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Effects of the bifunctional alkylating agents mitomycin C and nitrogen mustards on the structure of DNA

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EFFECTS OF THE BIFUNCTIONAL ALKYLATING AGENTS MITOMYCIN C AND NITROGEN MUSTARDS ON THE STRUCTURE OF DNA

by

ANIL KUMAR CHAWLA

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.

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ABSTRACT

EFFECTS OF THE BIFUNCTIONAL ALKYLATING AGENTS MITOMYCIN C AND NITROGEN MUSTARDS ON THE STRUCTURE OF DNA

by

Anil Kumar Chawla

Adviser: Professor Maria Tomasz

Mitomycin C (MC) can alkylate DNA monofunctionally or bifunctionally depending upon the activation of one or both masked functions of this drug. One can select monofunctional or bifunctional activation of MC using appropriate conditions of reducing agents. The mechanism which governs such activation was determined by analysis of adducts distribution as showm by high-performance liquid chromatography (HPLC) of enzymatically digested complexes of M. luteus DNA and poly(dG-dC). Effects of MC adducts on DNA conformation and dynamics were determined to assess the altered conformational behavior of selectively modified DNA and poly(dG-dC). We showed by means of circular dichroism (CD) and HPLC that all types of Z-DNAs are substrates for monofunctional alkylation by MC and remain in the Z-form. Upon bifunctional activation of MC, Z-form of poly(dG-dC)/Co^{3+} reverts to B-form and crosslinked adduct is formed. No such Z->B shift is possible in poly(dG-m^5dC) thus in this case MC binding is terminated at monofunctional stage. Experimental results showed a good correlation with computer assisted energy minimized molecular models. These results indicate that Z-DNA can not be crosslinked by MC.

Effects of alkylation products of nitrogen mustards were also determined on B->Z transitions of poly(dG-dC). Bis (2-chloroethyl)methylamine (HN2) was used as a bifunctional and N,N-dimethyl 2-chloroethylamine (HN1) as a monofunctional alkylating agent. Extent of modification of the guanine was determined by HPLC of perchloric acid digested samples. Results showed a good correlation between the loss of intensity of 187 nm band of vacuum CD and the extent of modification of guanine in all complexes. B->Z inhibition was observed by all complexes and it may be due to the interruption of cooperative B->Z transition process as samples were denatured at the alkylated base pairs.

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Finally, I must acknowledge my parents and all my family members for their continuous moral support and blessings. With their feet firmly planted in Indian soil, they have taught me that battles hard won are long treasured. Once again I thank you.

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LIST OF ABBREVIATIONS

AAAF	N-acetoxy-2-acetyl aminofluorene
br	Binding ratio (mole MC/mol mononucleotide)
CHO	Chinese hamster ovary
HN1	N,N-dimethyl 2-chloroethylamine
HN2	Mechlorethamine, [bis(2-chloroethyl)methylamine]
CD	Circular dichroism
EDTA	Ethylenediaminetetraacetic acid
HPLC	High-performance liquid chromatography
MC	Mitomycin C
NADH	Reduced nicotinamide adenine dinucleotide
NMR	Nuclear magnetic resonance
OD	Optical density
Rf	Retardation factor
SVD	Snake venom diesterase
TEAA	Triethylammonium acetate
TFE	Trifluoroethanol
TE	Tris EDTA buffer
TLC	Thin layer chromatography
Tris	Tris (hydroxymethyl)aminomethane
UV	Ultraviolet
θ	Molar ellipticity

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ABSTRACT
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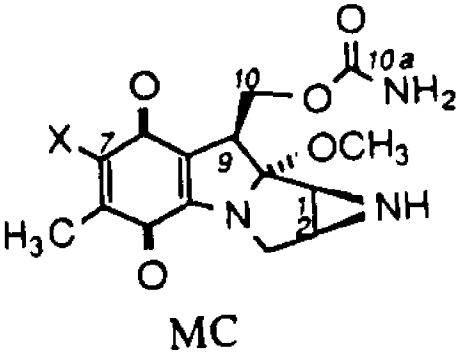
MECHANISM OF ALKYLATION OF DNA BY MITOMYCIN C
by
Anil Kumar Chawla

Adviser: Professor Maria Tomasz

Mitomycin C (MC) can alkylate DNA monofunctionally or bifunctionally depending upon the reductive activation of one or both masked alkylating functions of MC. We sought to determine the mechanism which governs the monofunctional or bifunctional reactivity of MC with DNA. Adduct distribution was analysed by High-performance liquid chromatography (HPLC) of nuclease-digested MC-DNA complexes. Complexes of M. luteus DNA were made under varying conditions in vitro using various reducing agents. Reducing agents such as H_2/PtO_2 and xanthine oxidase activated the MC mostly monofunctionally and $Na_2S_2O_4$ activated the MC bifunctionally under similar conditions. Excess MC suppressed the bifunctional activation of MC by H_2/PtO_2 but excess PtO_2 had promoting effect. 10-Decarbamoyl-MC reacted with DNA monofunctioanlly under all conditions. Oxygen inhibited bifunctional activation of MC in the $Na_2S_2O_4$ systems. We conclude from our results that MC is activated first at C-1 and then at C-10 position. Activation at latter position may be selectively inhibited by oxygen or other kinetic inhibitors. The DNA base sequence determines the ratio of crosslinked adduct (bifunctional activation) and its co-product (monofunctional decarbamoyl-MC) in O_2-free $Na_2S_2O_4$ system.

INTRODUCTION

The mitomycins which were discovered in 1950's are a series of chemically related antibiotics produced by Streptomyces verticillatus[1,2], and its related species. Among all members of mitomycins, mitomycin C (MC) (Figure 1) was developed rapidly as an antitumor agent for clinical use in cancer chemotherapy. Mitomycin C came in the United States in 1974 when Bristol Laboratories introduced it into the medical practice.



MC

Figure 1: Molecular structure of mitomycin C (MC).

Mitomycin C is known to interact covalently with DNA, both monofunctionally and bifunctionally. Bifunctional attachment of MC to DNA is manifested by the reversible melting behavior of MC-exposed DNA, attributed to covalent crosslinks between the complementary strands[3] and by the covalent association of the ultraviolet (UV) chromophore of MC to DNA[4]. These processes require reduction of MC into a transiently activated form, which is thought to occur in cells & can be mimicked easily in vitro

chemically or enzymatically[3]. Mitomycin C has been termed as prototypical bioreductive alkylating agent[5,6] as it requires activation by intracellular catalysts such as NADPH cytochrome P-450 reductase and xanthine oxidase [7-9]. These effects make MC unique among the known antibiotics. Such effects represent the ultimate molecular basis of biological activity of MC as indicated by the parallels with a number of known "DNA damaging agents": selective inhibition of DNA replication[3]; strong induction of SOS response[10], sister chromatid exchange[11], cross-resistance or hypersensitivity of bacterial[3,12] and mammalian[13] cells to UV light and MC.

The cytotoxic and antitumor activity of MC is believed to be a direct consequence of DNA alkylation; both binding modes, monofunctional & bifunctional have been implicated as biologically deleterious to the organism[3,14-18]. The crosslinks were suggested to be the direct cause of the observed inhibition of DNA synthesis and bacteriocidal effect upon exposure to drug[19].

Several investigators have concentrated their attention on understanding the chemical nature and the groups involved in the binding of mitomycin to DNA. It was hypothesized that C1 and C10 carbon atoms constituted the two reactive sites involved in the bifunctional cross-linking activity[3] and that C7 as a third reactive site could not be excluded[20]. Replacement of the amino group at C7 by methoxy or hydroxyl group was indicated not to greatly effect the lethal cross-linking activity of mitomycins[3].

In recent years, several bioreductive alkylation mechanisms have suggested C1 and C10 to be the binding sites during the active association of MC with DNA[6,21,22] or RNA[23]. Subsequently C1 has been confirmed to be one of the linking sites[24].

The C-1 aziridine and C-10 carbamate groups are the two masked alkylating functions which become "allylic" and therefore activated upon reduction of the quinone system & consequent spontaneous elimination of methanol from 9/9a position; then may be displaced by two nucleophiles of DNA, resulting in MC-DNA crosslink. This hypothesis was amended[6] by speculating that both displacements are of S_N1 types, facilitated by resonances with the indolhydroquinone system of reduced MC, taking place sequentially as shown in the Scheme I.

Most aspects of the scheme were verified by employing a large variety of enzymatic & chemical reducing systems as well as model low-molecular weight nucleophiles[22,25-30]. Evidence was also offered by several investigators that these model reactions take place in the semiquinone rather than hydroquinone reduction state of mitomycin C[29-32].

Although the striking covalent adduct association with DNA was discovered more than 20 years ago until lately the general assay for "cross-linked" DNA and association of the UV chromophore of MC with DNA were the only experimental indications of the alkylation process. But recently[24,33], the reaction products of reductively activated MC with DNA itself were isolated in the laboratory of Tomasz & their structures

elucidated in a collaborative effort between the groups of Tomasz at Hunter and Nakanishi at Columbia University as shown in the scheme II. It is apparent from the structures that reductively activated MC reacts with N2 position of guanine, in the minor groove of DNA, either monofunctionally (structures 1 & 2) or bifunctionally (structure 3).

The MC cross-links represent a novel antibiotic-DNA adduct and also play a key role in the antitumor activity of the drug. This crosslinked adduct was also shown to be formed in vivo upon injection of MC into rats[33].

The studies detailed herein have led to the finding that choice of the reducing system for MC activation has a profound influence on whether MC reacts as monofunctional or bifunctional DNA-alkylating agent. The distribution of the three adducts showed a striking dependence on the conditions of the reductive activation in vitro, employment of a system tailored to produce maximum monofunctional activation by H_2/PtO_2 and bifunctional activation by $Na_2S_2O_4$ as reducing agent. Studies herein determined the mechanism which governed the formation and distribution of these adducts <u>in vitro</u>.

MATERIALS & METHODS

MATERIALS:

Materials and their sources are as follows: Calf thymus DNA (type I; sonicated before use) and bacterial alkaline phosphatase from Sigma; M. luteus DNA (sonicated before use) from Worthington Biochemicals; mitomycin C from Bristol Laboratories, 10-decarbamoyl-MC was synthesized from MC[34]. Poly(dG-dC) from Pharmacia P-L Biochemicals; DNAse I and SVD (snake venom phosphodiesterase) from Cooper Biomedical.

METHODS:

Preparation and isolation of MC-DNA or MC-poly(dG-dC)complexes under various reductive activating conditions.

A: H_2/PtO_2 AS REDUCING AGENT:

(a) Standard Reaction: M. luteus DNA (0.67 umole/ml), MC (0.33 umole/ml) and PtO_2 as a catalyst (100 ug/umole MC) were mixed in 0.015 M Tris.HCl, pH 7.4 at room temperature and was deaerated by bubbling helium for about 10 minutes, followed by reduction by bubbling hydrogen[35] for 5 minutes and then helium again for 5 minutes. As a result, the blue color of MC changed to dark violet in the solution. The mixture was exposed to air, filtered and chromatographed on Sephadex G-100 (size 2.5 x 28 cm) using 0.02 M ammonium bicarbonate buffer.

Determination of the binding ratio (br; mole of MC per mole of mononucleotide) of the resulting MC-DNA complex or MC-

poly(dG-dC) complex was accomplished by a spectral method[4]: bound mitomycin was determined by uv absorbance of the complex at 310 nm using E310 of 11,500 for denatured DNA and 11,000 for native DNA. A more detailed method for determination of binding ratio has been reported elsewhere[4].

(b) Reaction Using 5-fold excess MC: Method is same as for the standard condition above, except that five fold excess MC (1.65 umole/ ml) was used in such reactions.

(c) Reaction using 10-fold excess of PtO_2: Same as the standard conditions above except that ten-fold excess PtO_2(1 mg/umole MC) was used in such reactions.

B: $Na_2S_2O_4$ AS REDUCING AGENT:

(a) Standard Reaction: DNA (0.67 umole/ml) and MC (0.33 umole/ml) in 0.015 M Tris.HCl buffer, pH 7.4 were treated with 0.06 M aqueous $Na_2S_2O_4$ (1.5 mole per mole of MC) under anaerobic conditions by bubbling helium gas for 25 minutes.

(b) Reaction in the presence of air: Same as the standard reaction above, except that air instead of the helium was bubbled through the reaction mixture.

C: XANTHINE OXIDASE/NADH AS A REDUCING AGENT:

(a) Standard Reaction: DNA (0.67 umole/ml), MC (0.67 umole/ml) and NADH (0.67 umole/ml) were incubated in 0.015 M Tris.HCL buffer, pH 7.4 with xanthine oxidase (0.5 unit/umole MC) for 20 minutes at 37 C under anaerobic (helium) atmosphere.

(b) Reaction with 10-fold excess NADH: same as above except that

10-fold excess NADH and 2-fold excess xanthine oxidase were used in this reaction.

Preparation of complexes of DNA with 10-decarbamoyl MC: The procedures were analogous to those of MC (as described above) in the standard reaction, except that the concentration of 10-decarbamoyl MC was 1.34 umole/ml in the reaction mixture and pH was 8.0 rather than 7.4.

Reactivation of DNA-bound monofunctional MC residues: MC-DNA complex (1 umole/ml) prepared by the H_2/PtO_2 activation method was treated with 1.5 umole/ml $Na_2S_2O_4$ under anaerobic (helium atmosphere) conditions in 0.015 M Tris.HCl buffer, pH 7.4 for 25 minutes at room temperature. The mixture was reexposed to air and then return of purple color was observable. The complex was isolated by Sephadex G-100 chromatography[4].

Digestion of MC-DNA complexes by nucleases: MC-DNA complexes (about 3 A_{260} units/ml) in 0.005 M Tris.HCl/0.001 M $MgCl_2$ buffer, pH 7.0 were digested to nucleosides and nucleoside-drug adducts at 37 C with enzymes according to the following protocol[24]: DNAse I (1.25 units/A_{260} unit) at 0 hr and 1 hr; SVD (1.25 units/A_{260} unit; pH increased to 8.2) at 2 hr and 5 hr; alkaline phosphatase(0.5 unit/A_{260} unit) at 7 hr and incubation continued until 24 hr.

HPLC separations: A reverse phase column (Beckman Ultrasphere

ODS; 1.0 x 25 cm) was used; flow rate was 2.0 ml/minute. Eluent was 8:92 CH_3CN/0.03 M potassium phosphate buffer, pH 5.0. Quantitation of peaks were accomplished by measurement of peak areas by triangulation.

RESULTS

Distribution of adducts 1-3 in MC-DNA complexes formed under various reductive conditions:

Brief treatment of a mixture of MC and calf thymus DNA in neutral buffer with either H_2/PtO_2, NADPH cytochrome c reductase, or xanthine oxidase resulted in covalent complex formation of MC with DNA. Such variation of the reductive activating agent did not significantly affect the binding ratio (br) which was typically in the range 0.04-0.07. Poly(dG-dC), however, formed complexes that exhibited a higher br (0.10-0.12). The digest of the calf thymus DNA-MC complexes and poly(dG-dC)-MC complexes were analysed by HPLC (Figure 2):

i) H_2/PtO_2 activation of MC : With the exception of the early-eluting unmodified nucleosides, only one major peak is evident in the pattern (Figure 2a) at 39 minute (adduct 2) and two minor adducts 1 and 3 were obtained. On the other hand, the presence of a larger excess of MC completely suppressed formation of 1 and 3 (Figure 2b); excess PtO_2 suppressed 2 (Figure 2c).

ii) MC-calf thymus DNA complexes formed under enzymatic activation (NADPH-cytochrome c reductase or xanthine oxidase) yielded patterns essentially identical to those obtained using H_2/PtO_2 (Figure 2d). When the amounts of enzyme and NADH were increased compared with the standard reaction, the relative amounts of 1 and 3 increased appreciably (Figure 2e).

Effect of O_2 on adduct distribution: Using $Na_2S_2O_4$ as activating agent, the standard anaerobic reaction yielded the pair 1 and 3 (Figure 3a); when air was bubbled through the reaction mixture, however, 2 became the predominant adduct (approximately 50% of the total three; figure 3b).

Adducts formed with 10-decarbamoyl-MC: 10-decarbamoyl-MC readily formed a complex with DNA under H_2/PtO_2, xanthine oxidase/ NADH, or $Na_2S_2O_4$ activating conditions. The single major adduct after enzymatic digestion was identified as 1 (Figure 2f); no trace of 3 could be detected. The only minor adduct, eluting later, is the 1^N- isomer of 1 (unpublished results).

Conversion of monofuctionally bound MC residues in DNA to bifunctional ones by reductive reactivation: MC-DNA complex, br 0.065, was prepared under H_2/PtO_2 activation (standard reaction; Materials and Methods), and its adduct distribution was determined by enzymatic digestion, followed by HPLC analysis of the digest (1:2:3 = 5:80:15). An undigested portion of the complex was reduced with $Na_2S_2O_4$ for a brief period and then allowed to be reoxidized by air. Compared with the original, this complex showed no change in br but a large change in adduct distribution (1:2:3 = 30:0:70). This indicates that the reduction of the MC residues, bound in the original, mostly monofunctionally substituted complex, converted all of the DNA-bound 2 type adducts into DNA-bound 1 and 3 (Figure 4). In an

analogous experiment using H_2/PtO_2 for the reduction of the complex, no change in the original adduct distribution was observable. All these results are summarized in the scheme III.

MC-DNA complexes in the presence of dicoumarol:

Calf thymus DNA (0.67 umol/ml), MC (0.67 umol/ml), 1 mM dicoumarol and PtO_2 catalyst (100 ug/umol of MC) were mixed in 0.015m Tris-HCl, pH 7.4 and hydrogenated for 16 minutes (The resulting complex gave br of 0.017). HPLC results yielded adduct 2 as the major adduct (Figure 5a). These results are similar to standard reaction where no dicoumarol was used. When reaction was carried out in the presence of air and 2 mM dicoumarol using $Na_2S_2O_4$ as reducing agent, HPLC patterns (Figure 5b) did not show any significant difference in adducts distribution as compared to the HPLC results of similar reaction but without the presence of dicoumarol (Figure 5c).

DISCUSSION

The differential binding of MC to DNA under reduction by $Na_2S_2O_4$, as opposed to reduction by H_2/PtO_2 or flavoenzymes, can be rationalized. Crucial for the rationale of this proposal was the discovery of autocatalytic activation of MC by Peterson and Fisher[28] in which a catalytic amount of electron equivalents induces stoichiometric activation of MC in a chain reaction to yield monofunctionally substituted mitosenes as final products. [A similar chain reaction in the case of mitomycin B was reported][30]. Accordingly, Tomasz and her collaborators[33] postulated that after an initial reduction and alkylation step the monoadduct species 2 may react further by either of two pathways: i) electron transfer to unreacted MC (autocatalytic activating conditions analogous to the Peterson-Fisher mechanism), thereby losing its activated state and giving monoadduct 2 as end product, or ii) retro-Michael elimination of the C-10" carbamate to give 3, which is receptive to a second nucleophilic attack giving bifunctionally alkylated end products 1 and 3 (Scheme IV). The actual balance of the two paths should depend on the particular reaction conditions in a predictable, experimentally testable manner. The results presented here represent such experimental testing, as follows.

In our system DNA is the substrate reacting with MC; adduct 2 is the product of monofunctionally activated MC (along

with <5% of its 1"-b isomer[24]), while adducts 1 and 3 are diagnostic coproducts of bifunctional activation formed as an obligatory pair[33]. The latter findings are explained by considering that in DNA only a fraction of the guanines are at cross-linking distance from another guanine, i.e., those in a GpC, CpG,or GpG sequence; only that fraction can give 3 upon reaction with MC. MC bound to other guanines will react with H_2O at its second activated position at C-10"; therefore, the product in that case will be 1 (Scheme IV)[33]. The present experiments consisted of varying the reaction conditions and observing their effects on the distribution of 1-3. The first conclusion drawn from the results is that the initial monofunctional step of DNA binding always occurs at C-1 of MC, yielding monoadduct 2. This simply follows from the fact that an alternative, C-10-linked monoadduct has not been observed. That 2 is precursor in its reduced form to 1 or 3 was also shown directly, by conversion of DNA-bound 2 in a second, separate reduction step to 1 or 3 (Figure 4,Scheme III).

Reduction kinetics determine the monofunctional/bifunctional activation ratio. Hydrogen/PtO_2, observed to act as a monofunctional activating agent to MC[22,25,35-37], was shown now to be fully capable of bifunctional activation, simply when a larger amount of PtO_2 was used (Figure 2a,c). Xanthine oxidase/NADH also switches from a purely monofunctional to a partly bifunctional activating catalyst when the enzyme + NADH/MC

ratio is increased (Figure 2d,e). In contrast, a larger excess of MC dramatically suppresses bifunctional product formation (Figure 2a,b). In these experiments the rate of reduction of MC is what varies, and the results indicate clearly that this determines the product distribution. Note that H_2/PtO_2 gives the same, full bifunctional activation as $Na_2S_2O_4$, under appropriate conditions (Figures 2c & 3a). These results constitute proof for the concept that underlies this mechanism (Scheme IV), which predicts that if the reduction rate is slow, the monofunctional, autocatalytic activation pathway predominates because $2^{\cdot -}$ is inactivated by excess MC faster than it is formed (as shown by Peterson and Fisher[28] for activation of MC in the absence of DNA; in their case the nucleophile was water). Alternatively, if the reduction rate is fast, $2^{\cdot -}$ will accumulate and have a long enough lifetime to undergo the second activation step ("bifunctional activation pathway").

S_N1 Displacement of the C-10" Carbamate Group. The second activation step of MC is indicated in Scheme IV as S_N1 displacement of carbamate, aided by iminium ion formation. This is more likely than S_N2 displacement on theoretical grounds. The net negative charge of the anion radical should greatly stabilize such an intermediate. Model experimental studies provided support for the iminium mechanism, at least in methanolic solutions and in the absence of DNA. That the displacement of the C-10" leaving group is indeed the critical step toward the

bifunctional pathway (Scheme IV) is confirmed by the finding that 10-decarbamoyl-MC and DNA yield 1 but not 3, even when the strictly bifunctional activating agent $Na_2S_2O_4$ is used (Figure 2f)[38], indicating the lack of bifunctional potential of 10-decarbamoyl-MC. This result is consistent with previous reports that this analogue does not cause cross-links in DNA in vitro[11] or in vivo[15]. Evidently, bifunctional activation requires a good leaving group at C-10", such as carbamate rather than OH-; i.e., elimination from C-10" is rate determining in the second activation step.

Effect of O_2 on the activation pathway. It is remarkable that the overall binding of MC to DNA in the presence of O_2 was decreased by only 20% as compared with the anaerobic counterpart of this experiment. Thus, it seems that, despite the potential inhibitory effect of O_2 on the reduction step itself, much of the MC has become monofunctionally activated. However, the overall activation pattern has changed from a purely bifunctional (1 and 3; Figure 3a) to a mixed mono and bifunctional one (1-3; Figure 3b). This result, as originally predicted from Scheme IV, indicates that O_2 indeed inactivated some of the initial monofunctional product 2 and thereby selectively decreased the extent of bifunctional activation.

These results, all taken together, confirm the proposed mechanism governing monofunctional versus bifunctional alkylation of DNA by MC. The main characteristic of this mechanism is the

potential for either autocatalytic or stoichiometric reduction kinetics. The results also apply to reductive alkylation reactions of MC and its analogues in general. Several additional comments regarding Scheme IV may be made :

i) The formulation of the reduction as one-electron type was adapted in light of several recent reports implicating the MC semiquinone radical anion ($MC^{\cdot -}$) rather than the previously assumed MC hydroquinone (Scheme I)[39] as the species giving rise to the subsequent activation steps[29,31,32]. The present findings have no bearing on this matter and adapt themselves to either formulation; i.e. if the MC hydroquinone proves to be the active species, then OH should be substituted for $O^{\cdot}$ in position 8" in the appropriate formulas in Scheme IV .

ii) A feature of the present mechanism is that, regardless of whether the semiquinone or hydroquinone is the active species, once it is formed it will suffice for activation of both alkylating functions of MC. Although it is demonstrated that $Na_2S_2O_4$ is capable of reactivating monofunctionally bound MC in a subsequent process to form cross-links (Figure 4), such second reduction is not necessary under conditions of the bifunctional pathway. It also seems a priori unlikely, on the basis of steric considerations, that in vivo the MC already bound to DNA monofunctionally could be a further substrate to a reductase[31].

DNA alkylation mechanisms and cellular toxicity of MC. Since MC-DNA adduct patterns are diagnostic of the partitioning of MC

between the monofunctional and bifunctional activation pathways, analysis of the adducts formed in vivo should give direct information on the course of activation in the cell. The cross-link adduct 3 has shown to form in cellular DNA upon injection of rats with a high dose of MC[33]; recent results in our laboratory[33] indicate formation of 1 and 3 in CHO cells treated with MC, also under high-dose conditions. Thus, in these cells bifunctional activation of MC has occurred, indicating the absence of excess (unreduced) MC at the MC-DNA binding sites, because excess MC would have inactivated the second function (Scheme IV). O_2 apparently did not cause such inactivation either. In accord with the latter point, the formation of cross-linked (i.e., bifunctionally alkylated) DNA in MC-treated tumor cells was detected by others[40,41] under aerobic conditions using the alkaline elution technique.

It is known, however, that MC is less toxic to aerobic cells than to hypoxic ones and the antitumor activity of MC against hypoxic solid tumors has been attributed to this selectivity[42]. The differential toxicity can only be observed at very low drug doses in cell cultures. In view of the mechanism (Scheme IV) it is attractive to speculate that the effect is due to increased bifunctional activation of MC, and therefore more cross-linking of DNA, in the hypoxic cells than in the aerobic ones, since in the latter O_2 may inhibit selectively the bifunctional pathway. The present work demonstrates the existence of such inhibition in a chemical system (Figure 3), but the in

vivo systems tested so far (rat, CHO cells) showed fully bifunctional activation under aerobic conditions[33]. The latter 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". More sensitive adduct detection techniques, when developed, will allow the use of lower, physiologically more relevant doses of MC in order to probe the proposed correlation among cellular O_2 tension, adduct distribution, and MC toxicity.

