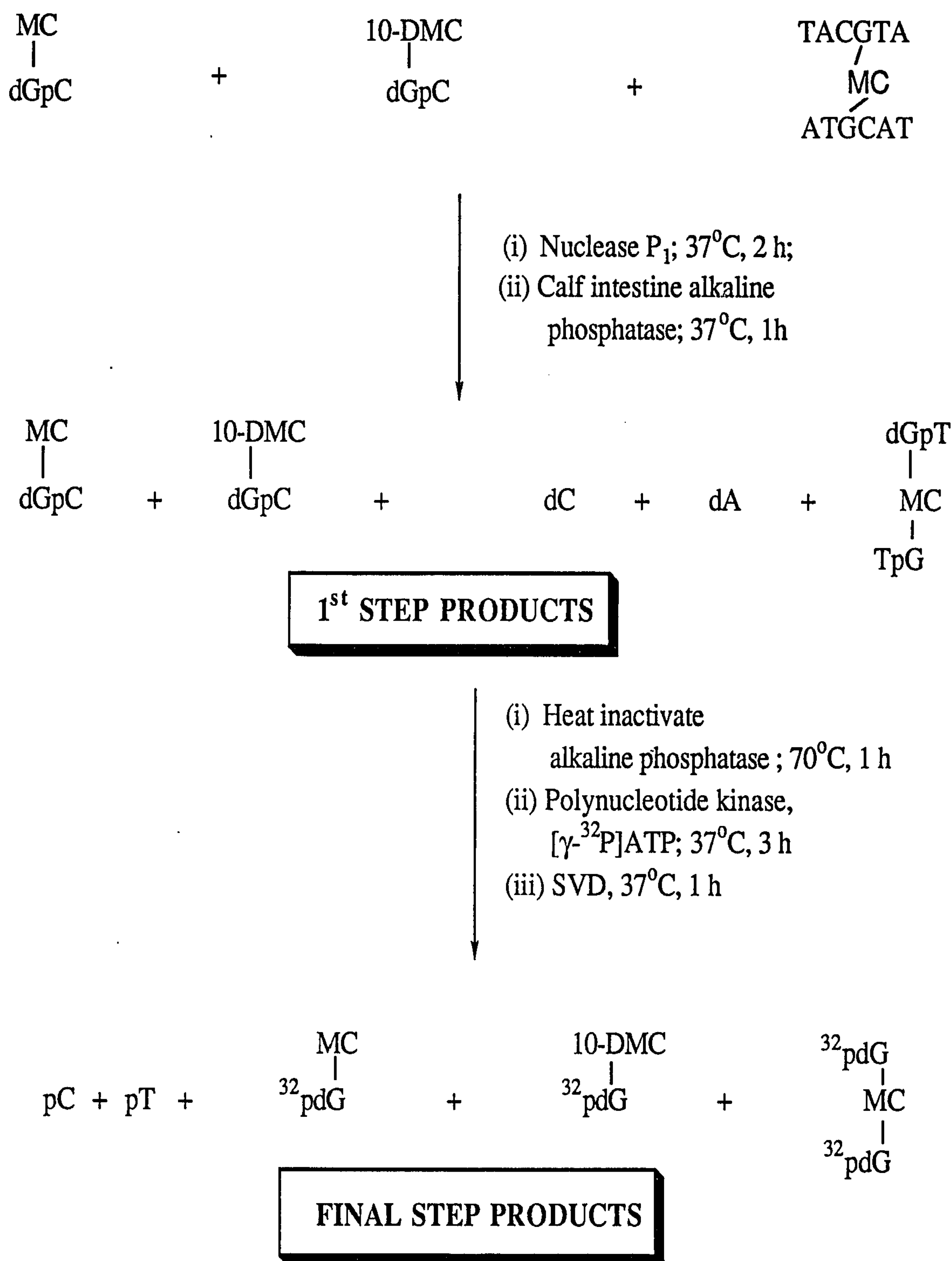
unmodified bases of the nucleosides from DNA are labeled with ^{32}P (*Scheme VIa*, page 89) which are then separated either by thin layer chromatography or HPLC. This technique was used to detect the lesions caused by various mutagens, for eg. safrole (1-allyl-3,4-methylenedioxybenzene), estragole (1-allyl-4-methoxybenzene)[80] and 7,12-dimethylbenz[a]anthracene.[81] The typical sensitivity of this technique is in the range of ~2 adducts in 10^8 nucleotides. Recently, the same authors[82] have reported a modified version of this technique with an enhanced sensitivity by including an incubation step of DNA digest with nuclease P_1 prior to ^{32}P-labeling (*Scheme VIb*, page 89). Nuclease P_1 cleaves deoxyribonucleoside 3'-monophosphates of normal nucleotides to nucleosides but not the adducted ones. Thus, digesting the DNA sample with nuclease P_1 prior to labeling will enrich the modified 3'-nucleotides, the only substrates of polynucleotide kinase. Accordingly, only the adducted nucleotides are ^{32}P-labeled whereas the normal nucleosides are free of the label. This technique was used in the detection of adducts of 7,12-dimethyl benz[a]anthracene, benzo[a]pyrene, 4-aminobiphenyl and MC and it has a typical sensitivity value of about ~1 adduct in 10^{10} nucleotides.[82] By using the ^{32}P-postlabeling assay method, Reddy and Randerath[42] as well as Kato et al.[65] have detected MC adducts in various tissues of rats and autopsy samples of cancer patients treated with MC. But neither of the reports have identified the adducts further. Reddy and Randerath[42] have reported that >90% of the adducts are guanine derived out of which a single adduct accounted for >70%. Adapting such a technique with the authentic *in vitro* standards will help in identifying the molecular lesions occuring in the cell. However, upon repeating the degradation of MC-adducted DNA as reported, we found that micrococcal nuclease and spleen exonuclease systems used in such a study do not cleave phosphodiester bonds of