No effect of dicoumarol on monofunctional or bifunctional activity of MC (in vitro). Dicoumarol is a potent inhibitor of DT-diaphorase, an enzyme which metabolizes MC to less toxic or nontoxic products. Dicoumarol was found to increase the toxicity of MC to cells in culture under hypoxic conditions [43]. It was then postulated that this is due to an increase in production of reactive metabolites from MC[43]. Our results indicated no effect of dicoumarol on the alkylating activity of MC under, anaerobic or aerobic conditions in vitro. Such results are consistent with the hypothesis that in vivo dicoumarol does not effect the activity of MC directly but through the involvement of DT-diaphorase.

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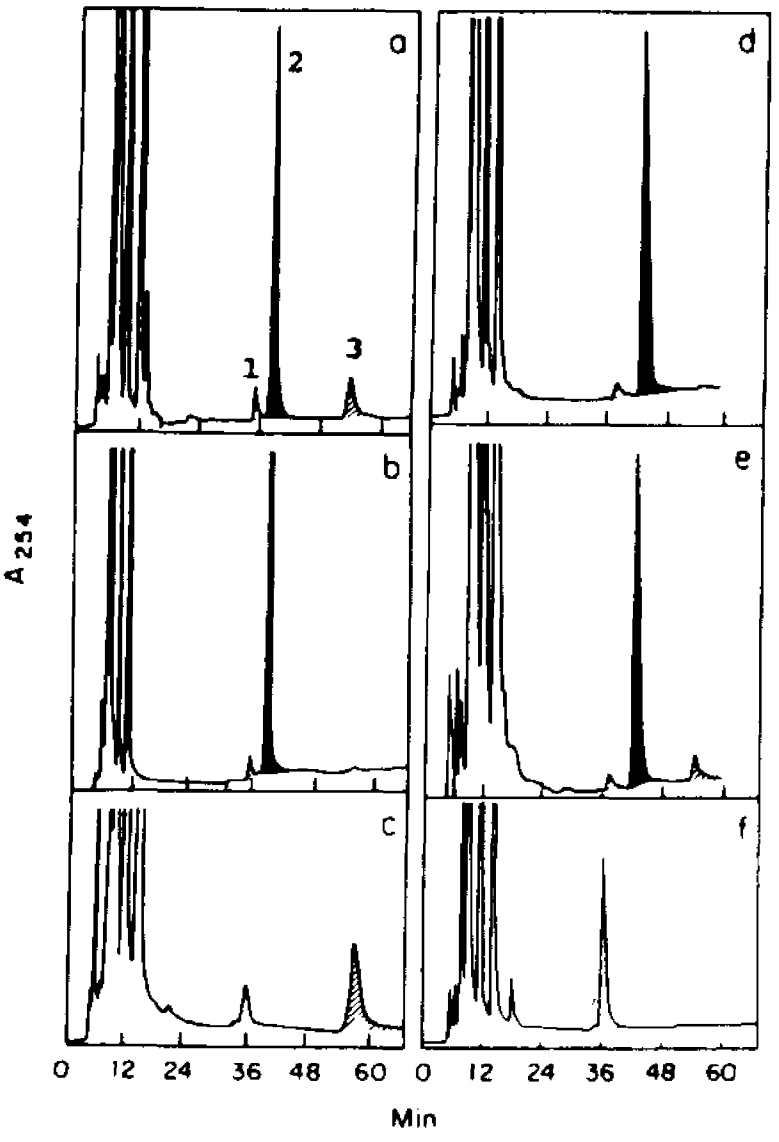


Figure 2: HPLC patterns from DNase I/snake venom diesterase/alkaline phosphatase digests of MC-DNA complexes formed under various activating conditions: (a) H_2/PtO_2 standard reaction; (b) H_2/PtO_2, 5-fold excess of MC; (c) H_2/PtO_2, 10-fold excess of PtO_2; (d) Xanthine oxidase/NADH, standard reaction; (e) Xanthine oxidase/NADH, 10-fold excess of NADH, 2-fold excess of xanthine oxidase; (f) H_2/PtO_2 standard reaction, analogue 10-decarbamoyl-MC used instead of MC for complex formation with DNA. The cross-identity of peaks in the various experiments is indicated by their identical shading.

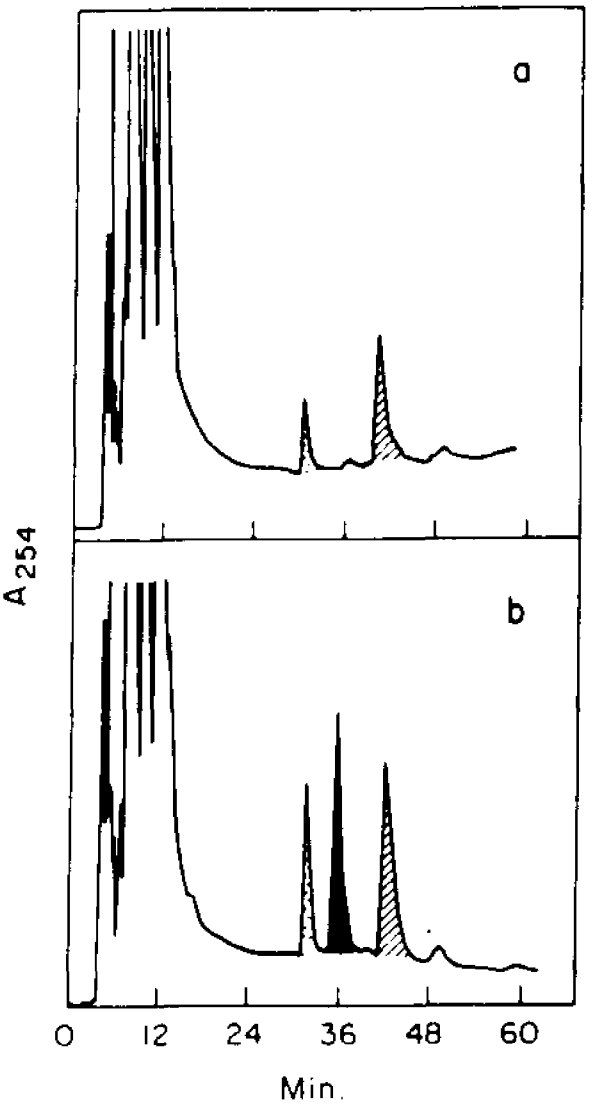


Figure 3: Effect of O_2 on adduct distribution: HPLC patterns from DNase/snake venom diesterase/alkaline phosphatase digests of MC-DNA complexes formed under (a) anaerobic $Na_2S_2O_4$ activation conditions and (b) $Na_2S_2O_4$ activation in the presence of air.

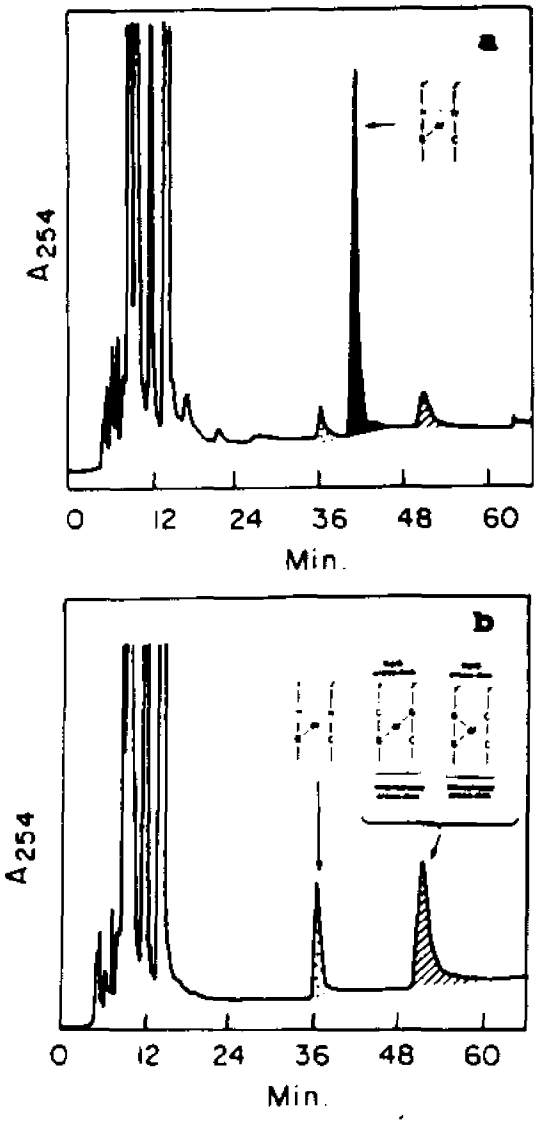


Figure 4: Conversion of monofunctionally bound MC residues in DNA to bifunctional ones upon reactivation: HPLC patterns of the nuclease digest of (a) MC-DNA complex and (b) same complex after exposure to $Na_2S_2O_4$ as reactivator.

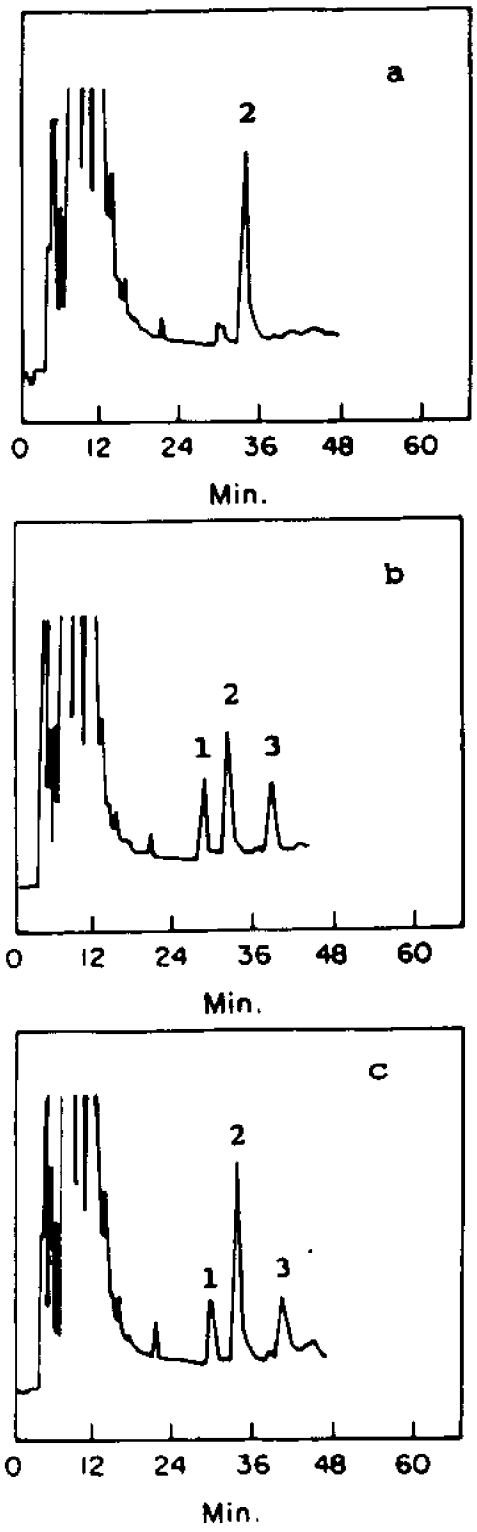


Figure 5: HPLC patterns from DNAse/snake venom diesterase/alkaline phosphatase digests of MC-DNA complexes made in the presence of dicoumarol. (a). H_2/PtO_2 activating conditions in presence of 1mM dicoumarol. (b). $Na_2S_2O_4$ activation in the presence of air. (c). $Na_2S_2O_4$ activation in the presence of air and 2mM dicoumarol.

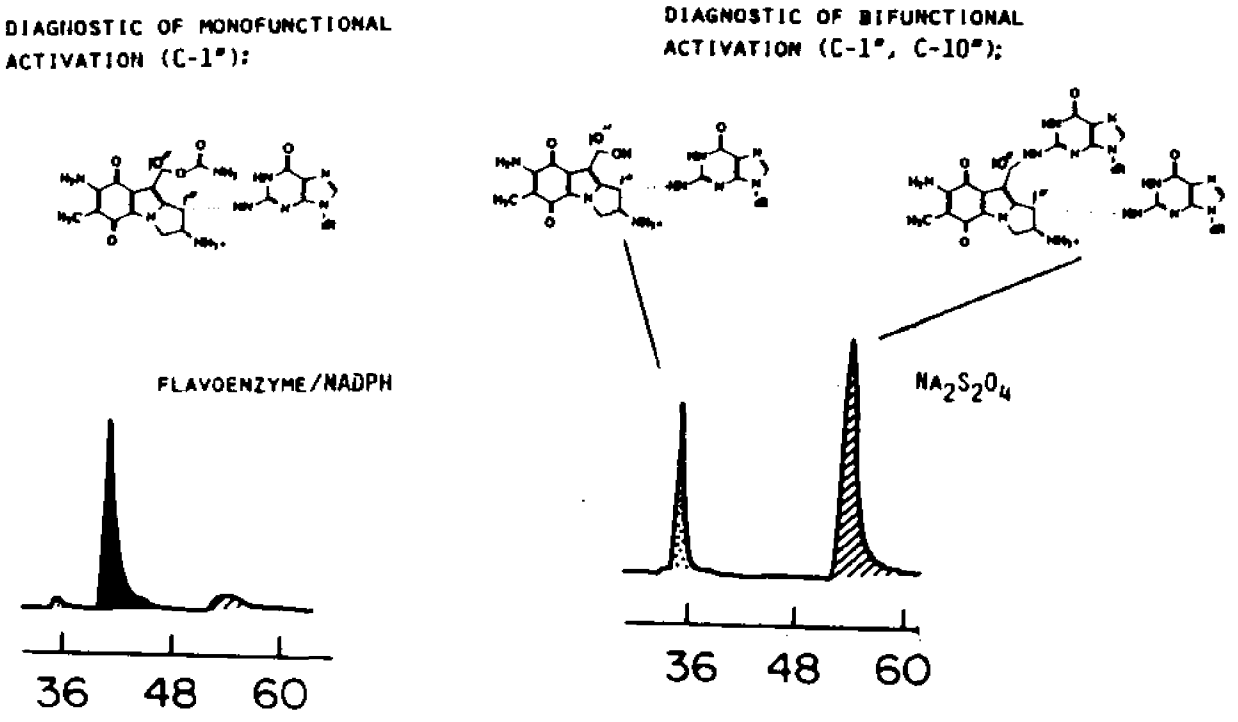
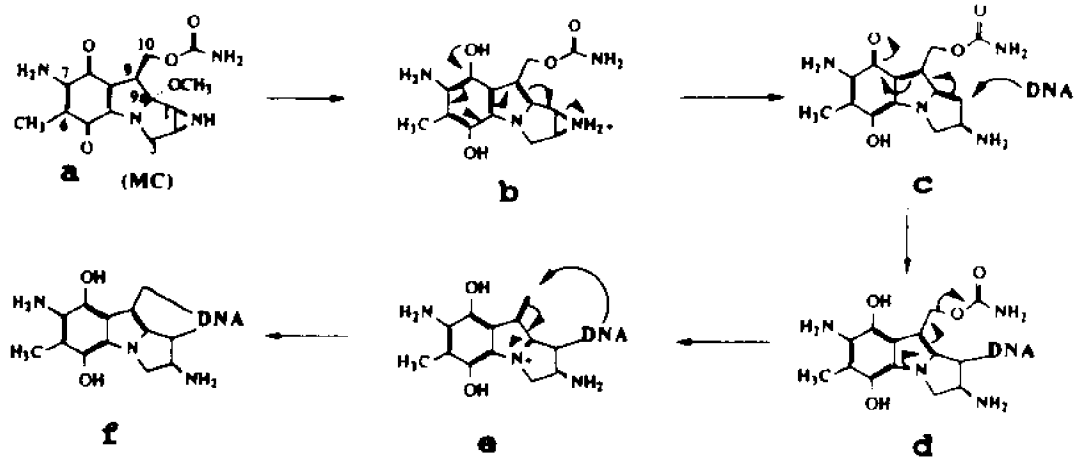
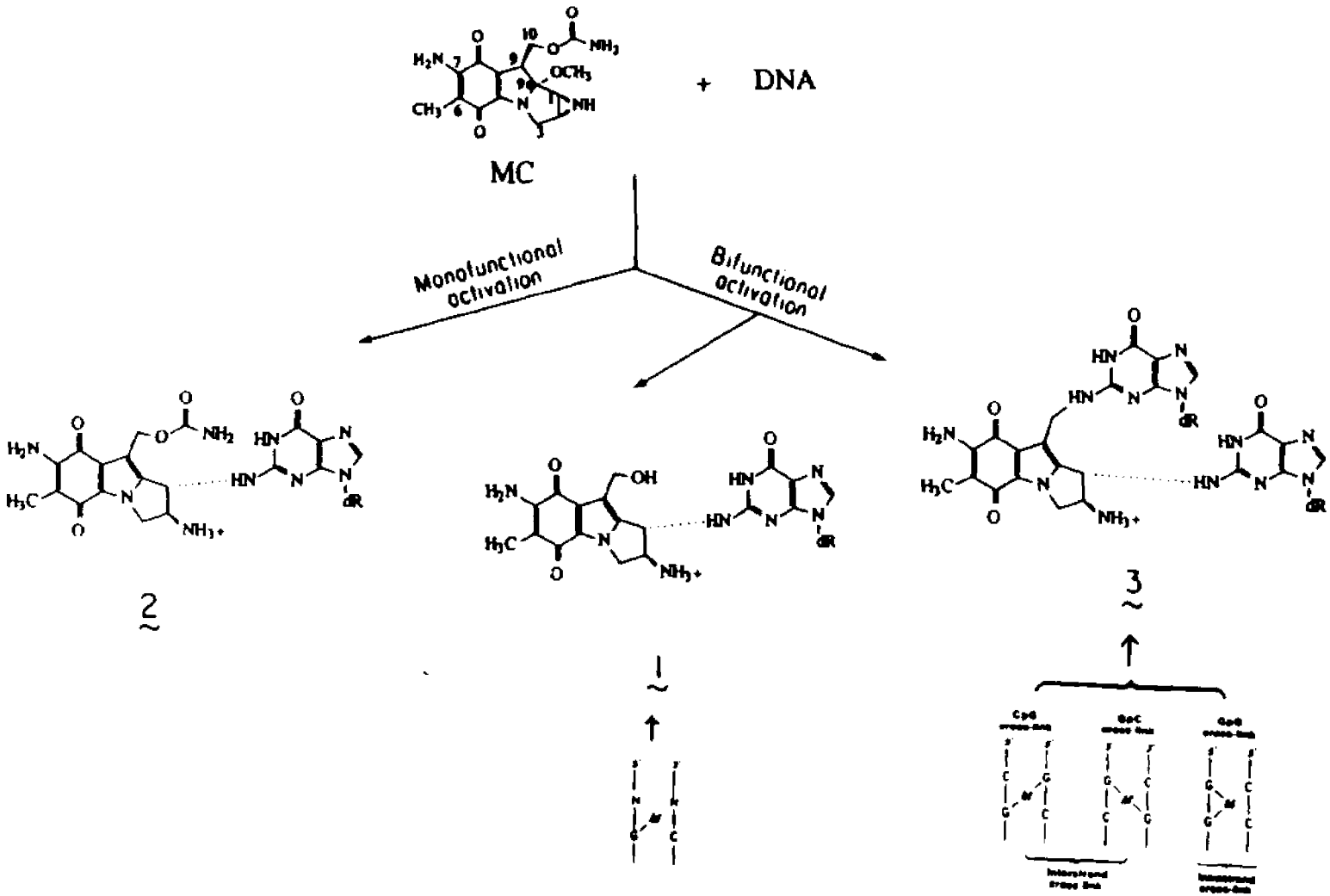


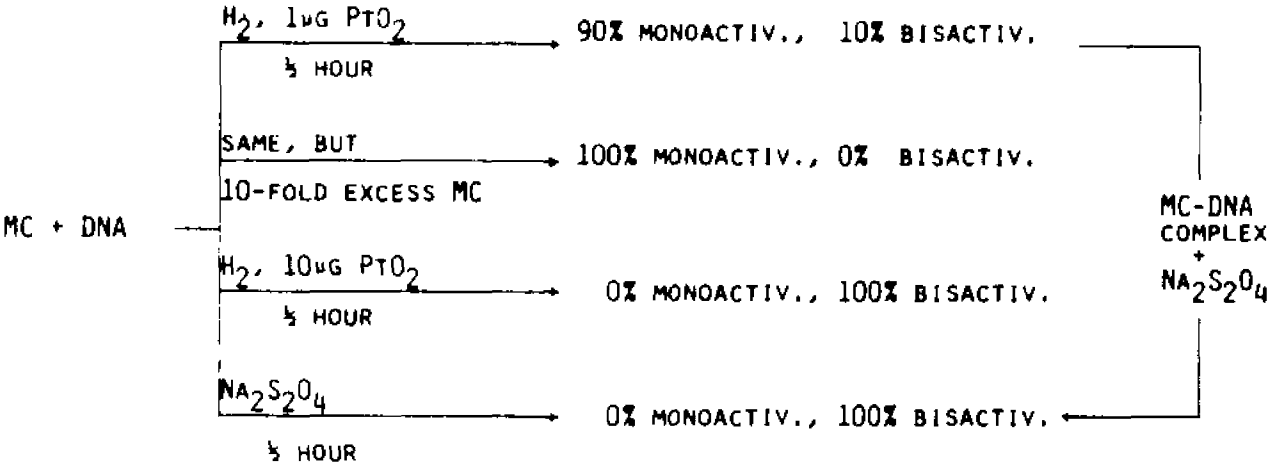
Figure 6: Diagnostic of monofunctional and bifunctional activation of MC showing the distribution of HPLC adduct (structures on the peak tops) formed under varying conditions of MC activation.

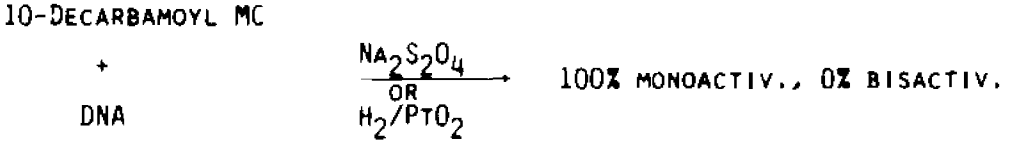


Scheme I: Displacement of C-1 aziridine and C-10 carbamate groups of mitomycin-C by two nucleophiles of DNA.

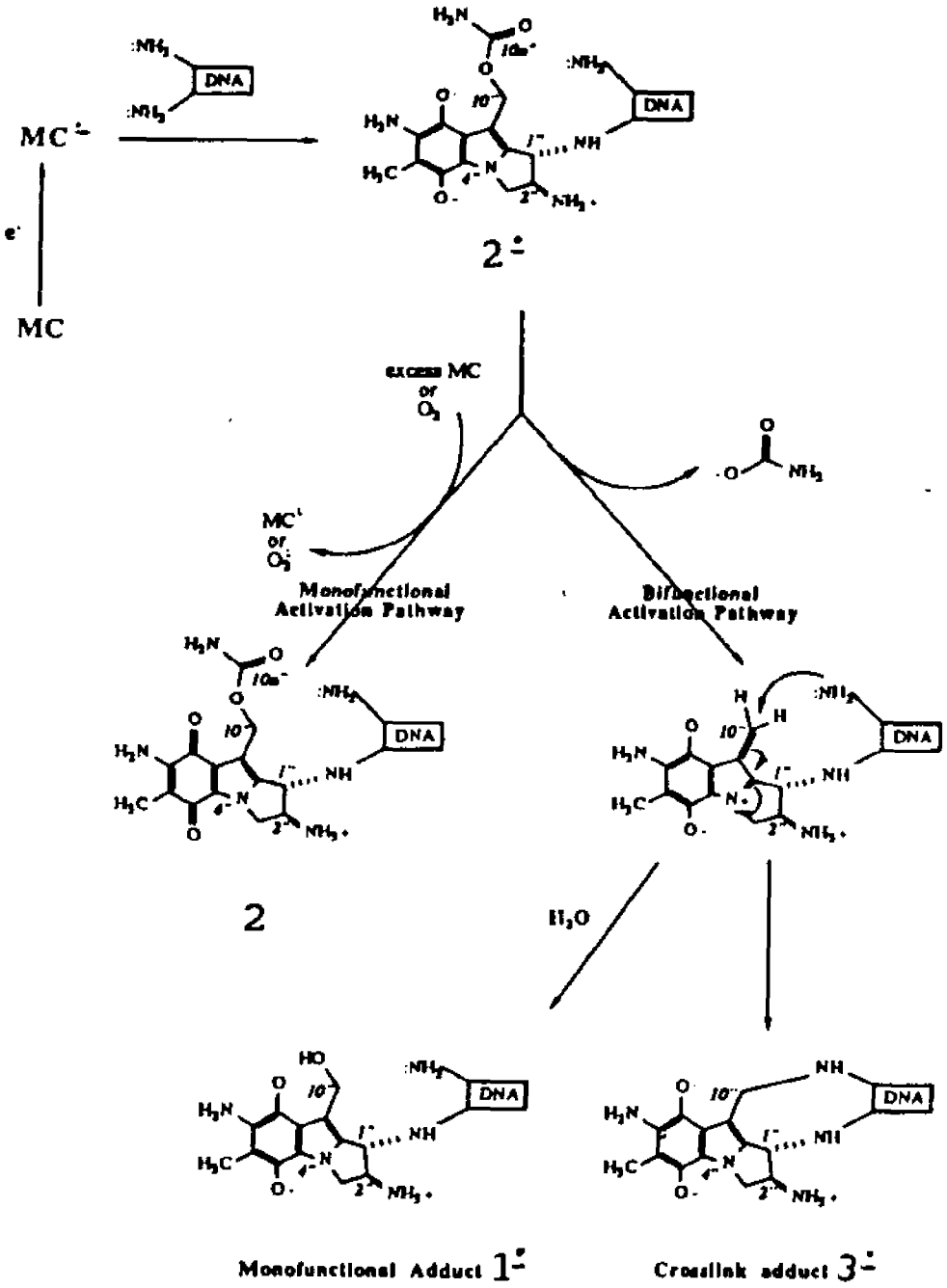


Scheme II : Summary of MC Adduct Distribution Under Various Reaction Conditions.





Scheme III: Summary of the distribution of MC-DNA adduct under various reaction conditions.



Scheme IV: Displacement of the C-10" carbamate group of MC during the second activation step.

SECTION II

EFFECTS OF MITOMYCIN C ON DNA CONFORMATION AND DYNAMICS

ABSTRACT
(SECTION II)

EFFECTS OF MITOMYCIN C ON DNA CONFORMATION AND DYNAMICS
by
Anil Kumar Chawla

Adviser: Professor Maria Tomasz

Mitomycin C (MC) can alkylate DNA monofunctionally or bifunctionally depending upon the activation of one or both masked functions of this drug; one can select monofunctional or bifunctional activation of MC using appropriate reducing conditions. In our work, we wanted to determine experimentally the effects of MC adducts on DNA conformation & dynamics and to assess the altered conformational behavior of selectively modified DNA and poly(dG-dC). Our results showed that all types of adducts stabilize poly(dG-dC) to much greater extent than DNA and the crosslinked (bifunctional) adduct is more effective in stabilizing poly(dG-dC) than mono adducts. We showed by means of circular dicroism (CD) and high-performance liquid chromatography (HPLC) that Z-DNAs such as poly(dG-dC)/Co^{3+}, poly(dG-m^5dC)/Mg^{2+} and brominated poly(dG-dC) are substrates for monofunctional alkylation by MC and remain in Z-form. Upon bifunctional activation of MC, Z-form of poly(dG-dC)/Co^{3+} reverts to B-form and crosslinked adduct is formed. In contrast, more stable Z-DNA such as poly(dG-m^5dC) remains in the Z-form under bifunctional activating conditions of MC, but only monofunctional adducts are detected. Crosslinking of the originally small amount of the B-form of poly(dG-dC) shifts the equilibrium from mostly Z-form poly(dG-dC)/Co^{3+} to B-form upon further crosslinking. No such Z->B shift is possible in poly(dG-m^5dC) thus in this case MC binding is terminated at monofunctional stage. Covalent interactions of MC with a Z-DNA hexamer were also studied using computer assisted energy minimized molecular modeling. The results showed that Z-DNA binds MC monofunctionally at N^2 of guanine in the minor groove with minimal distortion of the Z-DNA geometry but bifunctional (crosslink) type binding is not possible to Z-DNA. These findings are fully in accord with the above experimental results.

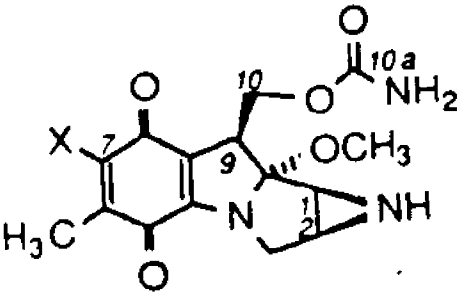
INTRODUCTION

The interconversions between B- and Z-forms of DNA are of central concern to biochemists and molecular biologists who are attempting to relate structural and functional attributes of various forms of DNA at the level of cellular processes and gene expression. Z-DNA which is the first crystallographic structure established for DNA[1-3] exists in solution, fibers, chromosomes, cells and supercoiled plasmids[4].

Drug-DNA interactions are generally quite complex in terms of thermodynamic and kinetic features. It is even more difficult to interpret their effects on B-Z conformational equilibria. Most of the drugs are known to stabilize B-form of DNA thus inhibiting B->Z forward reaction or such drugs promote reverse transition to B-form in synthetic polynucleotides such as poly(dG-dC) and poly(dG-m^5dC). Such drugs include a) covalent DNA modifiers: enantiomeric dihydroxy-anti-epoxybenzo[ ] pyrene which reacts at N2 of G with local inhibition but distal facilitation of the B->Z transition[5]; aflatoxin B1 (reacts at N7 of G)[6]; b) nonintercalators: netropsin[7] (but not distamycin A_3[8]; the steroid diamine dipyrandium[9]; c) intercalators: ethidium bromide[10-14];actinomycin D and actinomine[10,14]; porphyrins[15]; the anthracyclines daunomycin and adriamycin[16,17]; chloroquine[18]. There are a few drugs which stabilize Z-form DNA such as N-acetoxy-N-2-acetylaminofluorene which reacts at N7 of G[19,20];

cis-diaminedichloroplatinum(II)[21] and chlorodiethylenetriamino platinum(II) chloride[22,23]; DL-diepoxybutane, which crosslinks strands of the Z-form through N7 of G[24]; dimethylsulfate (alkylates at N7 of G)[25]; bromine (reacts at C8 of G and C5 of C)[26]; penta aziridino-cyclophosphazene[27] reacts with both with B- or Z-form of DNA and stabilizes it.

In the present project we studied the effects of the covalent binding of mitomycin C (MC) to DNA conformation, conformational stability, and equilibrium between the B and Z-forms. Mitomycin C (MC) differs from most other antitumor antibiotics in two respects: It must be activated by reduction before it interacts with DNA and it forms covalent bonds with DNA[28,29].



MC

Figure 1: Molecular structure of mitomycin C (MC).

The major biological consequence of MC binding to DNA is the inhibition of DNA replication. Secondary inhibition of RNA synthesis is evident due to losses in DNA integrity and template function upon binding of MC[29]. The formation of crosslinks by MC

between complementary strands results in true lethal effects. However, alternate lethal effects must exist since at higher doses, bacteriocidal action has been observed by mitomycin analogs without azirdine ring[29]. Monofunctional alkylation of DNA was thought to be lethal where the organisms have defficient repair mechanisms[28].

At physiological pH, the latent DNA-alkylating function of MC must be unmasked either by enzymatic or chemical reduction; thus MC represent a prototypical example of the class of the substances which function as "bioreductive alkylating agents"[30,31]. The covalent activity of MC is triggered by the reduction of its quinone system. The active species M* then attacks DNA monofunctionally or bifunctionally (scheme I). These processes can be easily mimicked in vitro, in the presence of chemical or enzymatic reducing agents[28]. Reductively activated MC reacts with the N2 position of guanines, in the minor groove of DNA, either monofunctionally or bifunctionally resulting in the latter case in the formation of covalent crosslinks between the complementary strands[32].

The formation & distribution of three different major adducts (Figure 2) show a striking dependence on the conditions of reductive activation in vitro: monofunctional adduct 2 is formed when MC is activated by H_2/PtO_2 or reductases and the pair of monofunctional and bifunctional adducts 1 and 3 when MC

is activated by $Na_2S_2O_4$. Figure 5 shows the scheme for inducing selective formation of single type of MC adducts in poly(dG-dC).

These adducts were obtained after digesting modified DNA's to nucleosides using enzymes such as DNAse, snake venom diesterase and alkaline phosphatase and the digests were analysed by HPLC[32]. The HPLC patterns (Figure 3) indicated the three different MC-nucleoside adducts in various proportions, depending on the particular reducing conditions and on DNA sequence. These three adducts were isolated and their structures were determined rigorously using spectroscopic methods.

In early studies on the conformation of MC-DNA complexes characteristic progressive changes of CD were observed to a much greater extent in poly(dG-dC) than either with DNA or RNA complexes[33,34]. Hydrodynamic studies[35] and transient electric dichroism studies of DNA and synthetic polynucleotides in water/ethanol mixture[36] suggested that mitomycin C was a strong factor for inducing Z-DNA conformation in polynucleotides which are rich in G-C content. Later, however, Z-DNA conformation in mitomycin C-polynucleotide complexes has been ruled out by vacuum CD[37], radioimmunoassay and ^{31}P NMR[34]. The CD spectrum of MC-DNA complexes has been interpreted in terms of two possibilities: (1) induced left-handed non-Z conformation; (2) drug-base interaction influencing the CD spectrum of B-DNA[34] ("induced CD"). In the present work we show evidence ruling out the first possibility,

based on vacuum CD measurements.

Most of the CD studies of nucleic acids have been carried out between the wavelengths 200-400 nm. CD studies below 200 nm have been ignored for two reasons; 1) wavelength limit of conventional Xe arc source dichrometers; 2) interesting and convincing spectra obtained in the 200-400 nm region. In most of the recent CD instruments however, it is possible to take CD spectrum below 200 nm, this region referred traditionally as vacuum ultra violet region. As quoted from Sutherland et al.[37], "vacuum ultraviolet-circular dichroism has proven, however, to be an important tool for studying the conformation of nucleic acids and DNA bound drugs in solution". For these reasons we carried out conformational studies of MC-DNA complexes using CD both in the uv region as well as vacuum uv region.

Sequence dependent effects on the conformations and dynamic properties of DNA may be important factors in determining the reactivities of chemical carcinogens with nucleic acids. Since guanine is the primary target for various drugs, studies of the interactions of MC with the synthetic polynucleotides poly(dG-dC) and 5-methylcytosine derivative poly(dG-m^5dC) are of special interest. The influence of conformational effects on the reactivities were investigated, since these polynucleotides are known to undergo salt concentration dependent conformational changes from B-form to Z-form. The existence of segments of

left-handed helices in living cells has been demonstrated[38] and these phenomena may thus be important in vivo. In order to understand the structure-effect correlations more clearly, it was desirable that the oligo- and polynucleotides be modified selectively by a single type MC adduct rather than by a mixture of the three adducts 1-3. This was made possible recently by devising MC-activating conditions, which suitably modulated the reactions of MC with DNA[39]. In the work presented here, we determined and compared the effects of all three selectively modified MC-polynucleotide complexes on the conformation of DNA and poly(dG-dC). Mono-linked and cross-linked covalent interaction of MC and DNA were also analysed with energy minimized models and results were compared with experimental data.

MATERIALS & METHODS

MATERIALS:

Materials and their sources are as follows:

Calf thymus DNA (type I, sonicated before use) and bacterial alkaline phosphatase from Sigma; M.luteus DNA (sonicated before use) from Worthington Biochemicals; poly(dG-dC) and poly(dG-m^5dC) from Pharmacia P-L Biochemicals; DNAse I and SVD (snake venom diesterase) from Cooper Biomedicals; Mitomycin C from Bristol Laboratories; 10-decarbamoyl mitomycin C was synthesized from mitomycin C[40]. Synthetic hexanucleotide d(TACGTA) was a gift of Dr. Dinshaw Patel (Columbia University); octanucleotide d(CTTACGTAC) was a gift of Dr. David Norman (Columbia University), d(GTACGTAG) was made at Hunter College (Applied Biosystems, Model 350A DNA synthesizer). Brominated poly(dG-dC) was a gift of Greg Hodgins, Cornell University Medical School.

METHODS:

Octamer [d(GTACGTAG)] purification: Freshly made tritylated octamer (10 umol scale) in concentrated ammonia was kept at 55 C for overnight (20 hrs). Purification of tritylated octamer was carried out by HPLC, on a C-3 column(1.0 x 25 cm), in 0.1 M TEAA buffer, pH 7.0, using acetonitrile gradient (20-35% in 20 minutes, 35-50% in 5 minutes and then 50% for additional 5 minutes) (Figure 4). For each run approximately 70-80 A_{260} units

of octamer were injected. Tritylated octamer (pooled from all fractions) was concentrated to about 5 ml using rotary evaporator and lyophilized (it looked oily and after lyophilization it had slightly yellow color). Detritylation was carried out by treating it with 5 ml of 80% glacial acetic acid. Mixture was stirred at room temperature for 20 minutes. Excess of acetic acid was removed by rotary evaporator (at temperature not more than 30 C). Detritylated octamer was separated from tritylated and other impurities by HPLC (same as above but using different gradient of acetonitrile, 0-20% in 40 minutes; after elution of the detritylated octamer peak, gradient was increased to 50% in 10 minutes and then back to 0% for another run). In each run approximately 70-100 A_{260} units of detritylated octamer were injected. All HPLC peaks of detritylated octamer were pooled, concentrated to about 10 ml at room temperature using rotary evaporator and then desalted by passing through Sephadex G-25 (5 x 10 cm).

Bromination of poly(dG-dC)[26] (carried out by Greg Hodgins, Cornell Medical College): Poly(dG-dC) from Pharmacia was disolved in 20 mM sodium citrate, 1 mM EDTA and 4 M NaCl, pH 7.2. The solution was kept at room temperature to facilitate the B->Z transition. Aqueous bromine reagent was prepared by adding bromine to distilled water and separated until the water became saturated with bromine at room temperature. The bromine saturated water was added to the polymer in bromine/nucleotide molar ratio of 19:1. The reaction was allowed to proceed at room

temperature for 10 minutes. Excess bromine was subsequently removed by bubbling air through the mixture which was kept in ice-water for 10 minutes. The solution was dialysed against two changes of TE buffer. Continued in our laboratory: to analyse the extent of the modification of polymer, it was precipitated with ethanol and the dried pellet was resuspended in 25 ul of 70% perchloric acid at 100 C for 1 hr. The solution was neutralized wih KOH and bases were separated by C-18 reverse phase column (Ultrasphere ODS; 1.0 x 25 cm), eluting with a methanol gradient (0-50% in 20 minutes) in 20 mM sodium hydrogen phosphate buffer, pH 4.5. The influence of bromination on the spectroscopic properties of poly(dG-dC) was monitored by A_{295}:A_{260} ratio.

Computer-assisted models of MC-Z-DNA: Z-DNA hexamer was built with Macromodel[41] "grow" mode, using Brookhaven National Laboratory Protein Bank[42]. 2"B,7"-diaminomitosene (4) or its 10"-decarbamoyl derivative (5) was placed into the minor groove region of the computer-generated duplex Z-DNA hexamer CGCGCG. Such work was carried out according to the published work Tomasz et al. where these mitosenes were incorporated into the central CpG sequence of a B-DNA type decamer[32,43]. For monofunctional attachment of MC, a bond was linked between C-1" position of mitosene 4 and N2-atoms of guanine of Z-DNA hexamer. For bifunctional attachment, two bonds were linked (C-1" & C-10" of mitosene 5) to the N2-atoms of either the central CpG or GpC sequence of Z-DNA hexamer. Before energy minimization, O^- was

added to the 5'-phosphates and a hydrogen atom to the 3'-hydroxyl (containing missing hydrogen) of the Z DNA duplex. These structures were then energy minimized using the Macromodel program and energy refinement was terminated at root-mean-square (rms) gradient of about 1.5 K Joules/mol A.

Reactions of DNA and synthetic polynucleotides with MC under variations in the reductive activating conditions:

(a) H_2/PtO_2 as reducing agent: (i) Standard reaction: M.luteus DNA and calf thymus DNA (0.67 umol/ml), MC (0.33 umol/ml), and PtO_2 as a catalyst (100 ug/umol of MC) were mixed in 0.015 M Tris-HCl (pH 7.4) and the mixture was deaerated by bubbling helium for 10 minutes, followed by reduction by bubbling hydrogen for 5 minutes and then helium again for 5-7 minutes. As a result, the blue color of MC changed to purple in the solution. The mixture was exposed to air, filtered and then chromatographed on Sephadex G-100 (25 x 2.5 cm) using 0.02 M ammonium bicarbonate as a buffer. (ii) Reaction using 5-fold excess MC: this is the same as the standard reaction above, except for 5-fold excess of MC (1.65 umol/ml) used in this reaction.

(b) $Na_2S_2O_4$ as reducing agent: DNA(0.67 umol/ml) and MC (0.33 umol/ml) in 0.015 M Tris-HCl (pH 7.4) were treated with 0.06 M aqueous $Na_2S_2O_4$ (1.5 mol/mol of MC) under anaerobic conditions (bubbling helium gas) for 30 minutes.

Reaction of DNA with 10-decarbamoyl MC[44]: This is the same as standard reaction above (a) except that 10-decarbamoyl MC was used rather than MC and pH of Tris-HCl buffer was 8.0 rather than 7.4.

Reactions of Z-DNAs with MC:

a) H_2/PtO_2 as reducing agent:

i) Z-form of poly(dG-dC)/Co^{3+}: Poly(dG-dC) (0.77 umol/ml containing 0.13 mM cobalt hexamine chloride, MC (3.5 umol/umol nucleotide) and PtO_2 (20 ug/umol MC) were mixed in 15 mM Tris-HCl buffer (pH 7.4). Mixture was deaerated by bubbling helium for 10 minutes, followed by bubbling hydrogen for about 15 minutes (till appearance of purple color). After passing helium for additional 5 minutes, mixture was exposed to air, filtered and the CD was determined in 0.2 cm cuvette. The mixture was then chromatographed on Sephadex G-100 (2.5 X 28 cm) using 0.02 M ammonium bicarbonate as buffer.

ii) Z-form of brominated poly(dG-dC): Brominated poly(dG-dC) (0.77 umol/ml), MC (3.5 umol/umol nucleotide) and PtO_2 (20 ug/umol of MC) were mixed in 15 mM Tris-HCl buffer pH 7.4. Reaction was carried out in the same way as above in (i).

iii) Z-form of poly(dG-m^5dC)/Mg^{2+}: Poly(dG-m^5dC) (0.77 umol/ml), MC (5 umol/umol nucleotide) and PtO_2 (20 ug/umol MC) were mixed in 50 mM NaCl, 15 mM Tris-HCl & 2mM $MgCl_2$ buffer, pH 7.2. Reaction was carried out in the same way as above in i).

b) $Na_2S_2O_4$ as a reducing agent:

Reaction of MC with all Z-forms of DNA was carried out in similar

way as above (i to iii) except that 5 times less MC was used and $Na_2S_2O_4$ (1.5 umol/umol MC) was used as a reducing agent. $Na_2S_2O_4$ was added in one installement under anaerobic conditions for reaction time of 30 minutes.

Selective adduct formation of MC with M.luteus or calf thymus DNA or poly(dG-dC): This was accomplished by manipulating the reductive activating conditions of MC (or its 10-decarbamoyl analog), in the presence of nucleic acid substrate. The principle of the selective modification is shown in figure 5.

(a) Monoadduct 1-DNA complexes: 10-decarbamoyl MC was reductively activated by H_2/PtO_2 in presence of DNA or poly(dG-dC) or oligonucleotides. The resulting complexes were isolated and characterized for drug/nucleotide binding ratio[44].

(b)Monoadduct 2-DNA complexes: MC was reductively activated by H_2/PtO_2; a large excess of MC (5-fold increase) ensured full selectivity of the modification[39].

(c) Crosslinked adduct 3-DNA or crosslinked adduct 3-poly(dG-dC) complexes: MC was reductively activated by $Na_2S_2O_4$ anaerobically. In this case, the DNA complex contained monoadduct 1 and the crosslinked adduct 3 in about 1:1 ratio; the poly(dG-dC) complex contained exclusively the crosslink adduct 3[32].

Selective adduct formation with synthetic oligonucleotides:

(a) Monoadduct 2-octamer [d(CTACGTAC)] complex: MC (5 umol/umol octamer), Octamer (1 umol/ml) and PtO_2 (20 ug/umol MC) as a catalyst were mixed in 0.1 M Tris-HCl buffer, pH 7.2 at room

temperature and deaerated by bubbling helium for 20 minutes followed by reduction by bubbling hydrogen for 45 minutes and then again helium for 5 minutes. The mixture was exposed to air, filtered and chromatographed on Sephadex G-25(2.5 X 56 cm) using 0.02 M NH_4HCO_3.

(b) Monoadduct 1-octamer [d(CTACGTAC)] complex: Same as above (a) except that 10-decarbamoyl MC was used at pH 8.0 . However monoadduct 1-octamer could not be made exclusively; the complex contained another adduct which has'nt yet been characterized.

(c) Crosslinked adduct 3-octamer complex: This was made by reactivation of monoadduct 2-octamer complex above using $Na_2S_2O_4$ as a reducing agent. Monoadduct 2-octamer complex (1 umol/ml) (prepared by H_2/PtO_2 activation) was mixed with its complementary strand [d(GTACGTAG)] (1:1 ratio) and treated with $Na_2S_2O_4$ (10 X 1.5 times the umol of MC in complex) under anaerobic conditions (He atmosphere) in 0.1 M Tris-HCl buffer, pH 7.2 for 60 minutes at 0 C (low temperature ensured octamer in its duplex form). Disappearance of the purple color of MC was noticed after few minutes of reduction. The mixture was reexposed to air for 10 minutes and complex was filtered and chromatographed on Sephadex G-25 (size 1.5 x 25 cm) Crosslinked octamer was separated from unmodified octamer strands by HPLC (C-3 column, 1 x 25 cm) using 0.1 M TEAA buffer, pH 7.0, and 5-15 % acetonitrile gradient in 60 minutes.

Characterization of the adduct-octamer complexes:

All complexes were digested to nucleoside and nucleoside-adducts by DNAse I, snake venom diesterase, and alkaline phosphatase in combination (methods, section I), followed by HPLC analysis of the digest; each particular adduct (1-3) was identified by comparison with authentic standards[32].

RESULTS

Bromination of poly(dG-dC): Figure 6 shows the influence of bromination on the spectroscopic properties of modified poly(dG-dC). It can be seen that bromination leads to an increase of the intensity of the absorption band at 295 nm. The change in the absorbance spectrum of the brominated polymer (A295/260 = 0.52) is evident as compared to the unmodified poly(dG-dC) (A295/260 = 0.1) (Figure 6). At an absorbance of A295/260 = 0.35, the brominated poly(dG-dC) is fully stabilized in the Z-form and degree of transition (O) is considered equals to 1.0. O value[26] of 1.0 normally corresponds to a level of 38% bromination of G, which produces the maximal change in the circular dichroism; the entire polymer is in the Z- conformation and exposure to the increasing concentration of NaCl does not result in further change in the A295/260 absorbance ratio. HPLC results (Figure 7) of perchloric acid digested sample of brominated poly(dG-dC) showed 56% modification of G's, which ensured full conversion of polymer to Z-conformation.

Preparation of MC-crosslinked octanucleotide: MC readily formed a monofunctional complex with octamer 5'-CTACGTAC-3' (2-octamer complex) when H_2/PtO_2 was used as an activating agent. HPLC of enzymatically digested sample showed (Figure 8a) adduct 2 as the major adduct (>90%). When 10-decarbamoyl MC was used under similar set of activating conditions, HPLC did not reveal the

formation of major adduct 1 as expected, but rather two adducts were formed (1 and an unknown) as shown in figure 8b. When the 2-octamer complex was reactivated using $Na_2S_2O_4$ as a reducing agent (without the presence of complementary strand), a different complex was formed and HPLC of the digest showed the appearance of an 18' adduct peak (not yet characterized) rather than adduct 1 (Figure 8c). The 2-octamer complex readily formed crosslink, however, when it was reactivated by $Na_2S_2O_4$ at 0 C in the presence of complementary strand 5'-GTACGTAG-3' (Figure 8d). Thus successful conversion of monofunctionally bound MC-octamer to crosslinked octamer was obtained by just reactivation of oligonucleotide-bound mono adduct 2 using $Na_2S_2O_4$ as a reducing agent. Crosslinked 3-octamer was separated from unmodified octamer using C-3 column of HPLC (Figure 9a) and its uv spectrum clearly showed the 310 nm peak, indicating bound MC (Figure 9b, spectrum # 3). The other two peaks of the HPLC (Figure 9a) are the unmodified complementary strands of octamer having no 310 nm peaks (Figure 9b).

Effects of the mitomycin adducts on the heat-stability of DNA, poly(dG-dC) and oligonucleotides:

There is an increase of T_m both in crosslinked and monofunctionally modified calf thymus DNA (Figure 10a). M.luteus DNA complexes exhibit larger (9-13) increases (Figure 10b). In the case of poly(dG-dC) the increase of T_m is even greater; crosslink adduct 3 has the greatest effect. At the higher level

(10-20%) of base modification all three adducts confer extreme heat-stability on this polynucleotide; there is hardly any melting observable below 100 C (Figure 10b). All crosslinked samples of oligonucleotides (hexamer, octamer and decamer) also showed drastic increase in T_m and samples did not melt fully even up to 85 C. All three crosslinked samples showed comparable stabilizing effects of the crosslink on duplex stability (Figure 11).

Vacuum CD of modified poly(dG-dC):

All modified complexes of MC with poly(dG-dC) display a large positive band at 187 nm in the vacuum CD region, characteristic of right-handed duplex DNA (or RNA)[37] (Figure 12). Although the CD of MC-poly(dG-dC) complexes in the near-UV region has the appearance of being "inverted"[33,34] the intensity of positive band at 187 nm is not diminished even for highly modified complexes, with 24-36% of G's modified .

Inhibition of B->Z transition of poly(dG-dC) by all three adducts

1-3: B->Z transition as measured by the changes in the absorbance ratio 295/260 are shown in figure 13a,b. At the level of 2-4% base modification, the transitions occur at higher ethanol concentration than that of control poly(dG-dC). The mid transition point for crosslink is at infinity (showing transitions even after adding 80% ethanol) as compared to the 55% and 62% ethanol for other two monofunctionally substituted

adducts 1 & 2, respectively, thus showing the greatest effect of crosslink among all three. At 10-20% level of base modification, the transitions are completely abolished in each case (Figure 13b).