MC-modified bases (Part I, page 41). Therefore, use of such an enzymatic scheme followed by 5'-end labeling with [γ-^{32}P]ATP/kinase reaction (*Scheme VII,* page 92) will generate a number of species that are 5'-labeled di-, tri-, and tetranucleotide phosphate adducts rather than 5'-mononucleotides.

In view of the above findings, this procedure is clearly unsuitable for MC adduct analysis. Therefore, a modified ^{32}P-postlabeling procedure was developed (*Scheme II,* page 93). The essential feature of this scheme is that we took advantage of our earlier finding[17,18] that nuclease P_1 does not cleave the -G(M)pN- phosphodiester bond.[17,18] Thus, the adducts can be 5'-labeled selectively eliminating the radioactive background due to the labeling of unmodified bases. We applied our modified version of ^{32}P-postlabeling technique to prepare MC-modified *in vitro* standards. The results indicated the formation of 5'-labeled MC-modified nucleotides (Fig. 24). The labeling efficiency was in the range of 2-6%. The low yields of ^{32}P-labeled adducts found here might be due to the presence of residual active alkaline phosphatase which removes the 5'-labeled phosphates from the nucleotides or due to the reduced efficiency of polynucleotide kinase towards MC-modified nucleotides. The first possibility is apparently ruled out after observing that heat inactivated alkaline phosphatase did not hydrolyze uridine monophosphate into uridine. Therefore, the reduced efficiency of polynucleotide kinase might be the cause for the low level labeling. It is reasonable to speculate such an alteration in the enzymatic activity towards MC-modified DNA since earlier studies in our laboratory showed such DNAs to be resistant to various nucleases.[17,18] The CD spectral studies also indicated changes in the conformation of MC-modified dinucleoside phosphates.[61] These results suggest that this modified assay can at least be applied to detect and identify the MC-modified lesions from