Effect of temperature on the CD of control & MC-modified oligonucleotides:

Figures 14 & 15 show the effect of increasing temperature on the CD of control and crosslinked hexamer. There is gradual decrease in the intensity of all CD peaks (215 nm, 249 nm, & 272 nm) of control hexamer with increasing temperature (Figure 14). On the other hand crosslinked hexamer showed slight shift in CD peaks but did not show significant decrease in the intensity of peaks except 215 nm peak (Figure 15). The decrease in the intensity of 272nm peaks of control and crosslinked hexamer were plotted viruses temperature (Figure 16). CD melting curves showed higher T_m (T_m = 28 C) for crosslinked hexamer than the unmodified hexamer (T_m = 22 C). At higher temperature (85 C) decrease in the in intensity of 215 nm peak of crosslinked octamer was also observed (Figure 17). The other CD region of crosslinked octamer did not show significant change except slight shift of CD peaks.

Interaction of MC with Z DNA:

A) CD of complexes formed in reaction of MC with Z-DNA:

i) Z-form of poly (dG-dC) in presence of 13 uM $Co(NH_3)_6.Cl_3$ as substrate: Characteristic CD spectrum of the Z-form of DNA was

not appreciably altered in "diagnostic" 220-300 nm region in the presence of activated MC using H_2/PtO_2 as a reducing agent (Figure 18a). Poly(dG-dC) isolated after the reaction showed 12% base modification. Surprisingly when MC was activated bifunctionally using $Na_2S_2O_4$ as a reducing agent, it resulted in a change of CD indicating reversion to the B-form (Figure 18b). Poly(dG-dC) isolated after the reaction showed 7.6% base modification.

ii) Z form of poly(dG-m^5dC) (in presence of 2mM $MgCl_2$) as substrate: When MC was activated either monofunctionally or bifunctionally, no change in the CD spectrum in the "diagnostic" 220-300 nm region was observed indicating that poly(dG-m^5dC) remained in the Z conformation (Figure 18c). Monofunctionally activated MC complex of poly(dG-m^5dC) showed 5% base modification of guanine, and 2% base modification was observed when MC was activated bifunctionally using $Na_2S_2O_4$ as a reducing agent (Table 1).

iii) Brominated poly(dG-dC) as a substrate: When this polymer was treated with MC (using H_2/PtO_2 or $Na_2S_2O_4$ as reducing agents), no change in CD spectrum took place (Figure 19). Binding of MC was observed in the former (9% base modification) but not in the latter case (Table 1).

B) Characterization of the covalent adducts (1,2 or 3) formed in above reactions of MC with Z-DNA.

i) Z-form poly(dG-dC)/13 uM $Co(NH_3)_6Cl_3$ as a substrate: Enzymatic digestion to nucleosides yielded crosslinked adduct 3

as the only type of modified nucleosides.

ii) Z form of poly(dG-m^5dC) as a substrate: When MC was activated monofunctionally using H_2/PtO_2 as a reducing agent significant binding of MC to this polynucleotide took place (Table 1) and HPLC analysis of this substituted poly nucleotide yielded exclusively adduct 2 (Figure 20c). When MC was activated bifunctionally using $Na_2S_2O_4$ as a reducing agent, HPLC analysis yielded monofunctionally substituted adduct 1(Figure 20d) rather than crosslinked adduct 3. All these results are summarized in table 2.

iii) Brominated poly(dG-dC) as a substrate: Brominated poly(dG-dC) in 15 mM tris buffer gave the CD spectrum characteristic of the Z-form (Figure 19). When this polynucleotide was used as a substrate for monofunctional binding of MC using H_2/PtO_2 as a reducing agent, 9% base modification was observed (Table 1) and HPLC analysis of this polynucleotide-MC complex yielded exclusively adduct 2 (Figure 21c). When brominated poly(dG-dC) was treated with MC using $Na_2S_2O_4$ as a reducing agent, no significant binding of MC was detected and correspondingly, HPLC analysis yielded no adduct.

Effect of denaturation on the vacuum CD spectrum of DNA and poly(dG-dC). Figure 22 shows the effect of increasing temperature on the hyperchromicity (uv absorbance) and the extent of decrease of intensity of the 187 nm peak of vacuum CD of calf thymus DNA and poly(dG-dC). Results show a good correlation in the T_m values

obtained by these two different methods. Figure 23 shows that at about 85 C, where both DNA and poly(dG-dC) are almost completely denatured the 187 nm peak shows essentially zero intensity. Denaturation of DNA and poly(dG-dC) was also accomplished by increasing the concentration of NaOH and the results showed that after addition of about 350 ul of 0.2N NaOH (pH nearly 12.0), almost complete denaturation of both DNA and poly(dG-dC) occured and this was accompanied by parallel decrease in the 187 nm peak of vacuum CD (Figure 24b).

Computer-assisted modeling of MC-Z-DNA: Figures 25 & 26 show the computer constructed and energy-minimized molecular models and stereochemical fit of the monofunctionally (Figure 25b) and bifunctionally bound MC to the central CpG (Figure 25c) and GpC (Figure 26b) sequence of Z-form $d(CG)_3$. It is evident from the view of the monofunctional fit of the mitosene moiety in the Z-DNA hexamer that sugar-phosphate backbone in the vicinity of binding is not distorted; all other backbone features at the locus of MC binding are similar to that of unmodified control Z-form $d(CG)_3$ (Figure 25a). In contrast the integrity of G-C base-pairing is completely distorted for crosslinked MC in the central CpG sequence (Figure 25c). Distortion of G-C base-pairing is also evident in GpC sequence (Figure 26b).

DISCUSSION

EFFECTS OF MITOMYCIN C BINDING ON B-DNA CONFORMATION:

a) Previous evidence that MC does not induce Z- conformation:

In previous results on the conformation of MC-DNA complexes, characteristic progressive changes of CD were observed to a much greater extent in poly(dG-dC) than either with DNA or RNA complexes[33,34]. Hydrodynamic studies[35] and transient electric dichroism studies of DNA and synthetic polynucleotides in water/ethanol mixture[36] suggested that MC was a strong factor for inducing Z-DNA conformation in polynucleotides which are rich in G-C content, Later, however, Z-DNA conformation in MC-polynucleotide complex has been ruled out by vacuum CD[37], radioimmunoassay and ^{31}P NMR studies[34]. The CD spectrum of MC-DNA complexes has been interpreted in terms of two possibilities: 1) Induced left-handed non-Z conformation; 2) drug-base interaction influencing the CD spectrum of B-DNA[34] ("induced CD"). In the present work a large positive band at 187 nm peak in vacuum CD region of all modified complexes of poly(dG-dC) (Figure 12) show evidence ruling out the first possibility i.e., any left handed conformation, therefore it must be "induced CD" that inverts the 200-300 nm region.

The CD spectrum of the crosslinked hexamer looked very different from the unmodified hexamer (Figures 14 & 15). This difference can also be accounted in terms of induced CD by bound drug in the crosslinked hexamer. The chiral environment of the

chromophore when bound to DNA or RNA are well known to induce CD, or modify the CD of the ligand compared to its unbound state[45]. Many drugs, mutagens and carcinogens, which bind to DNA, absorb ultraviolet wavelength less than 300 nm and CD spectrum of such complexes are sometimes interpreted as suggesting that ligand is inducing change in the conformation of the polynucleotide when, in fact, the observed spectrum may be the sum of the unperturbed spectrum of the polymer plus the induced CD of the ligand[45].

b) Melting behavior:

There are two surprising features for the observed increased heat stability of the complexes of MC with M. luteus DNA or poly(dG-dC) or oligomers (Figures 10 & 11): 1) It might have been expected that since other structural features are roughly equal (compare 1,2 & 3), the crosslinked DNA or crosslinked poly(dG-dC) or crosslinked oligonucleotides exhibited greatly enhanced duplex stability as compared with those of the monofunctionally modified ones. Actually, all three modifications show strong stabilizing effects on duplex stability of all complexes, although the crosslink has always the highest effect. This may be explained by the snug fit and H-bonding interactions of the drug in the minor groove, common to all three adducts; the additional second covalent bond, unique to adduct 3, may be secondary in importance in influencing the melting process. 2) The stabilizing effect on poly(dG-dC) is much greater than DNA, at the comparable (10-20%) base-substitution levels. This is apparently a sequence specific phenomenon which may be

explained by the cooperative binding of MC to poly(dG-dC).

c) No denaturation by MC binding:

As shown in figure 12, a large positive band of 187 nm peak of vacuum CD was displayed by all modified (24-36% base substitution levels) poly(dG-dC) complexes. The positive sign and large intensity of this band indicated that the modified polynucleotides remain right handed[37]. The advantage of the great magnitude of this peak was extended for sensitive diagnosis of denaturation of MC-modified poly(dG-dC) complexes. As seen in figure 23, both DNA and poly(dG-dC) lose the 187 nm CD peak upon heating. We correlated both heat-induced and alkali-induced denaturation of these polynucleotides with the loss of the 187-nm CD band quantitatively (Figures 22 & 24): Denaturation curves as followed by the customary hyperchromicity measurements were perfectly proportional to the curves where the 187-nm CD intensity was followed instead. (Actually, for DNA, CD at 191 nm is the best indicator). Based upon these results, we conclude, that loss of 187- (or 191-)nm CD intensity is directly proportional to the extent of denaturation, i.e. loss of basepairing. It then follows, that the MC-poly(dG-dC) complexes (Figure 12) are not denatured, since the 187-nm CD peaks are undiminished.

d) Inhibition of B->Z transitions:

The B->Z transitions of all three adducts 1-3 of poly(dG-dC) occured at higher ethanol concentration than that of control

poly(dG-dC) (Figure 13). The effect of adduct 3 is much greater than the other two monofunctionally substituted adducts 1 & 2, showing maximum effect of crosslink on B->Z transitions. At higher base substitution (10-20%), the B->Z transitions were completely abolished for all three adducts (Figure 13). These results suggest that significant stabilization of B-form of polY(dG-dC) is achieved upon MC binding.

e) Computer models:

The three-dimensional features of adduct 2 in double stranded DNA were studied previously in our laboratory by space filling molecular models, leading to the remarkable results that in one unique conformation the N^2-guanine bound M residue fits snugly into the minor groove without appreciable pertubation of DNA structure[46]. The stabilizing effect of bound MC on DNA duplex is analogous to that of anthramycin, a molecule of similar size and binding site[46]. Steriochemical fit of the MC crosslink in duplex DNA was also examined by inspection of computer-constructed and energy minimized molecular models[32]. It is evident from these results of Tomasz and coworkers, that mitosene moiety fits in the minor groove of B DNA, without distortion[32] of the sugar-phosphate backbone in the vicinity of the crosslink. All glycosidic bonds had the usual anti configuration; all other backbone features at the locus were similar to those observed in unmodified B-DNA molecules. The integrity of G-C base pairing was maintained in both of the modified pairs[32].

f) Conclusions:

It is apparent from all these results that MC-modified B-DNA lost its conformational dynamism with respect to B->Z transitions, regardless of whether the modification is monofunctional or bifunctional. It is also evident from the results that all adducts (1 & 2 accompanying the crosslinked adduct 3) are non-distortive of B-DNA geometry. One might speculate that each adduct can act as regulatory inhibitors of gene expression. Since neither of the adducts causes significant distortion of the DNA geometry, it is possible that only the crosslink inhibits DNA replication, simply by connecting the two strands together covalently at the replication fork.

INTERACTION OF MC WITH Z-DNA

a) Binding of activated MC to each of three different Z-DNAs:

It is apparent from the results summarized in table 1 that the covalent binding affinity of the activated MC to Z-DNAs is diminished as compared to that of the B form. The CD results (Figure 18a) clearly show that monofunctionally activated MC interacts with Z-DNA without distorting the B-Z equilibrium i.e., Z-form of poly(dG-dC)/Co^{3+}, poly(dG-m^5dC) and brominated poly(dG-dC) remain in Z conformation upon MC binding. In contrast the observed Z->B reversion of poly(dG-dC)/Co^{3+} in presence of bifunctionally activated MC (Figure 18b) may be due to the binding of the second active site of the monofunctionally bound MC to another guanine only in the B regions; this is followed by

further crosslinking, thereby shifting the equilibrium entirely and irreversibly to the B form. However when a more stable Z-form poly(dG-m^5dC) was used as substrate for binding with MC, the second activated site of MC reacted with water, because the other guanine was too far, resulting in the formation of monofunctional adduct type 1.

b) Interaction of other drugs with Z-DNA:

There are many other well known examples of shift of Z->B equilibrium induced by other drugs. The binding of actinomycin D sequentially converts the left handed polynucleotides, switching four to six base pairs to a right handed form per bound actinomycin D and thus making the regions of the right-handed poly(dG-dC) nearly saturated with actinomycin D[10]. Under such circumstances, the formation of energetically unfavorable B-Z interfaces would be minimized if the binding of such drugs including MC occured primarily by the extension of regions of previously bound drug, as opposed to random binding along the duplex. It is interesting to speculate that the cooperative binding observed in several drug-DNA systems may be result of the affinity of these ligands for a small fraction of the native DNA existing in an altered conformation under physiological conditions[48]. This possibility is the essence of a "two-site" binding model proposed by Rosenberg and Krugh in which the DNA is considered to consist of structurally distinct regions, of which one type bind the ligands in a highly cooperative manner while

the remaining regions bind the ligands by a neighbor-exclusion process. The cooperative binding sites saturate first, with subsequent binding at higher bound drug to DNA ratios occuring at the remaining sites in neighbor-exclusion manner. In case of mitomycin-Z DNA interactions, we believe that the conversion of left handed Z-DNA to right handed B- conformation occurs also in a sequential manner, the first step is the monofunctional binding of MC to Z-DNA (without any steric hindrance) and then in the second step, MC binds to the B- regions of poly(dG-dC) which is in equilibrium with Z-form and thus forming more of the regions of right-handed poly(dG-dC) that are nearly saturated with MC, and thereby shifting the equilibrium to B conformation. Consistent with our interpretation is the observation that the conformationally less mobile Z-form of brominated poly(dG-dC) and poly(dG-m^5dC) are neither reverted to B-form nor crosslinked in the presence of mitomycin C (results).

Adriamycin, also a cytotoxic antibiotic used widely in cancer chemotherapy binds preferentially to the B-form and converts the Z- into the B confirmation[16]. In this case it is presumed that the anthracycline ring of the adriamycin does not intercalate readily with the Z-form as compared to the B-form. This is mostly due to steric factors; for example, the Z-form has a smaller radius and syn G conformation. However, steric factors are not involved in case of MC; computer simulated molecular models show that monofunctionally bound MC fits excellently in the minor groove of Z-DNA. The bulky aromatic group of the MC

molecule projects mostly outside the Z DNA helix (see figure 27 in proceeding section 'Computer models'), thus resulting in minimum distortion of the backbone geometry & base pairing.

The carcinogen N-acetoxy-2-acetyl aminofluorene (AAAF) is also known to form covalent adducts with guanine residues of DNA. Unlike MC, AAAF modified DNA is locally denatured at the site of AAAF modification and the fluorene moiety is inserted between the base pairs adjacent to the modified guanine[49]. The characteristics of AAAF modified poly(dG-dC) led to the development of a model for the conformation of AAAF adduct in Z-DNA in which AAAF is located at the exterior of the helix with little denaturation or interruption of the base pairing at the site of modification and thus fascilitating B->Z transitions[49]. In contrast MC binds preferentially to B-form DNA for similar reasons: little denaturation and minimum interruption of base-pairing.

c) Computer models of MC-Z-DNA complexes:

In the present work the stereochemical fit of MC to Z-DNA was examined using energy minimized computer models with the mitosene moiety incorporated in the central CpG of Z-form of the duplex$(CG)_3$. We found extremely different results for monofunctionally and bifunctionally bound MC. Monofunctionally bound drug to Z-form of hexamer did not change the sugar-phosphate backbone at the vicinity of bound MC; no disruption of base-pairing was observed and all other features were similar to

that of unmodified Z-DNA (Figure 25a & 25b). The fit of the mitosene unit in Z-DNA was also demonstrated by examining of van der Waals radii as shown in the figure 27. It is evident from the view of van der Waals radii that the indoloquinone ring of monofunctionally bound MC molecule almost projects outside the helix thus causing least distortion to the overall geometry of Z-DNA. In contrast the computer model showed complete disruption of the both backbone geometry & base pairing when MC was

Figure 27: Computer generated molecular model showing van der Waals radii of monofunctionally bound MC-Z-DNA.

crosslinked into the central CpG sequence (Figure 25c). It is evident from the figure 28 that the closest distances for the second linkage of MC to nearest guanines is too large (9.2 A & 6 A) (Figure 28) to make the crosslink. Thus it was concluded that Z-DNA will bind MC monofunctionally but will not be crosslinked. All such conclusions were found to be fully consistent with experimental data.

Figure 28: Computer generated molecular model of monofunctionally bound MC-Z-DNA, showing the distances for the second linkage of MC to the nearest guanines of opposite strand.

e) Conclusions:

We draw the following conclusions from these results:

1. All Z DNAs are substrates for monofunctional alkylation by MC and remain in the Z-form without any distortion of DNA geometry.

2. Upon bifunctional activation of MC, Z-form of poly(dG-dC)/Co^{3+} reverts to B form resulting in the formation of crosslinked adduct 3.

3. No Z->B shift is possible in more stable Z-form such as poly(dG-m^5dC) and in this case MC binding is terminated at monofunctional stage.

4. One can speculate from such results that MC is an antibiotic tailor-made to crosslink only B DNA.

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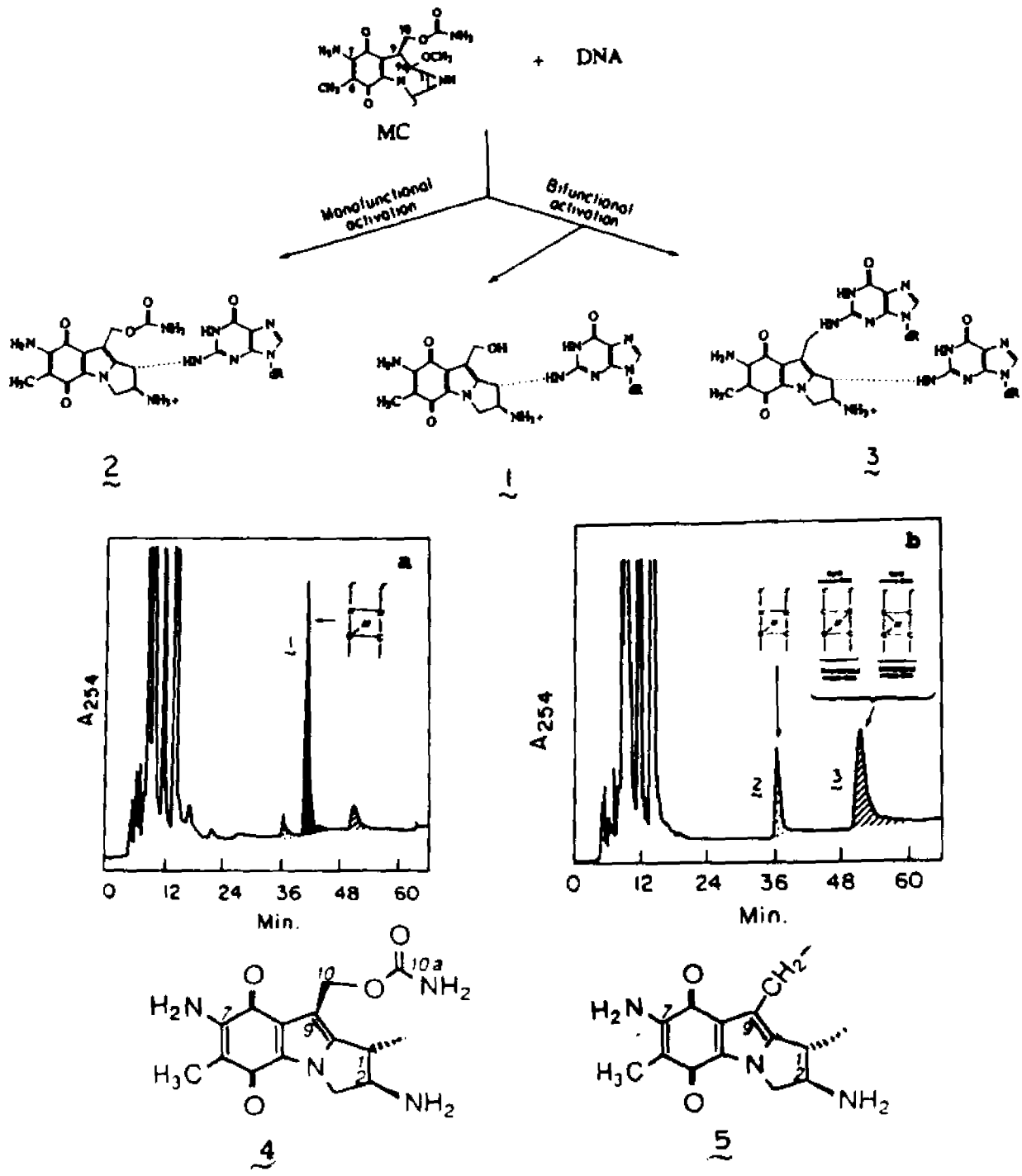


Figure 2: Structures of the mitomycin-nucleoside adducts from DNA (adducts 1-3), and 2"B,7"-diaminomitosene (adduct 4) & its 10"-decarbamoyl derivative (adduct 5).

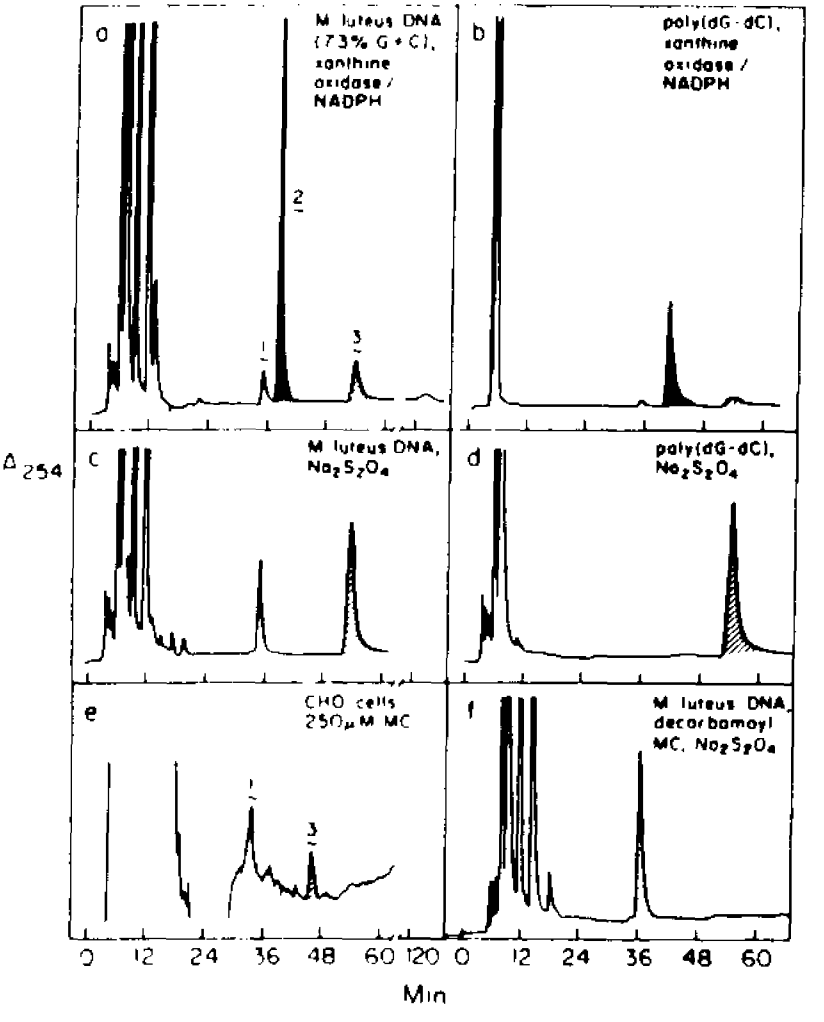


Figure 3: HPLC patterns from nuclease digests of various MC-DNA and MC-poly(dG-dC) complexes. The DNA species and the reductive activation method is indicated at the upper right of each segment. (a)-(d): Taken from ref.32 (e). Pattern from DNA from CHO cells treated with MC. (f). The analog, 10-decarbomoyl MC was used instead of MC. Cross-identity of peaks in the various experiments is indicated by their identical shading.

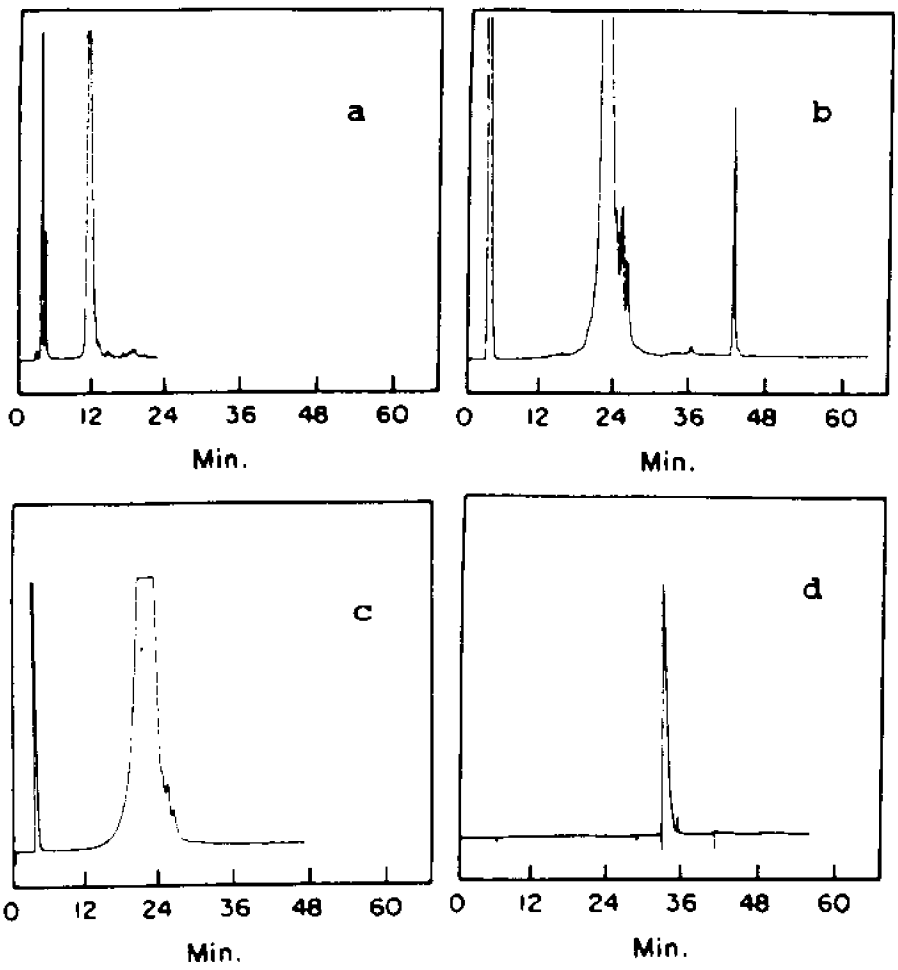


Figure 4: Purification of Octamer (5'-GTACGTAG-3') using C-3 column (1 x 25 cm) of HPLC. (a). Tritylated octamer in 0.1 M TEAA buffer, pH 7.0 with acetonitrile gradient of 20-35% in 20' -> 35-50% in 5' ->50% for 5' and then back to 20%. (b). Detritylated octamer (after treatment with 80% acetic acid) in the same buffer as above with acetonitrile gradient of 0-20% in 40' -> after getting the octamer peak gradient to 50% for 10' and then back to 0%. (c). same as (b) above except that detritylated octamer treated with additional 80% acetic acid for 2'; 44 minutes tritylated peak disappeared this time. (d). Purity of the isolated octamer (after steps a - c above, acetonitrile gradient of 5-12% in 60 minutes time.

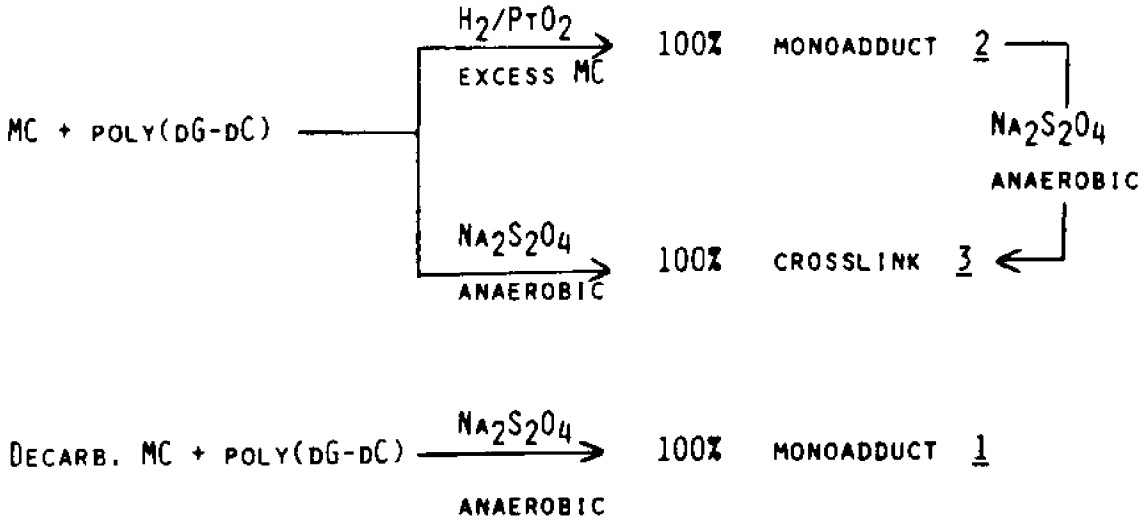


Figure 5: Scheme for selective introduction of a single type of mitomycin adduct into poly(dG-dC).

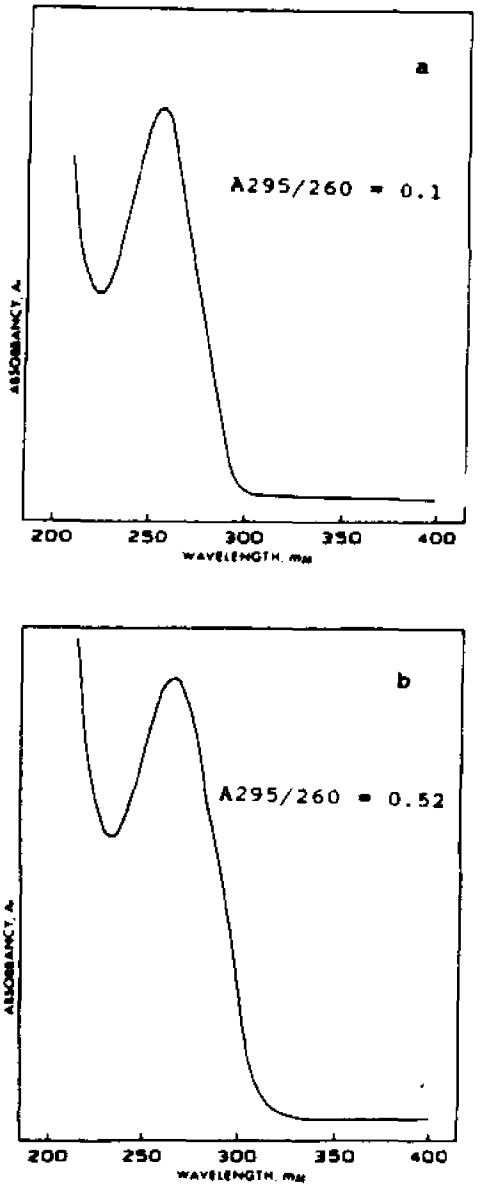


Figure 6: UV spectrum of (a) control poly(dG–dC) and (b) brominated poly(dG-dC) (56% 8-bromoguanine & 13% 5-bromocytosine) in 15 mM tris, pH 7.2.

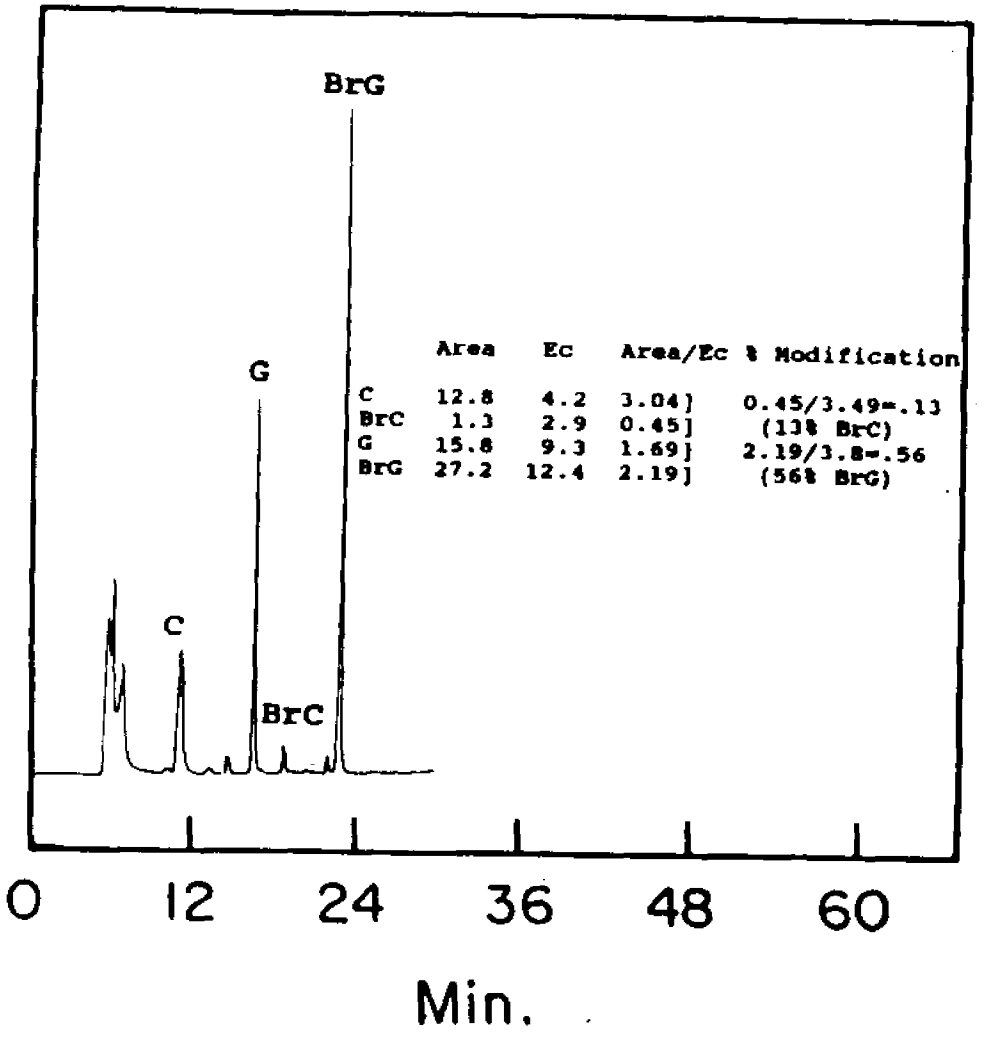


Figure 7: Reverse phase HPLC (C-18 column, size 1 x 25 cm) of perchloric acid digested sample of brominated poly(dG-dC) in 20 mM sodium phosphate buffer, pH 4.5 with methanol gradient (0-50% in 20' time). The molar extinction coefficients (Ec) for the individual bases at 254 nm and calculations for the %age modification of cytosine and guanine are also shown.

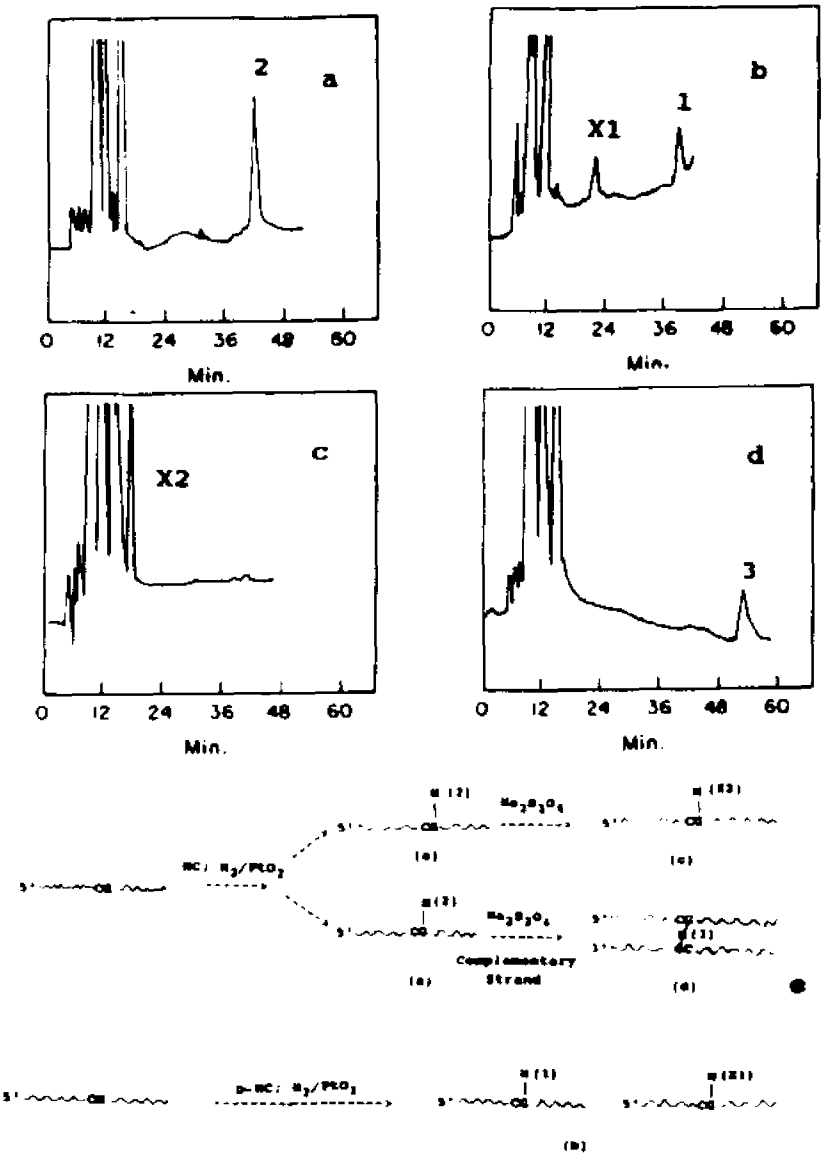


Figure 8: Reverse phase HPLC (C-18 ultrasphere ODS; 1 x 25 cm column) of digested MC-octamer complexes. (a): MC-octamer complex (52% modified) in 93% 0.03M phosphate buffer, 7% acetonitrile, pH 5.0. (b): 10 decarbamoyl MC-octamer complex (51% modified) in the same buffer as above (a). (c): MC-octamer complex reactivated with MC using $Na_2S_2O_4$ as reducing agent without the presence of its complementary strand. (d): Crosslinked octamer (same as (c) above except that reactivation was carried out in presence of complementary strand). (e): Diagram for the reactions which led to the digest patterns. The letters under the molecules identify the corresponding digest in the Figure. "M" signifies the bound MC residue in general. The bold-faced number identifies the formula of the adduct.

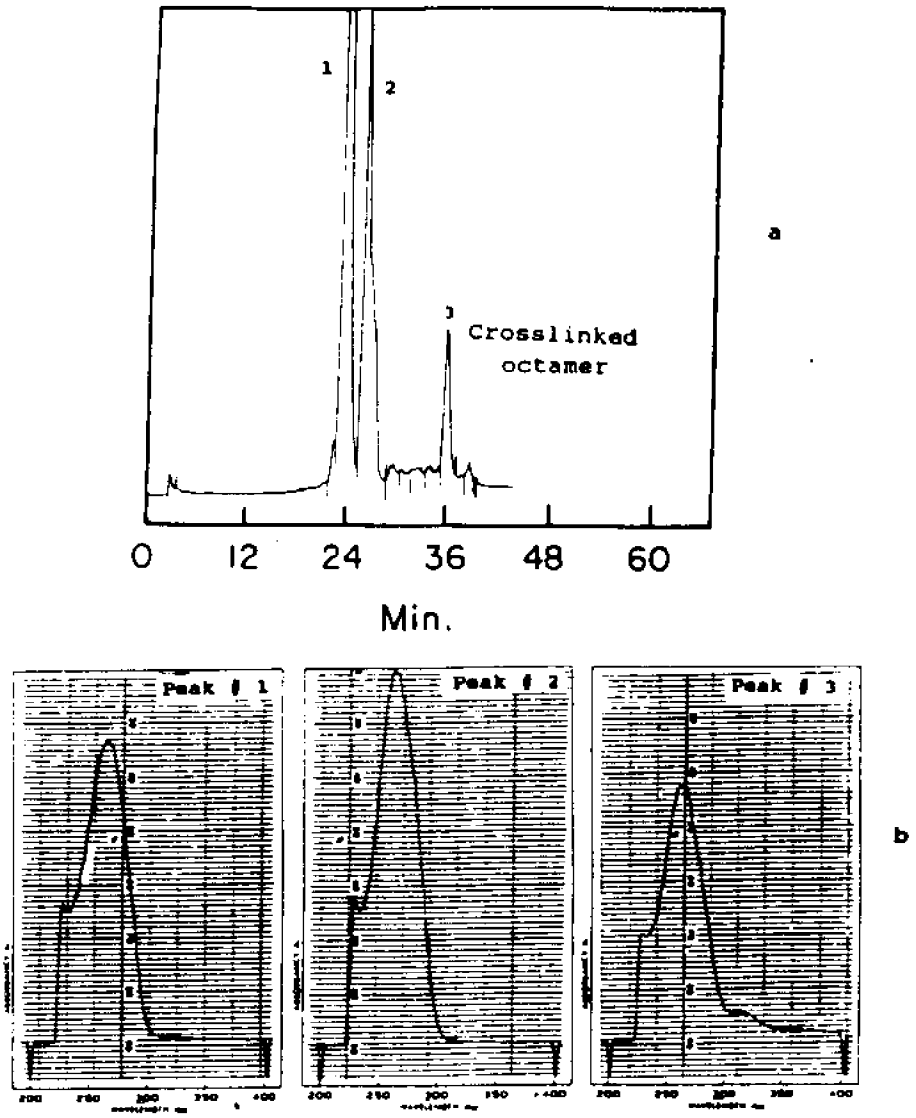


Figure 9: Isolation of crosslinked octamer from unmodified complementary strands using reverse phase C-3 column of HPLC. (a). Isolation of crosslinked octamer using 0.1 M TEAA buffer, pH 5.0 using 5-15% acetonitrile gradient in 60 minutes time. (b). UV spectrum of crosslinked octamer (36 minute peak of HPLC above) is shown in extreme right of fig b Other two uv spectra correspond to the unmodified complementary strands.

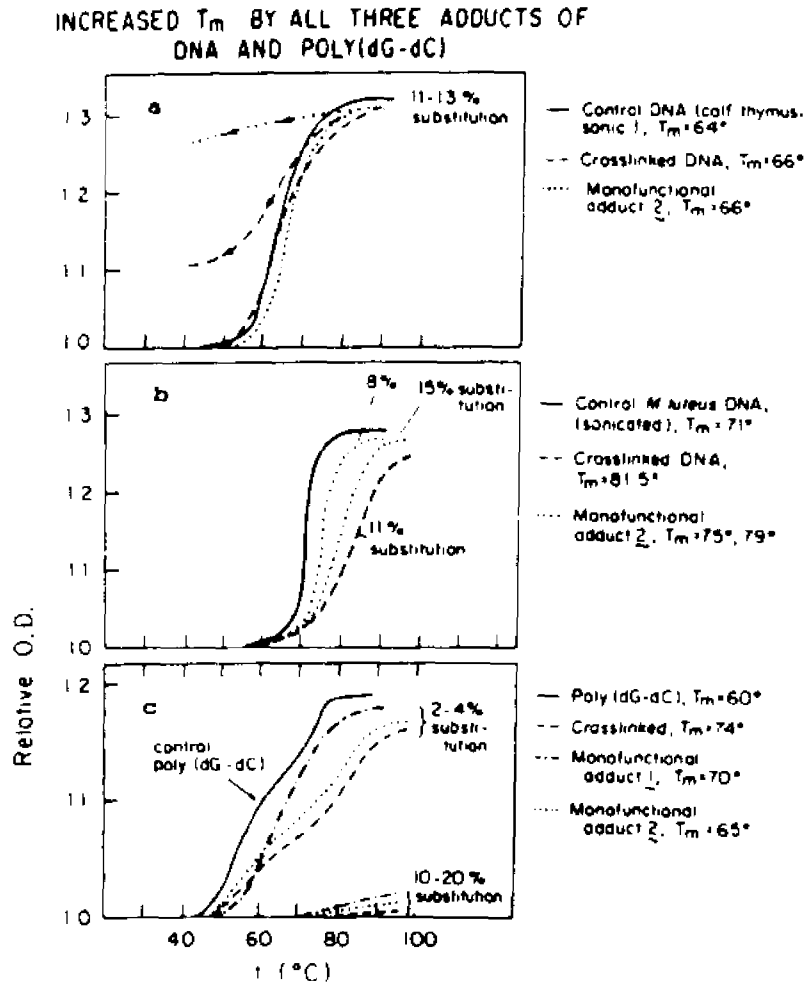


Figure 10: Melting curves of MC-DNA and MC-poly(dG-dC) complexes. (a). Control and modified calf thymus DNAs in 5 mM Na-phosphate,pH-7.2, 0.03 mM EDTA, were heated at a rate of approx. 2 C/min. in a Gilford Model 240 spectrophotometer equipped with automatic cuvette changer, 4-channel recorder, thermosensor and sample compartment heated by a circulating bath. Cooling curves were also recorded, as indicated by arrows. (b). Control and modified M.luteus DNA were subjected to heating as in (a). (c). Control and modified poly(dG-dC) in 0.05 mM NaCl 0.003 mM Na-phosphate, pH 7.2 0.03 mM EDTA, were subjected to heating as in (a).

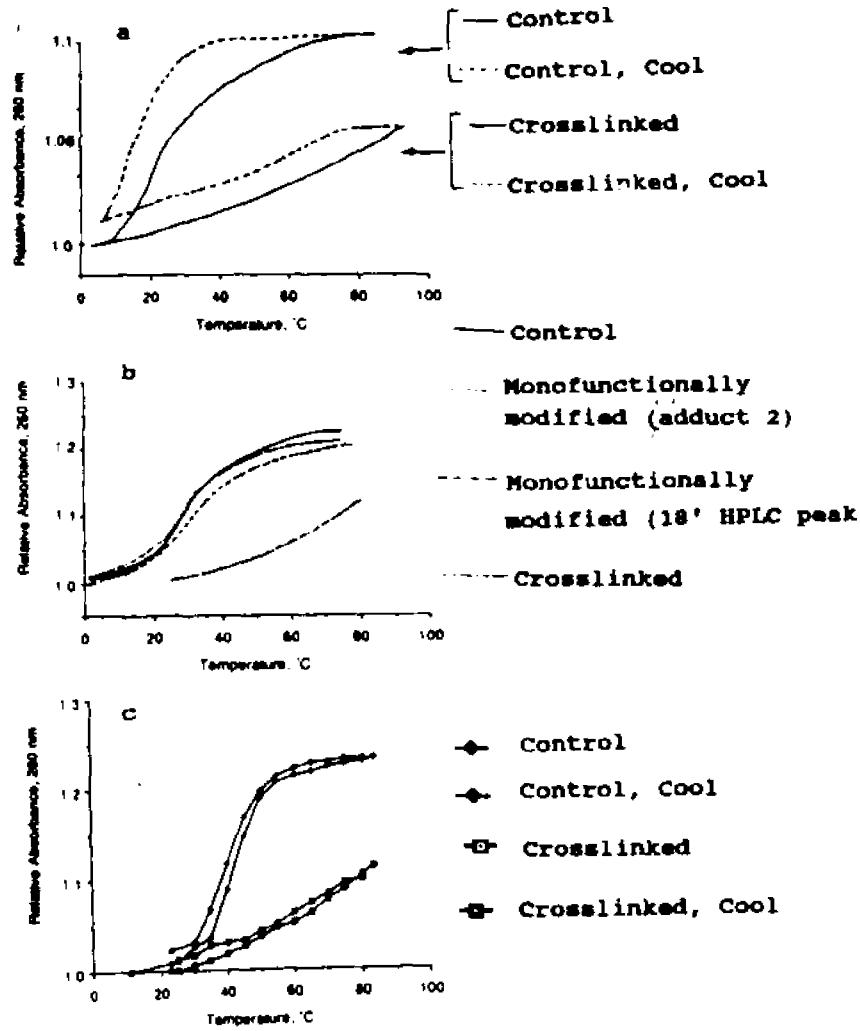


Figure 11: Melting curves of MC-oligonucleotide complexes. (a). Control (mM) and crosslinked d-TACGTA (mM) melting in 1M NaCl, 10 mM sodium phosphate, 0.1mM EDTA, pH 7.2. (b). Control and two monofunctionally substituted complementary octamer pairs (see text) (one having the adduct 2 & other has the adduct corresponding to 18' peak of HPLC which is not characterized) and crosslinked octamer pair in 1 mM NaCl, 10 mM sodium phosphate, 0.1 mM EDTA, pH 7.2. (c). Control and crosslinked d-TATACGTATA in the same buffer as of (a) above.

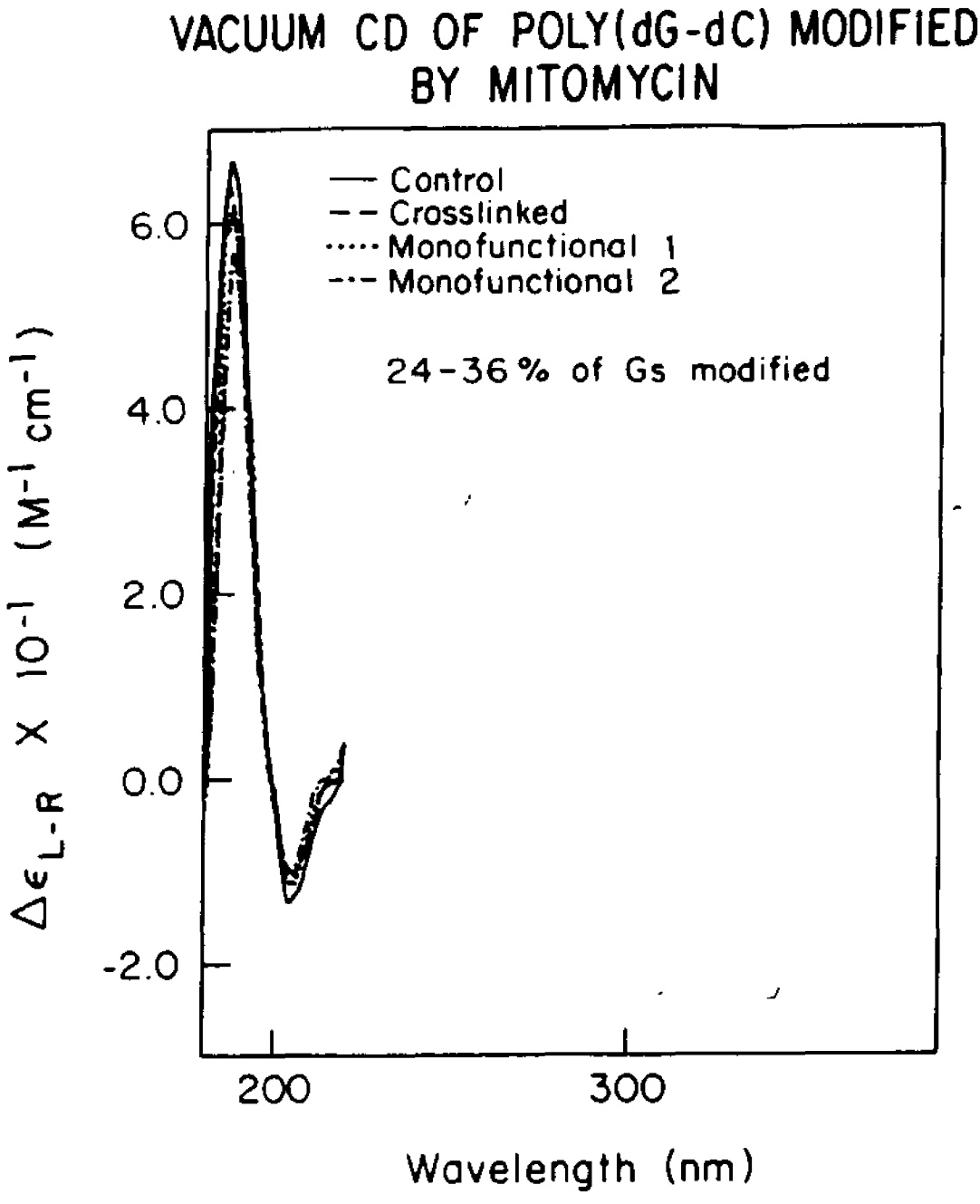


Figure 12: Vacuum CD of MC-poly(dG-dC) complexes. Buffer 10 mM Na-phosphate, pH 7.2, pathlength 0.2 cm. Sample solutions were not deaerated prior to the measurements. Instrument: Jobain-Yvon Auto-Dichrograph Mark V.