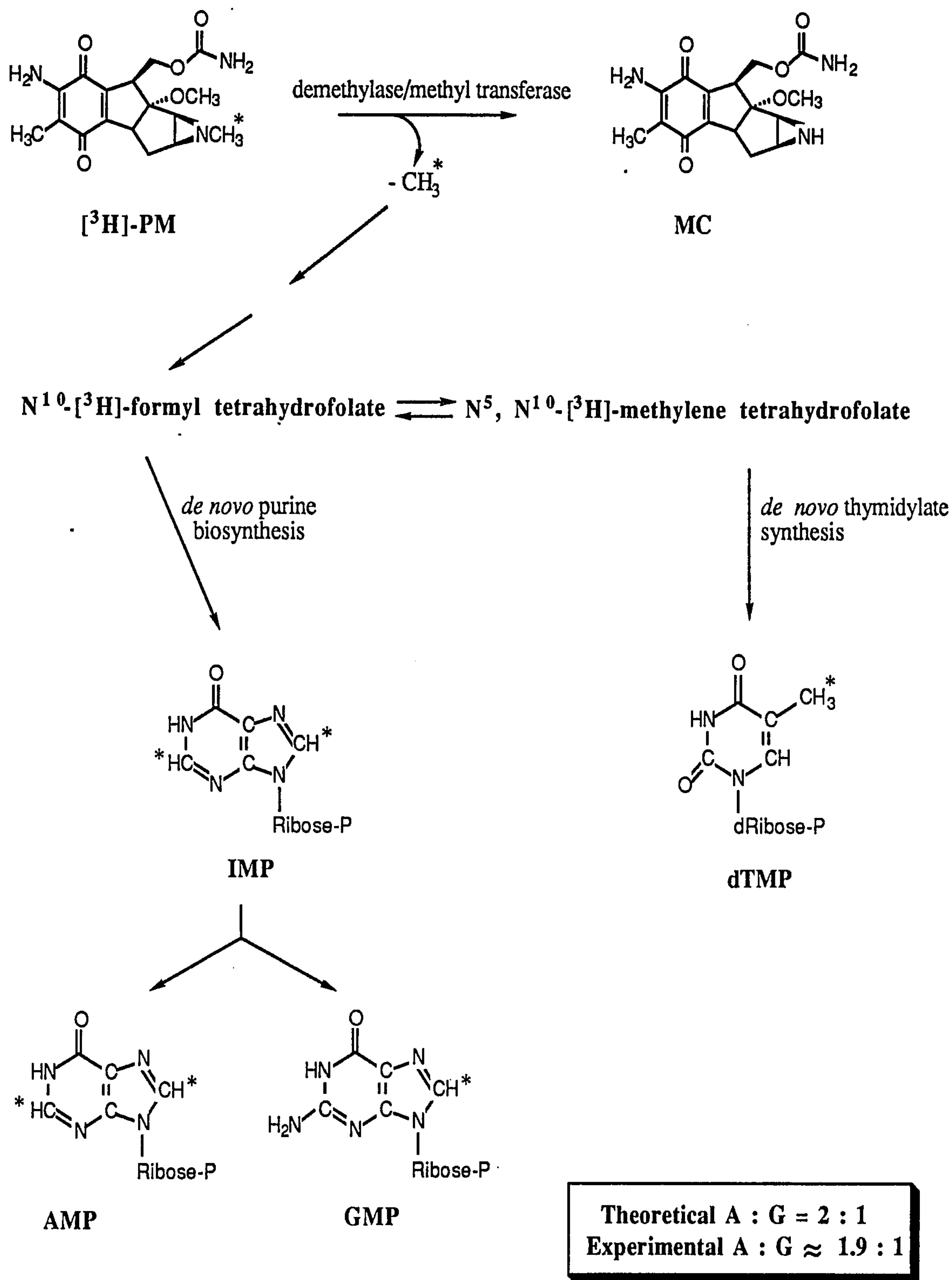
Scheme VI. ^{32}P-postlabeled adducts of MC using Reddy and Randerath's method.



Scheme II. ^{32}P-postlabeling of the MC-modified dinucleoside phosphates and hexamer.

in vivo samples, although it might not be suitable for absolute quantification of such lesions.

Use of PM, a close analog of MC, is increasingly popular for mode-of-action studies of the mitomycins, due to its availability in labeled form.[35] Therefore, in order to identify the PM-modified lesions, we took advantage of the labeled PM, [1a- ^{3}H]-porfiromycin **7** (page 11). The results obtained from the digests of DNA isolated from cell cultures exposed to [^{3}H]-PM were quite unexpected. The HPLC pattern and liquid scintillation counting plots (Figs. 21 & 22) showed [^{3}H] label in the unmodified nucleosides only. Among the unmodified nucleosides, the label was seen only in A, G, and T but none in C. When the cells were grown in "HAT" medium that blocks the dihydrofolate reductase (DHFR) pathway and thus the one carbon transfer involved in *de novo* purine biosynthesis and thymidylate synthesis, no label was incorporated into the nucleosides. These data clearly showed that the incorporation of [^{3}H] is occuring *via* the N^{5}-methyl tetrahydrofolate and the related intermediates. One conclusion that can be drawn here is that the [-CH_3^*] group is removed from PM by the action of some demethylases or methyltransferases thus converting it into MC. The [-CH_3^*] group might get into the tetrahydrofolate (THF) pools and lead into N^{10}-formyl THF* and N^{5},N^{10}-methylene THF* from where the [^{3}H] label gets into the bases. Interestingly enough, the distribution ratio of the label between A and G was about 1.9 : 1.0 which is very similar to the theoretical value of 2.0 : 1.0 calculated based on *de novo* synthesis pathway of purines, assuming that the [^{3}H] label is coming from N^{10}-formyl THF* (*Scheme VIII,* page 95 and Table II, page 80). The demethylation of [^{3}H]-PM is seen in a variety of cell lines tested ranging from mouse, hamster (CHO cells, Balb/c-3T3