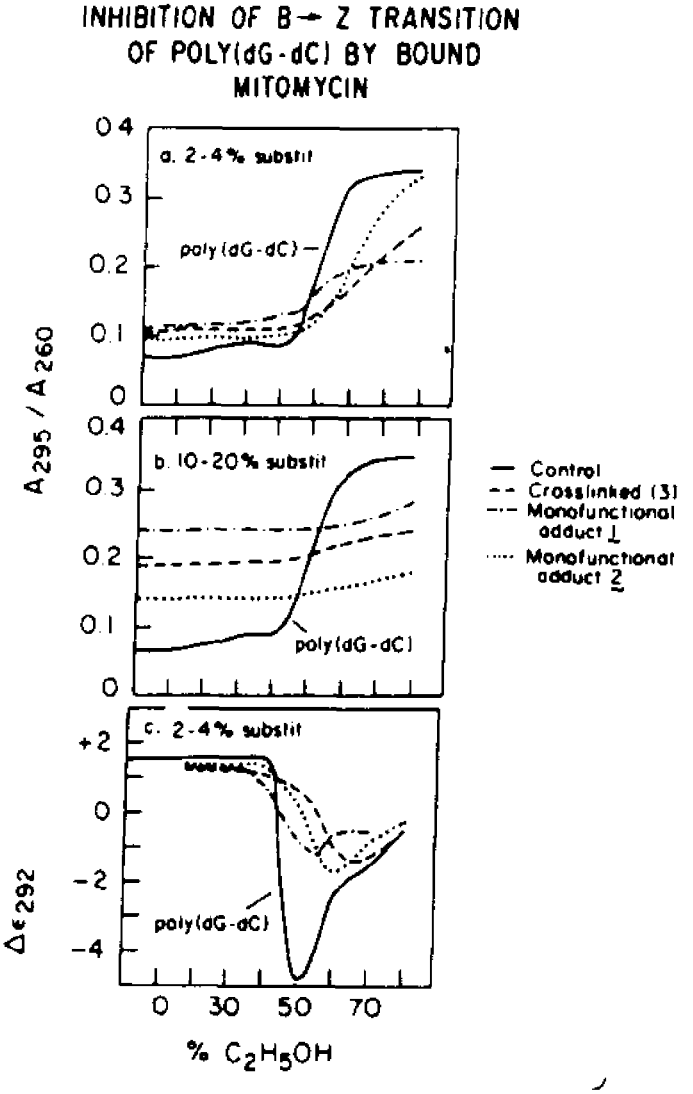


Figure 13: Inhibition of the B->Z transition of MC-poly(dG-dC) complexes. (a). Control and modified poly(dG-dC) complexes (2-4% base substitution) in 1 mM sodium phosphate, pH 7.2-0.1 mM EDTA buffer were diluted with increasing amount of absolute ethanol and absorbance of the solutions at 260 and 295 nm was monitored, according to the B->Z transition assay of Pohl. (b). The same experiment, employing complexes having higher levels of base substitutions by MC (10-20%). (c). Control and modified (polydG-dC) (2-4% base substitution) in the same buffer as (a) and B->Z transitions were monitored by circular dichroism.

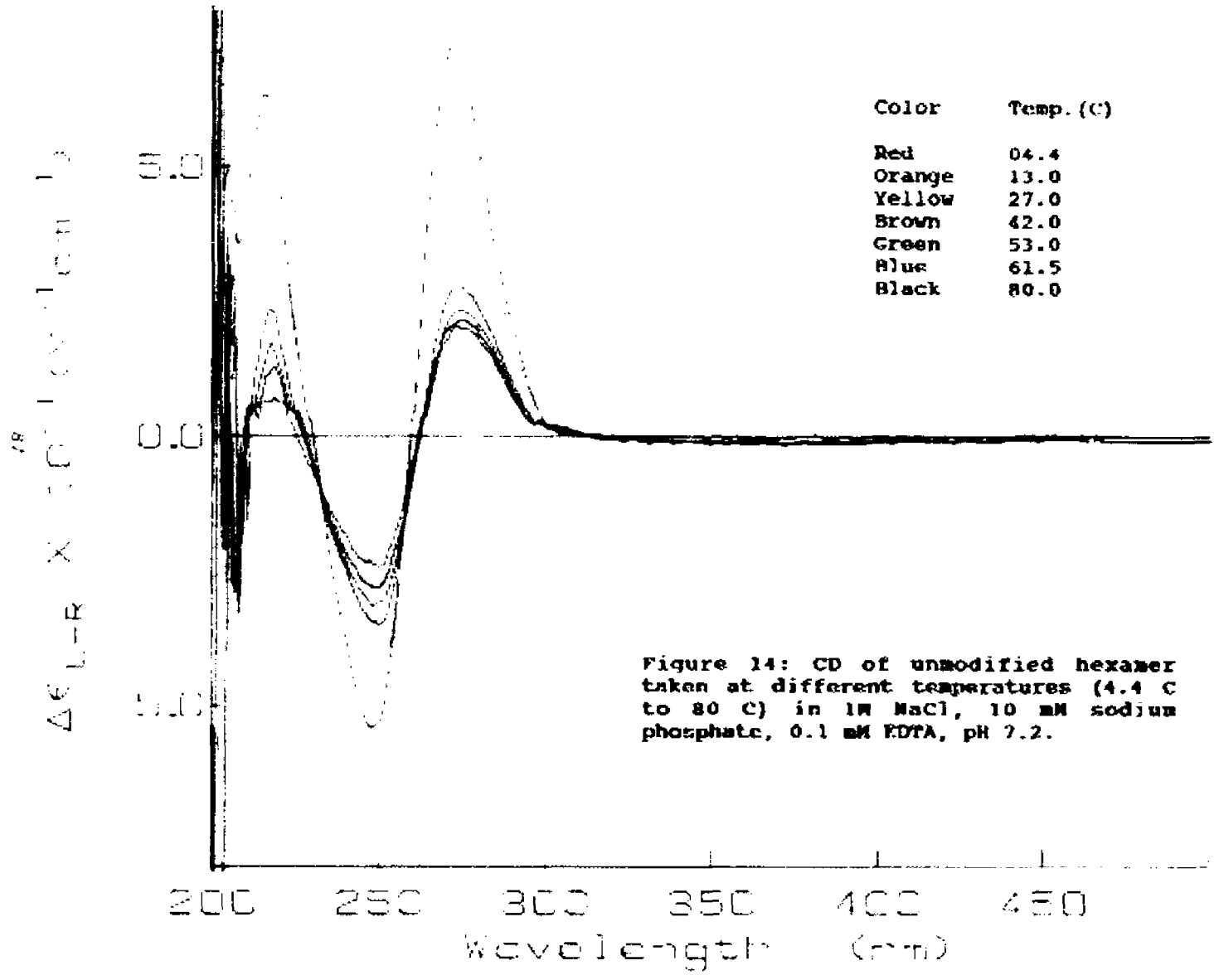


Figure 14: CD of unmodified hexamer taken at different temperatures (4.4 C to 80 C) in 1M NaCl, 10 mM sodium phosphate, 0.1 mM EDTA, pH 7.2.

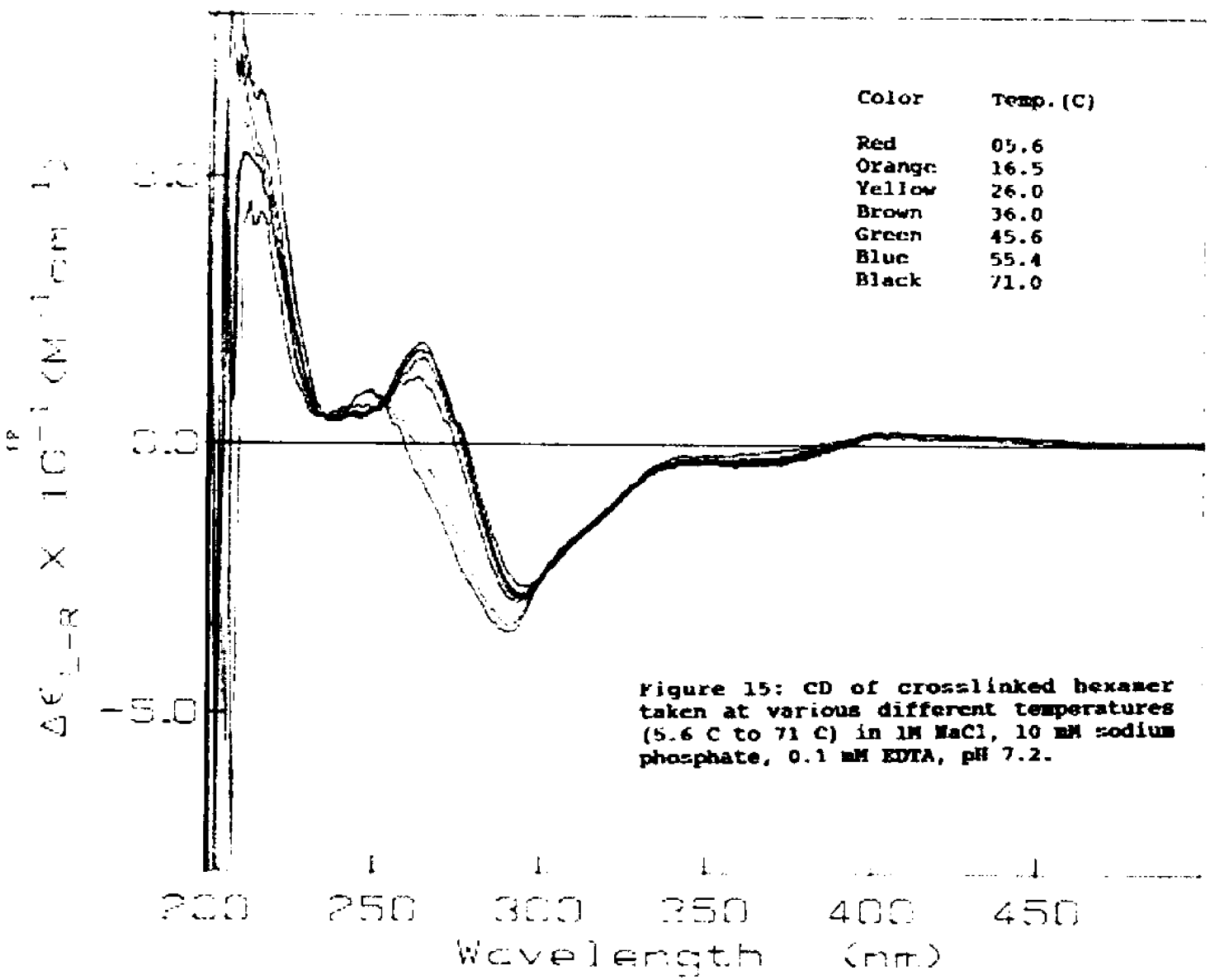


Figure 15: CD of crosslinked hexamer taken at various different temperatures (5.6 C to 71 C) in 1M NaCl, 10 mM sodium phosphate, 0.1 mM EDTA, pH 7.2.

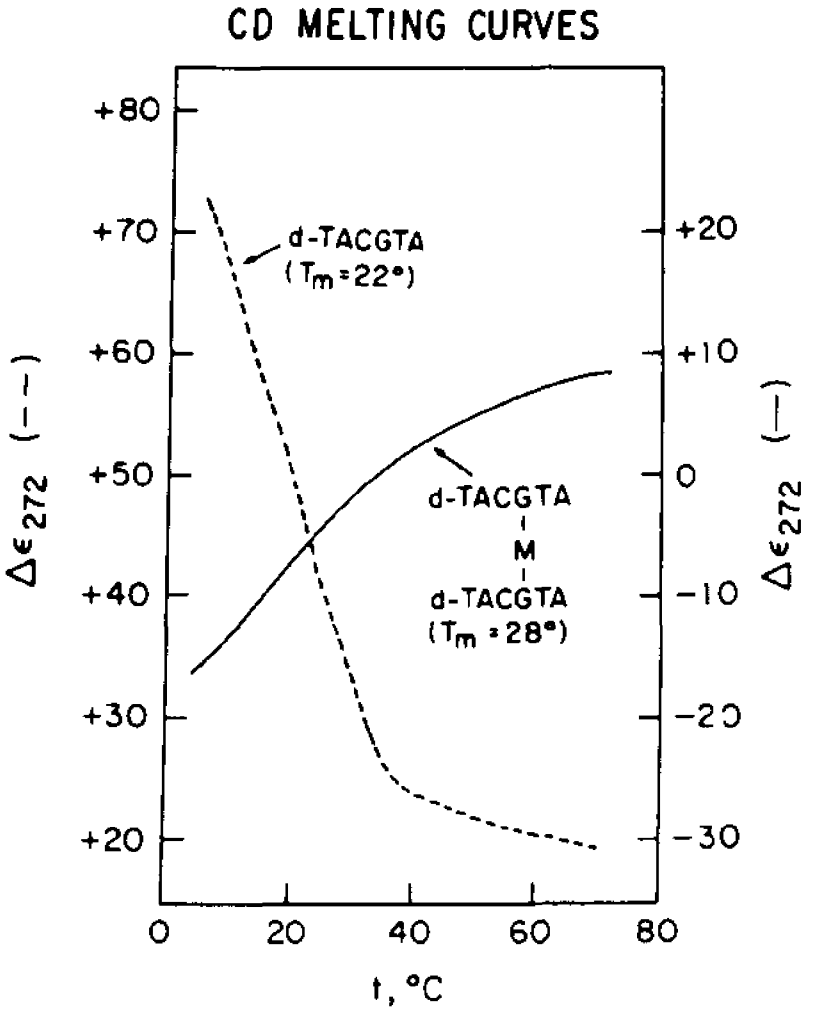


Figure 16: CD melting of control and crosslinked hexamer in 1M NaCl, 10 mM sodium phosphate, 0.1 mM EDTA, pH 7.2.

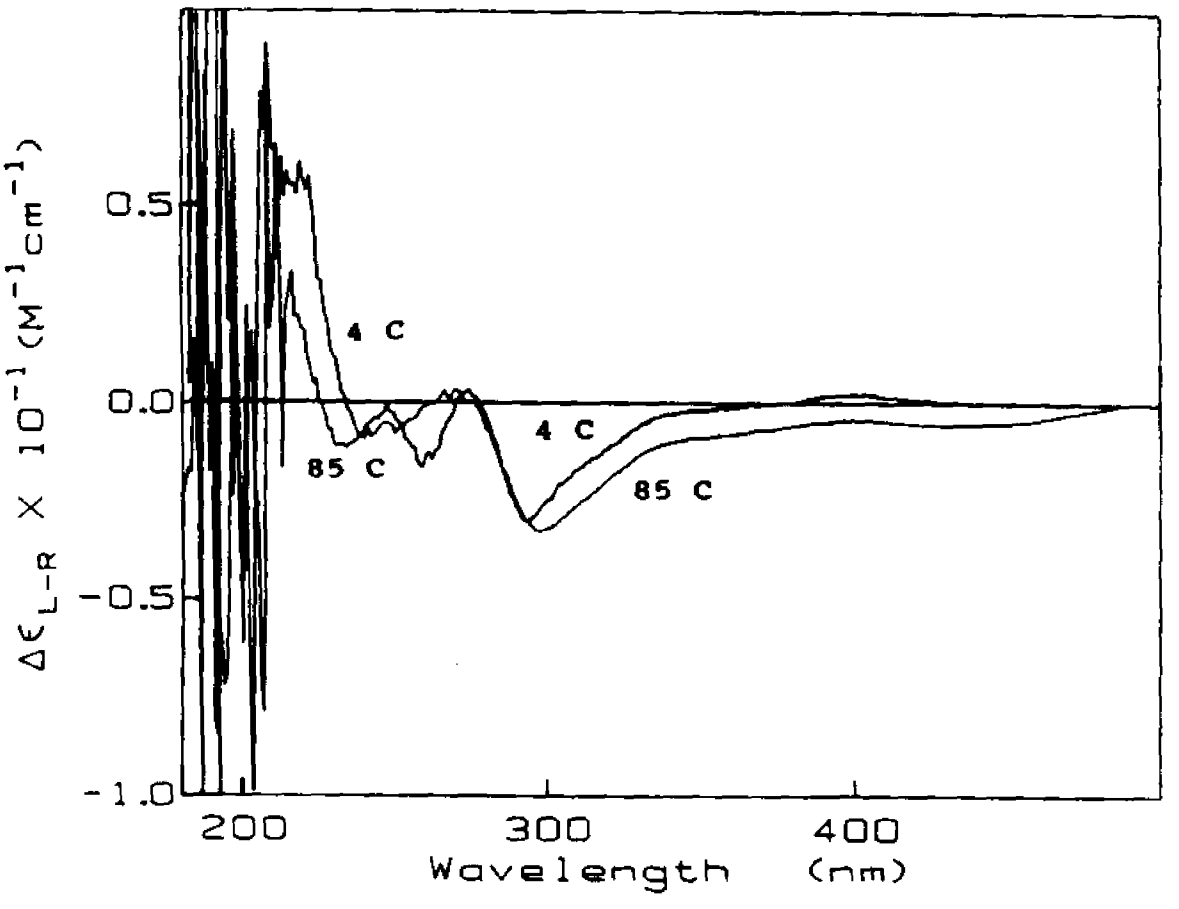


Figure 17: CD of MC crosslinked octamer taken at two different temperatures (4 C and 85 C) in 1M NaCl, 10 mM sodium phosphate, 0.1mM EDTA, pH 7.2.

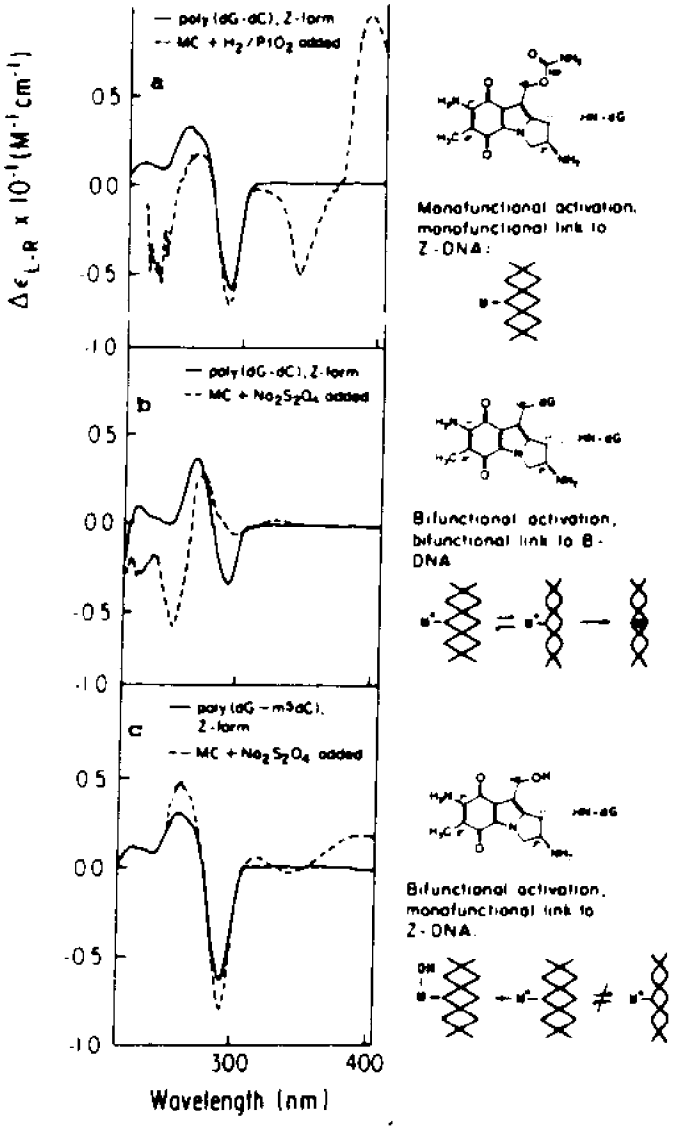


Figure 18: Effect of activated MC on the CD of Z-DNAs. (a). CD of poly(dG-dC) (0.66 mM in 15 mM Tris-0.13 mM $Co(NH)_3Cl_3$, pH 7.2), before and after addition of MC and H_2/PtO_2 (3.5 umol/umol nucleoside and 20 ug/umol MC respectively). (b). same as (a) except that the reducing agent is $Na_2S_2O_4$ (0.46 mM MC & 0.66 mM $Na_2S_2O_4$). (c). CD of poly(dG-m^5dC) in 50 mM NaCl, 5 mM Tris, 2 mM $MgCl_2$, pH 7.2, before and after the addition of MC and $Na_2S_2O_4$ (1:1 umole ratio).

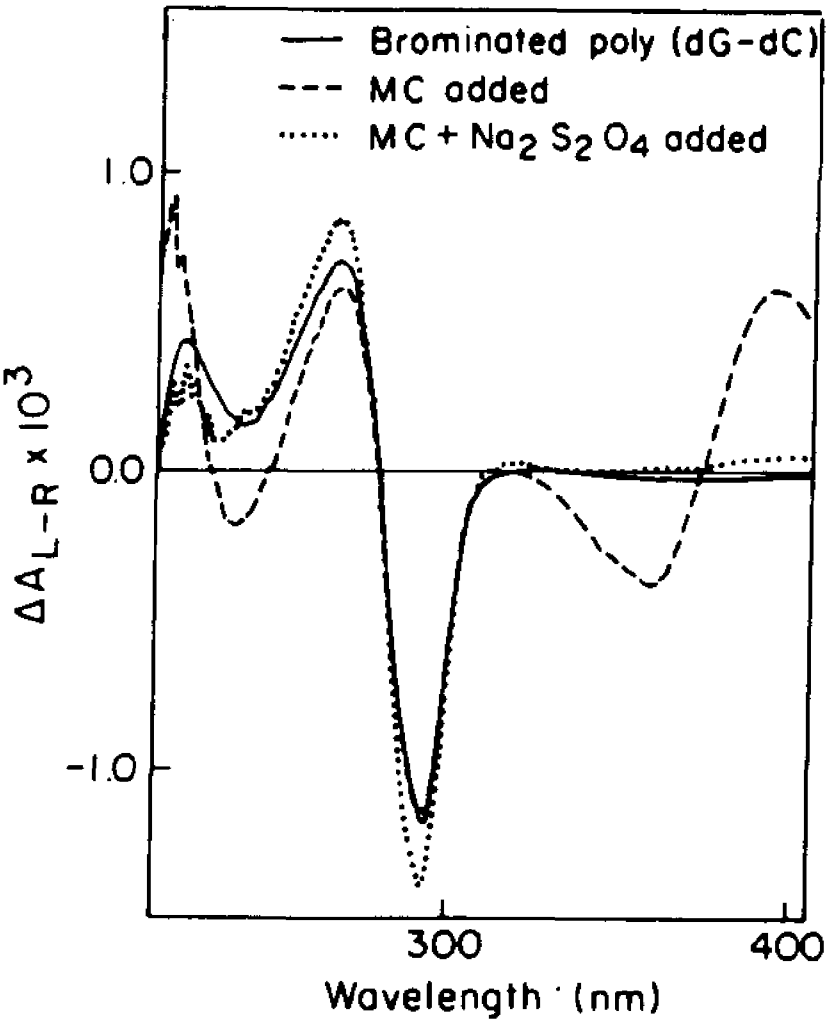


Figure 19: CD of brominated poly(dG-dC) (0.66 mM) (56% 8-bromoguanine, 13% 5-bromocytosine, (θ = 1.0 in 15 mM Tris, pH 7.2), before and after the addition of MC (0.46 mM) followed by the addition of $Na_2S_2O_4$ (0.66mM).

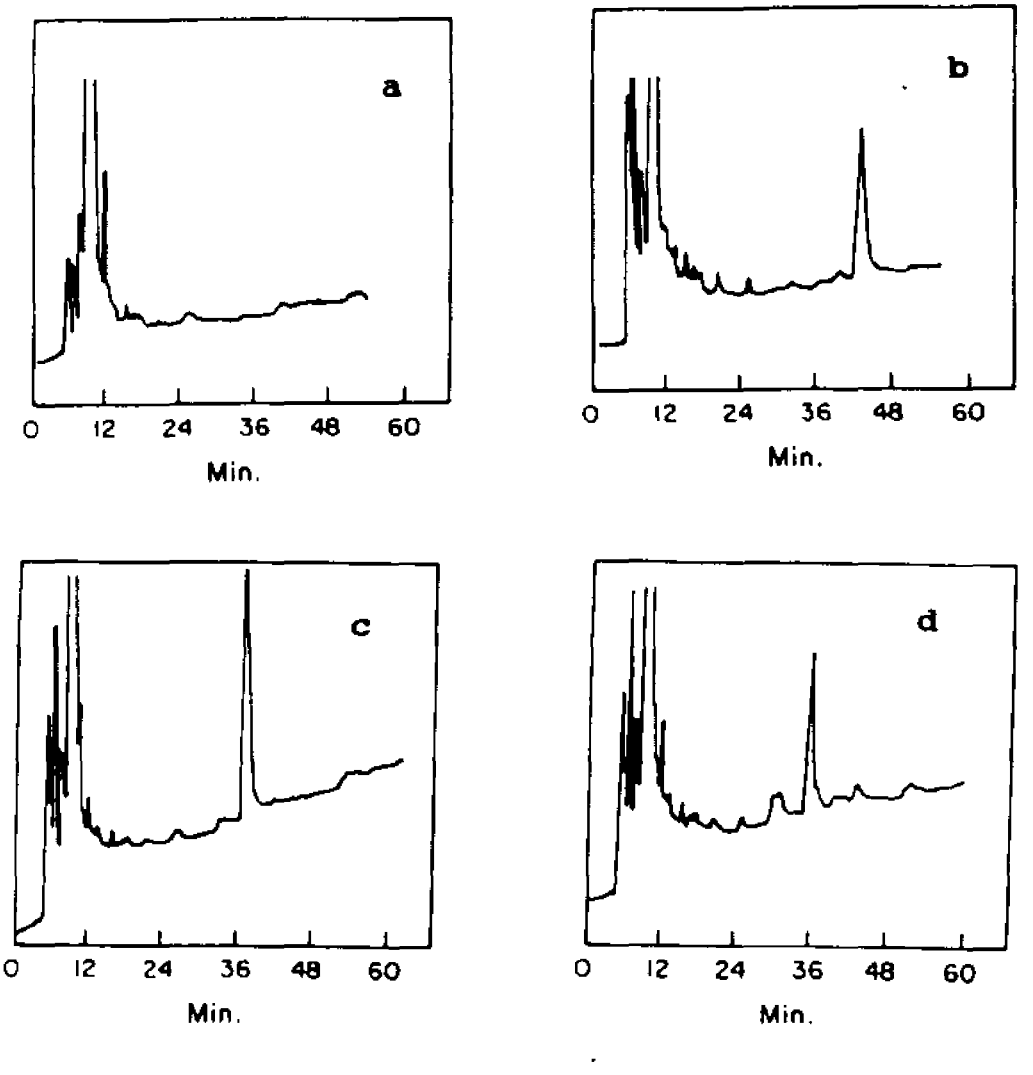


Figure 20: HPLC patterns from DNAase I/snake venom diesterase/alkaline phosphatase digests of MC-poly(dG-m^5dC) complexes. (a). control poly(dG-m^5dC) (no treatment with MC). (b). B form of poly(dG-m^5dC) treated with MC using $Na_2S_2O_4$ as reducing agent (1.5 mol/mol of MC). (c). Z form of poly(dG-m^5dC) treated with MC using H_2/PtO_2 (100 ug/5 u mol of MC). (d). Same as (c) but MC was reduced using $Na_2S_2O_4$ (1.5 mol/mol of MC).

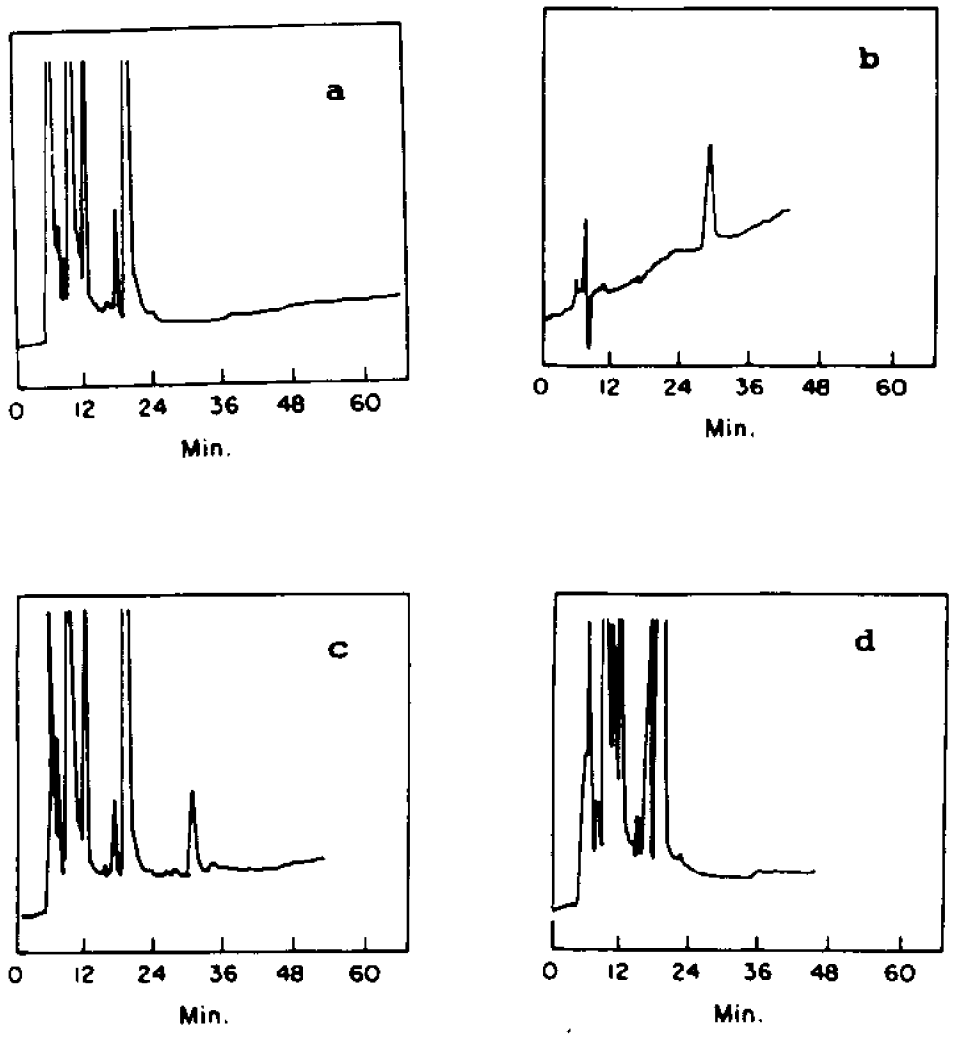


Figure 21: HPLC patterns from DNAase I/snake venom diesterase/alkaline phosphotase digests of control and MC modified brominated poly(dG-dC). (a). MC untreated brominated poly(dG-dC). (b). Standard adduct 2 (c). Z form of brominated poly(dG-dC) in 15 mM Tris, pH 7.2 treated with H_2/PtO_2 (100 ug/mol of MC) using 5 fold excess of MC. (d). Same as (c) above except that $Na_2S_2O_4$ (1.5 mol/mol of MC) was used as reducing agent.

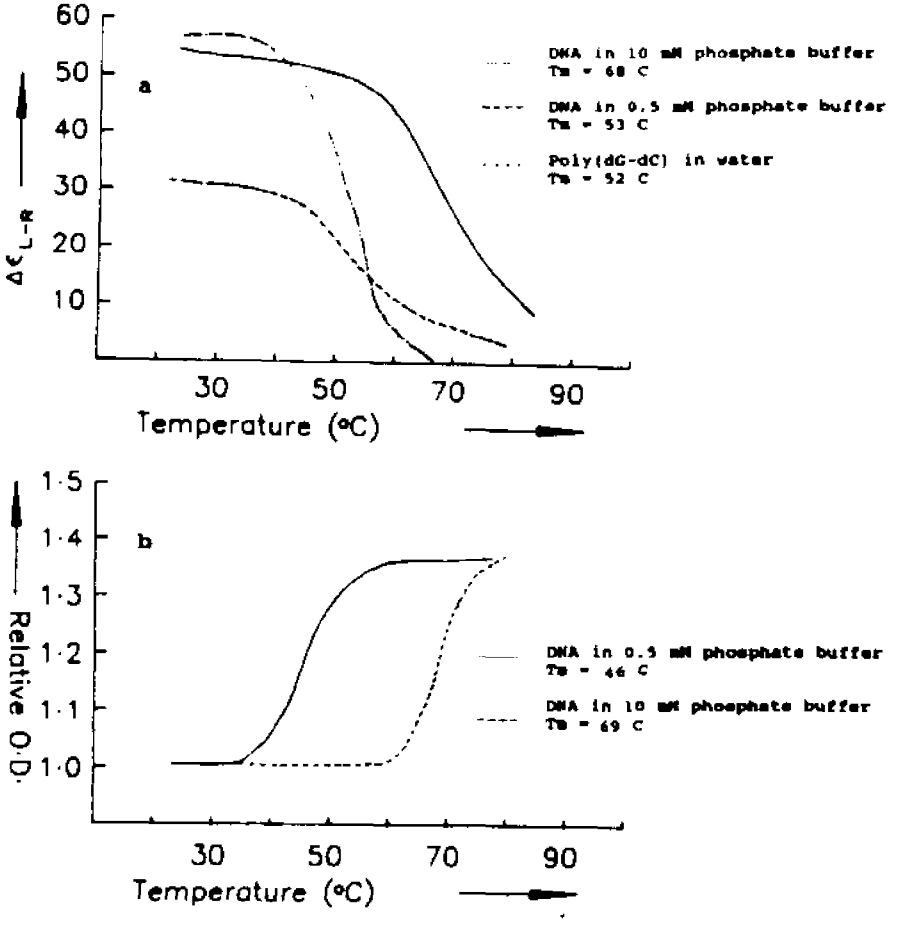


Figure 22: Effect of increasing temperature on the relative absorption & 187 nm peak of vacuum CD of poly(dG-dC) and calf thymus DNA. (a). Effect of increasing temperature on the intensity of 187 nm peak of vacuum CD of poly(dG-dC) (in water) and calf thymus DNA (0.5 mM & 10 mM phosphate buffer, pH 7.2). (b). Increasing temperature vs. reletive absorbance of calf thymus DNA in same two buffers as above.

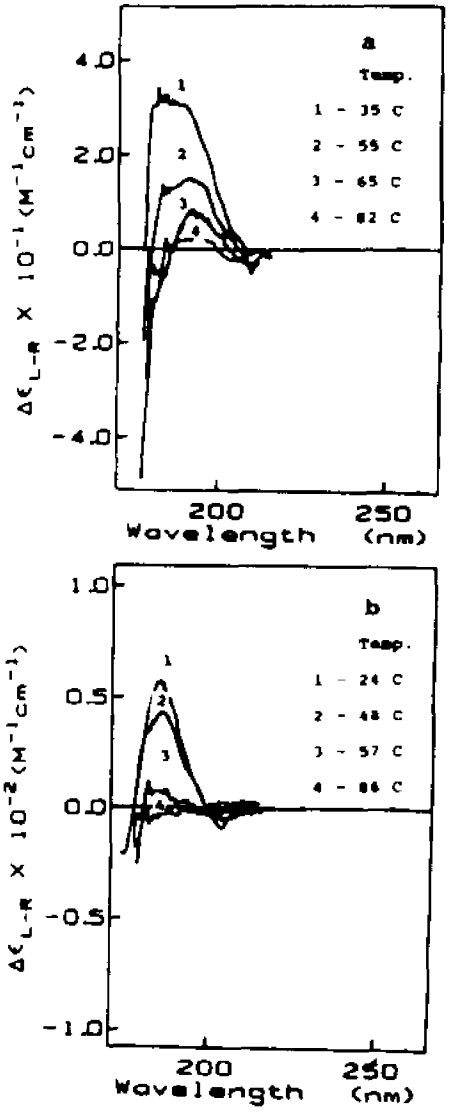


Figure 23: Effect of increasing temperature on 187 nm peak of vacuum CD of DNA and poly(dG-dC). (a). Calf thymus DNA in 0.5 mM phosphate buffer, pH 7.2. The effect of four different temperatures on the intensity of 187 nm peak is shown here. (b). same as above except that poly(dG-dC) in water was used.

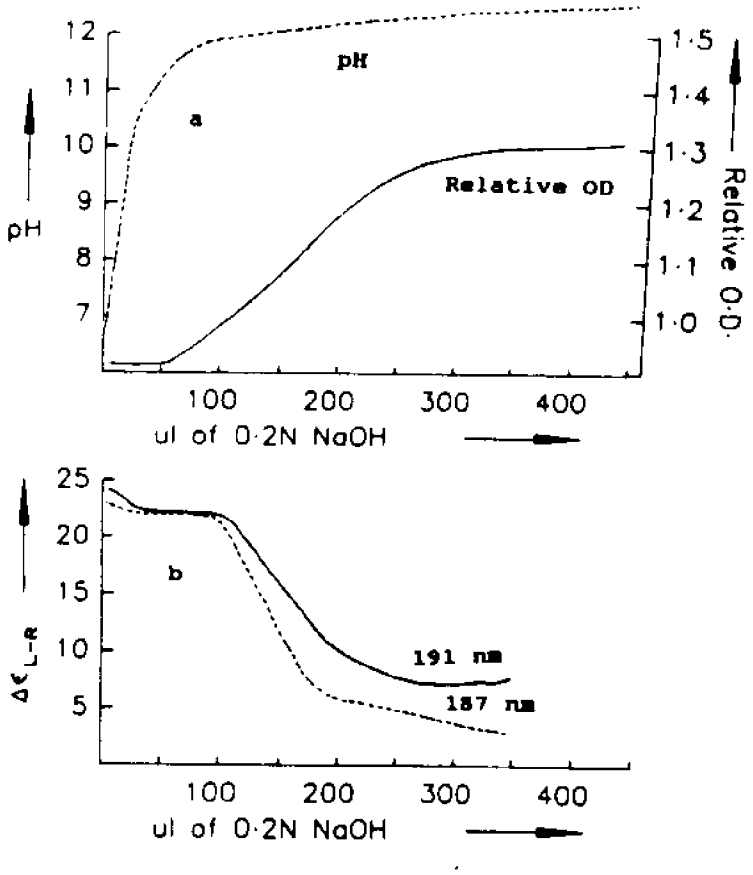


Figure 24: Effect of increasing concentration of 0.2N NaOH on pH, relative OD & vacuum CD of poly(dG-dC) and calf thymus DNA. (a). Effect of increasing concentration of 0.2N NaOH on pH & relative absorbance of calf thymus DNA. (b). Effect of 0.2N NaOH on 187 nm & 191 nm peak of vacuum CD.

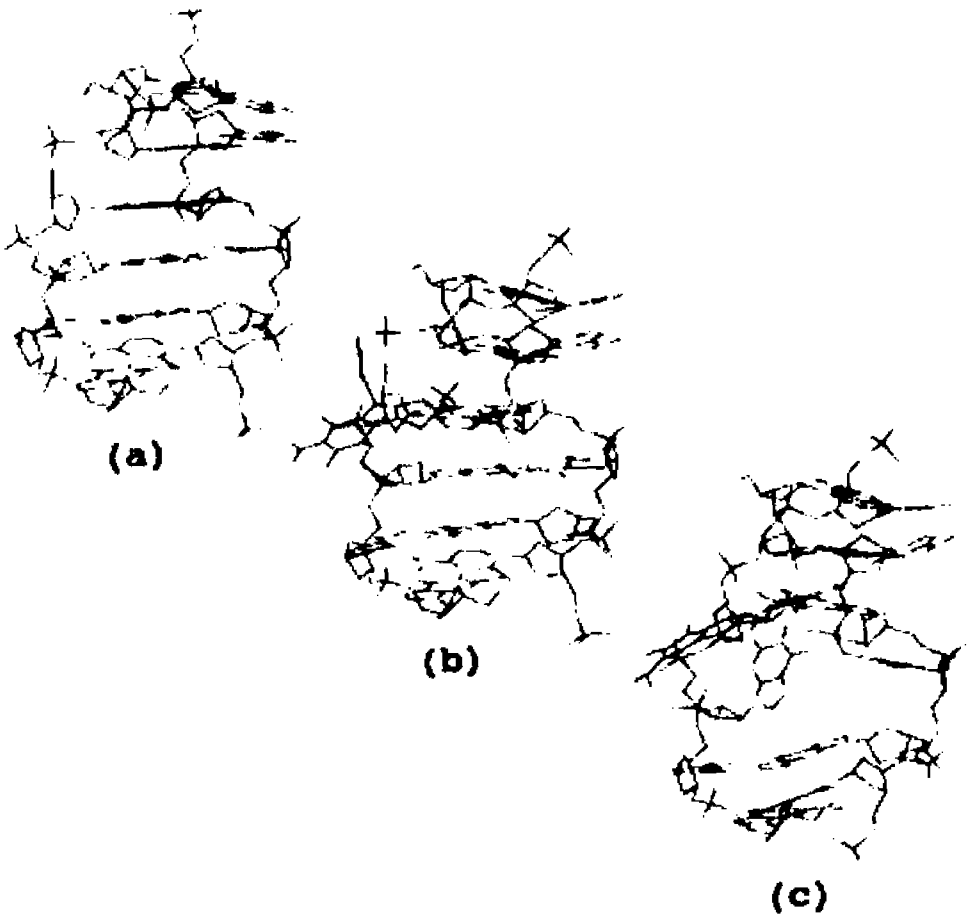


Figure 25: Computer constructed energy minimized molecular model of control and MC bound Z-DNA hexamer. (a). Control Z-DNA (b). Monofunctionally bound MC (shown in purple color) at control guanine. (c). MC incorporated bifunctionally into the control CpG sequence.

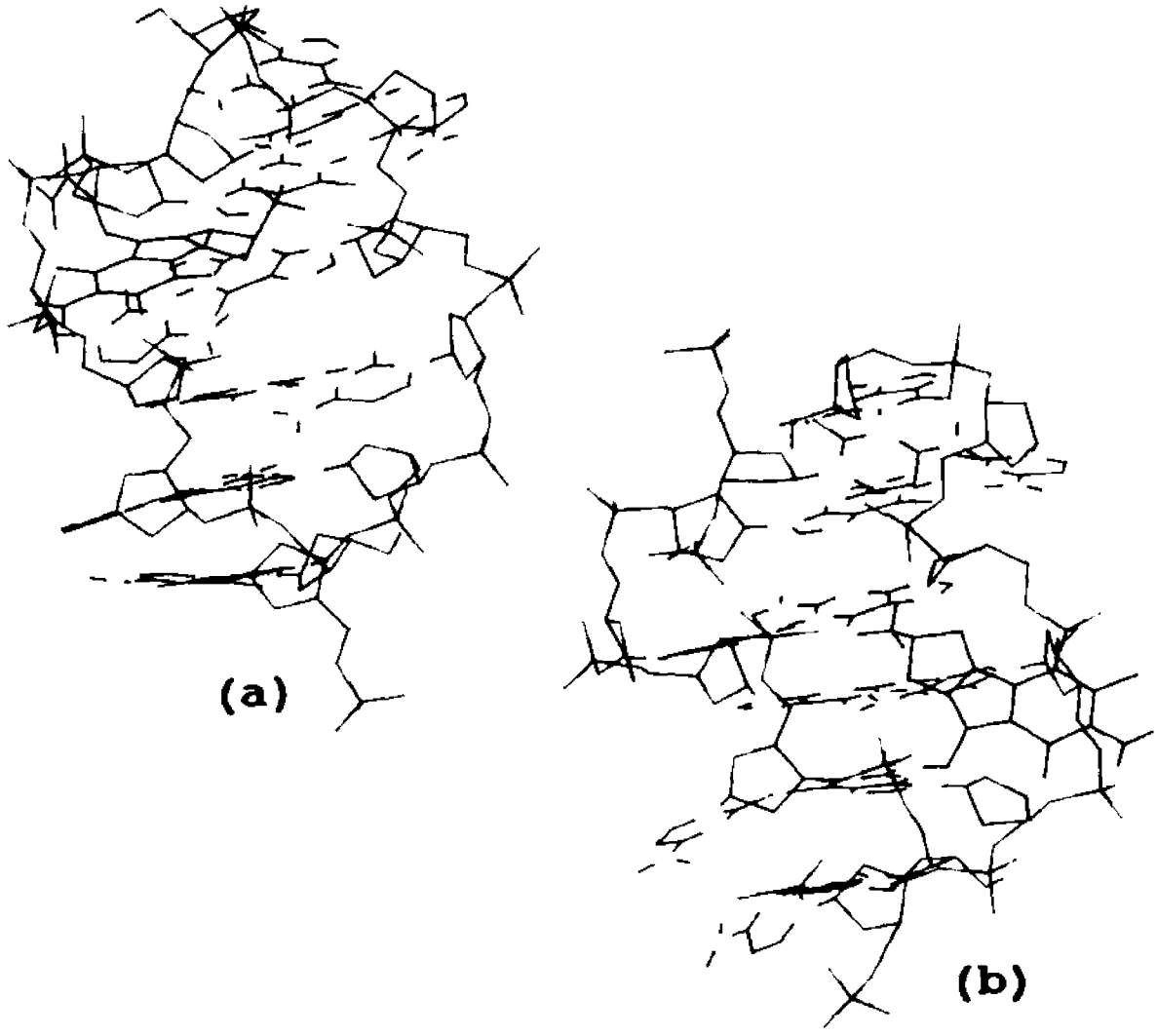


Figure 26: Computer constructed energy minimized molecular models of mono- and bifunctionally bound MC-Z DNA. (a). monofunctionally bound MC (shown in purple color) at central guanine of Z-DNA (b). MC incorporated bifunctionally into the central GpC sequence.

COMPLEXES OF MITOMYCIN WITH Z-FORM OF POLY(dG-dC)

Various forms of Z-DNA	Reactants Ratio (moles) DNA:MC	Reducing agent	Position of B-Z equilibrium (with reduced MC)	Binding Ratio (Z-DNA complexes)	Binding Ratio* (B form poly(dG-dC)-MC complexes)	Adducts distribution (HPLC)
Poly(dG-dC) (0.77 u mol/ml) in 0.13 mM cobalt hexamine	1:0.7	$Na_2S_2O_4$	Shifts from Z to B	0.076	0.1	Adduct 3 (crosslink)
		PtO_2	Stays in Z form	0.12	0.18	Adduct 1
Brominated poly(dG-dC)	1:0.7	$Na_2S_2O_4$	Stays in Z form	—	0.1	—
		PtO_2	Stays in Z form	0.09	0.18	Adduct 1
Methylated poly(dG-dC) (poly dG-m^{5}dC)	1:1	$Na_2S_2O_4$	Stays in Z form	0.02	0.13	Adduct 2
		PtO_2	Stays in Z form	0.05	0.2	Adduct 1

* For comparison with binding ratios of MC-Z DNA complexes

Table 1: Correlation between the mode of activation of MC with various forms of Z-DNA and the HPLC adducts from DNAase I/snake venom diesterase/alkaline phosphatase digestion.