Scheme VIII. Path of incorporation of [^{3}H]-label from [^{3}H]-porfiromycin into purines and thymidylate.

cells, and P388D_1 cells) to primate (CV_1) cells. In view of these findings, the data published by several investigators[34,36] using PM are questionable as to whether the results observed are due, in fact, to PM itself or MC. Furthermore, in 1965, Lisio and Weissbach[83] reported the localization of [^{14}C]-PM in E.*coli* and λ-particles after lysogenic induction of E.*coli* K_{12} (λ) with 1.9 μg/ml of [^{14}C]-PM for 30 min. No further analysis of the adducts of [^{14}C]-PM with DNA was reported from that study. Once again, the label observed by these authors might be from [^{14}C] associated with the unmodified purines and thymidine itself.

We wanted to get independent information as to whether any drug interacted directly with DNA in the cells in the above experiments regardless whether in the form of PM or MC. This can be assessed by the assay for crosslinks which does not require labeling of the drug.The results from alkaline ethidium bromide fluorescence assay of DNA samples from cells treated with MC and PM showed the presence of crosslinks (Table I, page 62). Thus, treatment of cells with 10 μM and 100 μM MC resulted in 4% and 13% crosslinking, respectively, whereas PM showed 5% crosslinking at 100 μM and none at 10 μM dose. The difference in percent of crosslinking seen in tissue cultures by such a study with PM and MC (5% *versus* 13% at 100 μM dose) is in agreement with the reports that PM is less toxic to aerobic cells than MC.[34] A difference in the degree of crosslinking by these two agents is reported by Fracasso and Sartorelli.[34] Thus, these results clearly indicated a difference between the control and the drug-treated DNA samples suggesting that these drugs do crosslink DNA in the cell.

In view of these findings, it is attractive to speculate the following, at least, in the systems tested using [^{3}H]-PM:

(i) it might be getting converted into MC by demethylases or methyltransferases,

(ii) the cytotxic effects and crosslinking effect of this drug seen in tissue cultures might be due to MC.

It would be interesting to see if this is the case in other cell lines including solid tumors. If this were to be the case, then the demethylases and/or methyltransferases might play an important role in the cytotoxic mechanism of PM. A detailed study of the distribution of concentrations of demethylases and methyltransferases among the normal *versus* the tumor cells and aerobic *versus* hypoxic cells and the rates of formation of cytotoxic lesions by MC and PM has to be carried out in correlating such effects with the cytotoxicity. If the amounts of demethylases and methyltransferases are different in the above mentioned cell lines, then it might be possible to explain the observed differential cytotoxic effects of MC and PM.[34,36].

CONCLUSION

The main objective of this last part of the thesis, viz., developing a sensitive *in vivo* adduct assay, has not been achieved completely because of the following problems:

(i) the radiolabeled drug, [^{3}H]-PM, could not be used due to the demethylation process seen in tissue cultures;

(ii) the ^{32}P-postlabeling assay, modified by us, when used with MC-DNA from *in vivo*, showed a background of ^{32}P-counts from unreacted [γ-^{32}P]ATP which made

detection of the low counts of adducts impossible. The method could be applied to detect MC-lesions from *in vivo* samples, once the unreacted [γ-^{32}P]ATP is removed. Such a process could be accomplished by the application of a combination of chromatographic and/or gel electrophoresis techniques. Accordingly, purification of the samples by anion-exchange chromatography prior to HPLC might retain [γ-^{32}P]ATP selectively, while the ^{32}P-labeled adducts may be eluted earlier thus eliminating radioactivity from the unreacted [γ-^{32}P]ATP in the HPLC eluate.

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