MITOMYCIN ADDUCTS FORMED WITH Z-DNA

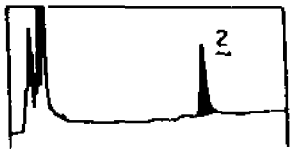
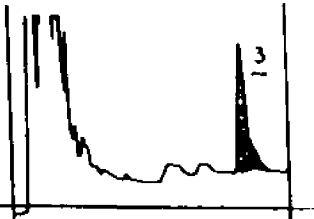
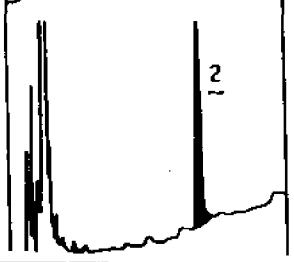
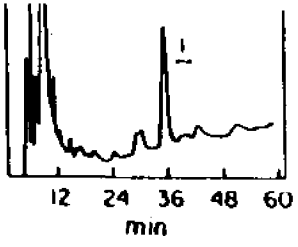
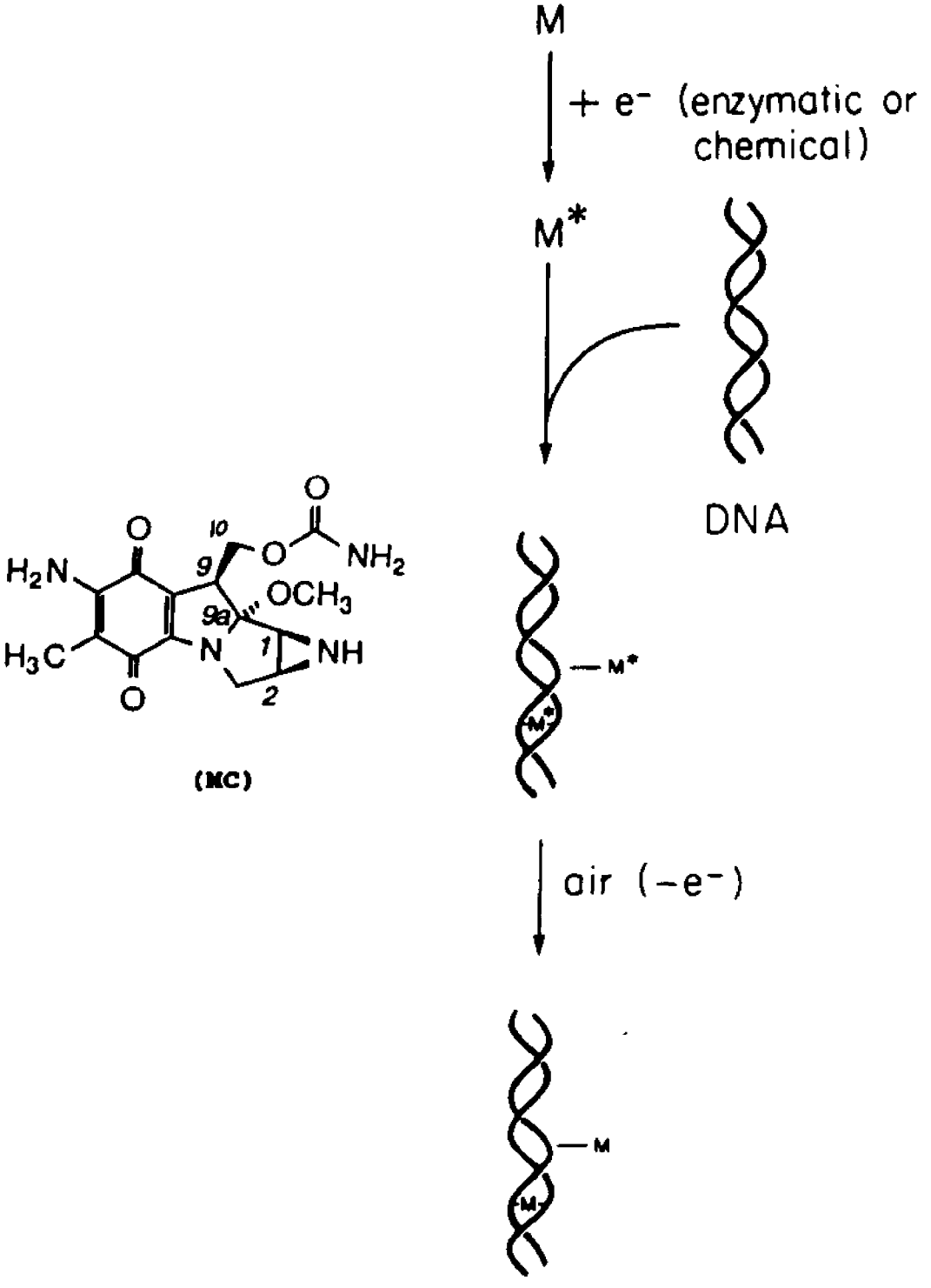
Z-DNA-substrate	Activation of MC	CD after the reaction	Adducts (HPLC)	Interpretation
Poly(dG-dC) with 0.13 mM $Co(NH_3)_6$ present	monofunctional (H_2)	Z-form		Monoactivated form binds to Z-DNA: Z-2
—"—	bifunctional ($Na_2S_2O_4$)	B-form		Crosslinking is possible to B-DNA only: Z ⇌ B → B; 2ᵃ 2ᵃ 3
Poly(dG-m^5dC) with 2 mM $MgCL_2$ present	monofunctional (H_2)	Z-form		Same as first entry above
—"—	bifunctional ($Na_2S_2O_4$)	Z-form		Crosslinking is possible to B-DNA only; H_2O competes: Z ⇌ B → B; 2ᵃ 2ᵃ 3; H_2O ↓ Z

Table 2: Summary of the mitomycin adducts formed with Z-DNA



(MC)

Scheme I: Scheme of reductive activation of MC to alkylate DNA monofunctionally and bifunctionally. M: a mitomycin residue in general; M*: activated form of mitomycin.

SECTION III

ALKYLATION OF DNA BY NITROGEN MUSTARDS AND THEIR EFFECTS ON B->Z TRANSITIONS

ABSTRACT
(SECTION III)

ALKYLATION FO DNA BY NITROGEN MUSTARDS AND THEIR EFFECTS ON B->Z TRANSITIONS
by
Anil Kumar Chawla

Adviser: Professor Maria Tomasz

Nitrogen mustards alkylate DNA primarily at the N7 position of the guanine of DNA. Bifunctional alkylating nitrogen mustards such as bis(2-chloroethyl)methylamine (HN2) are well known to crosslink DNA. We wanted to establish whether HN2 crosslinks poly(dG-dC) in analogy to its crosslinking action on DNA. We also wanted to determine experimentally the effect of alkylation products and the crosslinks if any, on B->Z transitions of DNA. N,N-diemethyl 1-2-chloroethylamine.HCl (HN1) was used to serve as a non-crosslinked modified control for HN2-modified complexes. Poly(dG-dC) was alkylated by HN1 and HN2 under various conditions. Extent of modification of guanine was determined by High performance liquid chromatography (HPLC) of perchloric acid digested samples. Conformational transitions were studied using circular dichroism (CD) in ultraviolet (UV) & vacuum UV region. Results showed a good correlation between the loss of intensity of the 187 nm peak of vacuum CD and the extent of guanine modification in all complexes. Alkali-treated samples of HN1- and HN2-poly(dG-dC) complexes showed complete inhibition of B->Z transitions measured in 60% TFE. Alkali-untreated samples at the same level of substitution of guanine showed CD in 60% Trifluoro ethanol (TFE) which was composite of B & Z forms as seen by comparison with computer generated CD of mixtures of B- and Z-forms. The observed inhibition of B->Z transition may be due to interruption of the cooperative B->Z transition process as both HN1- and HN2-modified samples were denatured at alkylated base pairs. Introduction of positive charge on the imidazole ring was also demonstrated for such B->Z inhibition.

INTRODUCTION

Nitrogen mustards are highly reactive alkylating agents which preferentially attack DNA. Major products of alkylation with DNA are the guanine-N7 adducts[1,2]. Mechlorethamine (HN2, bis(2-chloroethyl)methylamine is well known as an established cytotoxic drug which not only crosslinks DNA to DNA[3-6] but also forms crosslinks between DNA to protein[7], RNA to RNA[8,9] and RNA to protein[2,8]. Chloroethyl side chains of HN2 become highly reactive after cyclization and thereby forming immonium ion intermediates which alkylate DNA at N-7 position of guanine. The reaction of second chloroethyl side chain of HN2 with another guanine may result in the formation of DNA crosslink.

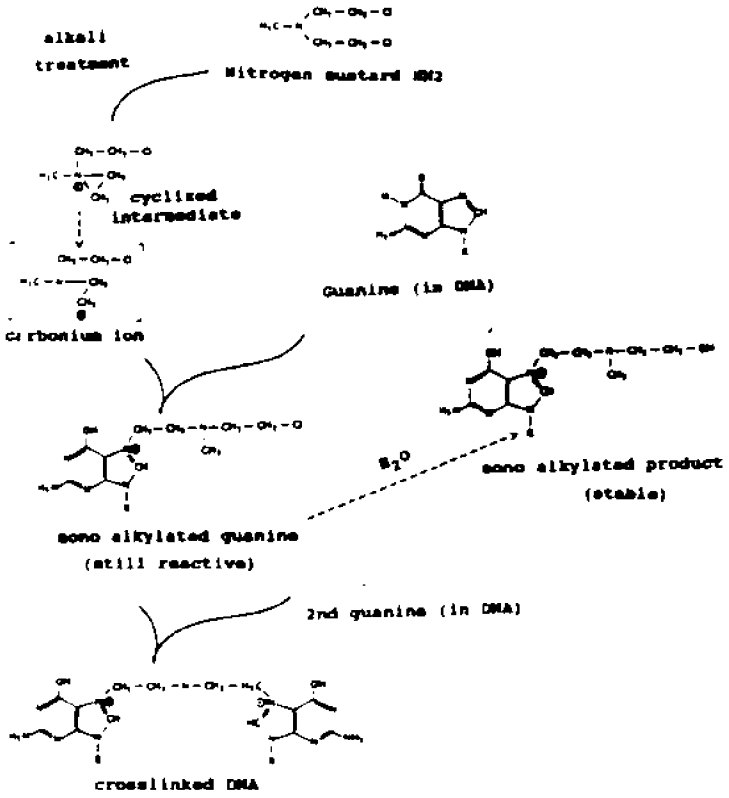


Figure 1: Monofunctional and bifunctional alkylation of HN2 with guanine residues of DNA.

Monoalkylated DNA may also result from alkylation of HN2 if the second side chain reacts with water rather than with another guanine (Figure 1). The cytotoxic effect of this drug is probably the consequence of such DNA crosslinks[2,10-12], which may be interstrand in nature, located between the two strands of DNA helix or intrastrand in nature, located within single strand of DNA. In general, the cytotoxicity of HN2 seems to correlate with the cross-linking efficiency[2,10,13-16].

The formation of crosslinks between two DNA strands in the double helix was suggested by the observation that HN2 treated DNA was converted to a reversibly denaturable form[3] as shown in the following figure :

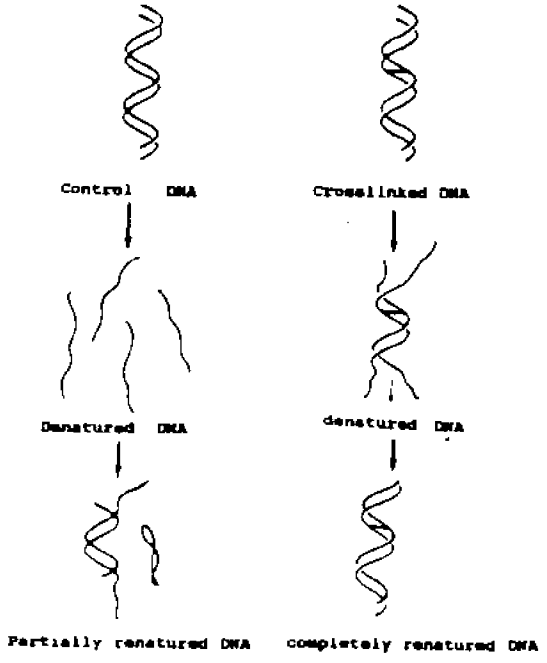


Figure 2: Effect of HN2 crosslinks on the ability of DNA to renature. (a). Denatured DNA after exposure to alkali and partial renaturation after neutralization. (b). Crosslinked DNA after exposure to alkali and complete renaturation after neutralization.

Denatured DNA or RNA yield a lower proportion of bis adduct (Figure 3) than the native DNA[17], which indicates that there is less tendency for the formation of such diguaninyl derivatives in single strands of nucleic acids.

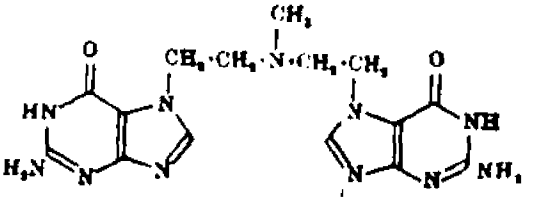


Figure 3: Structure of crosslinked adduct di-(B-guanin-7-ylethyl)amine.

It has been found[18] that 2.4 alkylations per molecule of calf thymus DNA by HN2 cause 10% reversible denaturation and nine alkylations of Bacillus subtilis DNA containing $3x10^4$ nucleotides constitute one crosslink hit which causes reversible denaturation of DNA[19]. Although it is assumed that the major mechanism of mustard cytotoxicity is due to cross-linking, there is no clear indication if intrastrand cross-linkage also leads to such toxicity. However, monofunctional alkylation has been known to contribute cytotoxicity due to depurination, imidazole ring opening, and DNA chain scission[12].

The purpose of our study of alkylating agents was to elucidate the effect of crosslinks on B->Z transitions. For the

latter investigation, a pair of nitrogen mustard was selected: HN2 (bis(2-chloroethyl)methylamine) as bifunctional and HN1 (N,N-dimethyl 2-chloroethylamine) as monofunctional agent. Such studies required various complexes of poly(dG-dC) with HN2 & HN1. Different levels of percent modification of guanine were achieved by varying the concentration of nitrogen mustards. The implication of such studies may be considerable, since the sequences of poly(dG-dC) exists in naturally occuring DNA and interconversions of B->Z forms may be involved in regulating DNA functions[20,21].

MATERIALS & METHODS

MATERIALS:

Materials and their sources are as follows:

Calf thymus DNA (type I; sonicated before use), bis(2-chloro--ethyl)methylamine (HN2), N,N-dimethyl 2-chloroethylamine.HCl (HN1), bacterial alkaline phosphatase and disodium guanylate from Sigma; M. luteus DNA (sonicated before use) from Worthington Biochemicals; poly(dG-dC) from Pharmacia P-L Biochemicals; DNAse I and SVD (snake venom diesterase) from Cooper Biomedicals.

METHODS:

Reaction of HN2 with disodium guanylate: Reaction of HN2 with disodium guanylate was carried out from the published procedure[22] with few changes: Bis-(2-chloroethyl)methylamine hydrochloride (2g) in water (10 ml) was added to a solution of disodium guanylate (2g) in water (10ml) at 37 C. Continued shaking gave a solution of pH 7.5 initially, falling to pH 6.5 after 1 hour. Concentrated hydrochloric acid (2 ml) was added and the solution heated at 100 C for 1 hour, cooled and applied to a column (23 x 2.5 cm) of Dowex 50 (H+ form; equilibrated with 2N hydrochloric acid). 700 drops were collected for each fraction. After obtaining two uv-absorbing peaks (140 fractions), HCl concentration was further raised to 5N, overall 300 fractions were collected. TLC-cellulose chromatography was carried out using solvent, methanol-ethanol-concentrated hydrochloric acid-

water (50:25:6:19).

Complexes of HN2 and HN1 with poly(dG-dC): Three complexes of poly(dG-dC) were made with HN2 using 0.3 mg, 0.5 mg and 1.0 mg HN2/OD_{260} poly(dG-dC). All reactions were carried out at the concentration of poly(dG-dC) of 5 OD_{260} /ml in 0.07 M phosphate buffer, pH 7.2. After 2 hours incubation at room temperature, the excess HN2 was neutralized by adding 250 ul of 1N $Na_2S_2O_3$. Samples were divided into two parts in each case: (1) Treated for 1 hr with NaOH/glycine buffer, pH 11.0, (2) untreated ("no treatment"). Mixtures were loaded to Sephadex G-100 column (28 x 2.5 cm) , using 0.02 M ammonium bicarbonate and fractions containing poly(dG-dC)-HN2 complexes were pooled, concentrated and lyophilized. Complex of poly(dG-dC) with HN1 (N,N-dimethyl-2-chloroethylamine) was made in the same way except that 3 mg HN1/OD_{260} poly(dG-dC) was used for incubation time of 20 hrs. Automatic pH titrator was used to maintain constant pH at 7.2. Excess of HN2 or HN1 in the reaction mixture was removed by dialysis against 3 changes of 1:200 volume of 10 mM sodium phosphate buffer, pH 7.2.

Complex of HN2 with calf thymus DNA: One complex of calf thymus DNA was made with HN2 using 0.15 mg HN2/OD_{260}DNA. Reaction was carried out at the concentration of 10 OD_{260}DNA /ml of 0.07 M phosphate buffer at pH 7.2. After two hours of incubation time at room temperature, the excess HN2 was neutralized by adding 500

ul/10 OD_{260} DNA of 1N $Na_2S_2O_3$. This reaction mixture was divided into two parts: (1) Treated for 1 hr with NaOH/glycine buffer, pH 11.3, (2) Untreated ("no treatment"). Mixtures were loaded to Sephadex G-100 column (28 x 2.5 cm), using 0.02 M ammonium bicarbonate. Fractions containing DNA-HN2 complex were pooled, concentrated and lyophilized.

Complexes of HN2 and HN1 with M.luteus DNA: An attempt was made to make these complexes by using 0.3 & 0.5 mg/OD_{260} DNA of HN2 and 2 mg/OD_{260} DNA of HN1 but no significant success was achieved in making such complexes.

Percent modification of guanine in HN2- or HN1-treated poly(dG-dC): This was determined by our standard procedure as follows: About 1 OD_{260} unit of HN1- or HN2-poly(dG-dC) complex was dried and the pellet was digested for 1 hour at 100 C containing 50 ul of perchloric acid. All samples were diluted to 1 ul with distilled water and pH was adjusted to 4.0 by KOH solution. White precipitates of potassium perchloride were separated by configuration. The resulting digests were separated by HPLC (reverse phase C-18 column) in 0.03 m phosphate buffer, pH 6.0. Quantitation of peaks (containing free bases) was accomplished by measurement of peak areas by triangulation and dividing the areas by the appropriate extinction coefficient at 254 nm (the wavelength of detection).

Conformational transitions of HN1- and HN2-poly(dG-dC) complexes: These complexes were studied by circular dichroism (Jobin Yvon Auto Dichrograph mark V) using 10 mM sodium phosphate buffer, pH 7.2 containing 60% trifluoroethanol .

Denaturation of poly(dG-dC)-HN1 or HN2 complexes: This was determined by measuring the extent of the loss of 187 nm peak of vacuum CD, as compared to the intensity of this peak of control poly(dG-dC). Vacuum CD of all complexes was taken in 10 mM sodium phosphate buffer, pH 7.2; pathlength 0.2 cm.

Digestion of HN1 & HN2-DNA complexes to nucleosides by nucleases: HN1 and HN2 complexes (about 3 A_{260} units/ml) in 0.005 M Tris.HCl/0.001 M $MgCl_2$ buffer, pH 7.0 were digested to nucleosides and nucleoside-drug adducts at 37 C with enzymes according to the following protocol: DNAse (1.25 units/A_{260} unit) at 0 hr and 1 hr; SVD (1.25 units/A_{260} unit; pH increased to 8.2) at 2 hr and 5 hr; alkaline phosphatase (0.5 unit/A_{260} unit) at 7 hr and incubation continued until 24 hr. HPLC separations were carried out by reverse phase column(Beckman ultrasphere ODS; 1.0 x 25 cm); flow rate 2.0 ml/minute and eluent 8:92 CH_3CN/0.03 M potassium phosphate buffer, pH 5.0. Quantitation of peaks were accomplished by measurement of peak areas by triangulation.

RESULTS

Complexes of HN1- and HN2-poly(dG-dC): Three complexes of HN2-poly(dG-dC) and one complex of HN1-poly(dG-dC) were made as described in Materials and Methods. Table 1 shows the percent modification of guanine of the four complexes. Results summarized in this table show that complexes of HN2 (1-3) exhibit increase in percent modification (29%, 44%, & 62%) with increase in concentration of HN2 (0.3 mg, 0.5 mg and 1.0 mg/ OD_{260} poly(dG-dC). On the other hand, one complex of HN1 (complex #4) which was made by using 0.3 mg HN1/OD_{260} poly(dG-dC) showed 11% modification of guanine.

Complex of HN2 with calf thymus DNA: The only one complex of HN2-calf thymus DNA which was made by using 0.15 mg HN2/OD_{260} DNA which showed 43% base modification of guanine residues. In contrast, same extent of percent modification of guanine was achieved by the HN2-poly(dG-dC) complex (complex # 2, table 1) which was made by using 0.5 mg HN2/OD_{260} poly(dG-dC). These results show that the concentration of HN2 required to modify guanine in poly(dG-dC) is much higher than the amount required to modify guanine by same extent in DNA.

CD of HN1 and HN2 complexes of poly(dG-dC): Figure 4a & 5a shows that there is gradual decrease in the intensity of 187nm peak of vacuum CD with increase in concentration of HN2 from 0.3 mg/OD_{260}

to 1.0 mg/OD of poly(dG-dC). CD in the non-vacuum range of all three complexes in 60% TFE show that there is complete inhibition of B->Z transition in alkali treated samples (Figure 4b) as seen by comparison with CD of control poly(dG-dC) which shows complete transition into Z-form. Alkali-untreated samples at the same level of modification of guanine residues show CD which resembles the composite of B & Z forms (Figure 5b) as seen by comparison with a series of computer generated mixtures of B- and Z-form CD of poly(dG-dC) (Figure 6). In comparison to HN2, HN1 was found to be quite resistant to form complexes with poly(dG-dC), thus only one complex of HN1 could be made (Table 1). This showed 13% decrease in 187nm peak of vacuum CD (Figure 7a). Non-vacuum CD of alkali-treated (poly(dG-dC)-HN1 complex in 60% TFE also showed complete inhibition of B->Z transition (Figure 7b) as seen by comparing with CD of Z-DNA. Untreated sample of poly(dG-dC)-HN1 complex at the same level of modification of guanine residue showed the CD which had (70%) right handed B form character (Figure 8b).

All CD results of HN1 and HN2 complexes of poly(dG-dC) are summarized in table 1. Results shown in this table indicate that there is a good correlation between percent modification of guanine residues as determined by HPLC of perchloric acid hydrolyzate and percent denaturation as seen by the loss of the 187nm peak of vacuum CD.

Preparation of Di(2-guanine-7'-ylethyl)methylamine ("Standard Crosslink"): Standard Di(2-guanine-7'-ylethyl)methylamine was prepared in order to compare its uv or other physical properties with those of HN2-poly(dG-dC) adducts (Materials and Methods). Figure 9 shows the Dowex 50 H^+ column chromatography of various adducts formed from reaction of disodium guanylate with HN2. Out of these six peaks, the third peak showed zero value of Rf as seen by TLC-cellulose chromatography. Paper chromatography has been previously proven to be valuable for identifying diguaninyl type adducts when bifunctional alkylating agents are used and these adducts (diguaninyl) have been reported Rf value of zero in all solvents of paper chromatography. UV spectrum of the fractions of the above third peak showed the maxima of 252 nm at zero pH (Figure 10a) and maxima of 284 nm at pH 7.0 (Figure 10b). These values of uv maxima at two different pH are fully consistent with the reported values as reported for the diguaninyl adduct (Figure 3), proving that this is the expected "standard crosslink".

Search for the crosslink from HN2-poly(dG-dC) complexes: Sephadex G-25 chromotography of perchloric acid digested sample of HN2-poly(dG-dC) gave three peaks (Figure 11a). The first peak corresponds to the standard crosslink (Figure 12b), and the second & third peaks correspond to cytosine & guanine respectively as seen by comparing with standards (Figure 12a). The first peak of figure 11a does not seem to be hydrolyzed

completely by perchloric acid. Thus, the fractions 5-9 which correspond to the standard crosslink were pooled, concentrated and re-treated with perchloric acid for further digestion. Digested sample was rechromatographed on same column (Figure 11b) and the first peak of this chromatographic run corresponded to the fractions of standard crosslink. It was presumed that this is the crosslink peak but the uv spectrum of it (Figure 11c) was not found to be identical to that of standard crosslinked adduct (Figure 10b) (maxima at 255 nm rather than 284 nm at pH 7.0). The small peak in figure 11b is probably due to cytosine as the fractions were pooled from the previous run and cytosine peak was very close to the pooled first peak.

Enzymatic hydrolysis of the complexes to the nucleoside level was also unsucessful in the search. A mixture of enzymes (DNAse I, SVD and alkaline phosphatase) were used for digestion of HN1- and HN2-poly(dG-dC) complexes and the resulting digest was submitted to HPLC. Samples HN2 and HN1-poly(dG-dC) complexes gave almost similar results (Figure 13a & Figure 14a). The two major peaks in both complexes corresponded to elution time of standard dC and dG. Other small peaks were ruled out for the crosslinked adduct (Figure 3) by comparing their uv spectra (Figure 13b,14b) with that of standard (Figure 10).

HPLC of enzyme digested samples of M.luteus DNA-HN1 and HN2 complexes did not show any changes in HPLC pattern (Figure 15b,c) as compared with control M.luteus DNA (Figure 15a).

DISCUSSION

The purpose of the preparation of HN1- and HN2-modified poly(dG-dC) was to study the effect of covalent crosslinks between N-7 atoms of guanine residues in the major groove on the B->Z transition. The HN1-modified polynucleotide was to serve as the non-crosslinked but otherwise similarly modified control for the HN2-modified samples. The B->Z transition was to be monitored by the characteristic change of the CD spectra in the 220-400 nm ("non-vacuum") range[23]. The "vacuum CD" (below 200 nm) spectra were to serve as additional indicators of the conformations of the samples, since bands in this region are highly characteristic of such[24] .

First we wanted to establish that HN2 does crosslink poly(dG-dC) in analogy to its crosslinking action on DNA. This has not been studied before. The structure of DNA shows that the N-7 atoms of guanine moieties are sterically well available for reaction. For a pair at these atoms to be situated at a distance apart such that they could be linked by an extended alkyl chain 4 or 5 atoms, i.e. about 8 A, it appears from the model shown in figure 16 that the sequence of bases along the DNA macromolecular chain must be guanine-cytosine along one strand and by the necessary complementary pairing of bases, cytosine-guanine on the other strand. With this sequence guanine moieties on each of the twin strands could be readily cross-linked, as represented

diagrammatically in figure 16. Linkage of adjacent guanine moieties on the same molecular strand of DNA cannot be eliminated on steric grounds, but would require the alkyl chain to assume a less probable non-extended configuration[17]. Support for this model of cross-linking comes from consideration of the proportion of cross-linked guanine resulting from alkylation in the decreasing order of DNA, denatured DNA and RNA.

On the basis of these considerations, we hoped for the formation of maximum amount of crosslink in poly(dG-dC) upon reaction with HN2. The first surprise was that poly(dG-dC) was much less reactive towards HN2 and HN1 than DNA: as seen in Results, concentration of HN2 required to modify guanine in case of poly(dG-dC) is much higher than the amount required to modify guanine by same percentage in DNA. 0.5 mg of HN2 per OD (at 254 nm) was needed to achieve modification of 44% of guanine in poly(dG-dC) while only 0.15 mg HN2/OD modified guanine by 43% in case of DNA as revealed by HPLC results. In case of poly(dG-dC)-HN1 complexes, 30 times more concentration of HN1 (as compared to HN2) and 10 times longer incubation time at constant pH was needed to get the same percentage modification of guanine. The changes in absorption ratio 295/260 were quite significant. This ratio was found to be 0.28 in sample where poly(dG-dC) was treated with 0.5 mg HN2/OD sample as compared to the control value of 0.11. In addition to the lower overall binding affinity of HN1 and HN2 toward poly(dG-dC) than toward DNA, apparently no

crosslink was formed with the bifunctional mustard HN2, despite of the supposedly favorable -GC- base sequence in the polynucleotide. This was concluded from our failure to detect any of the crosslink (Figure 3) in our $HClO_4$ or nuclease hydrolysates of HN2-treated poly(dG-dC). The authentic crosslink (Figure 3) was obtained from reaction of HN2 with guanosine, by a published procedure[22], for direct comparison.

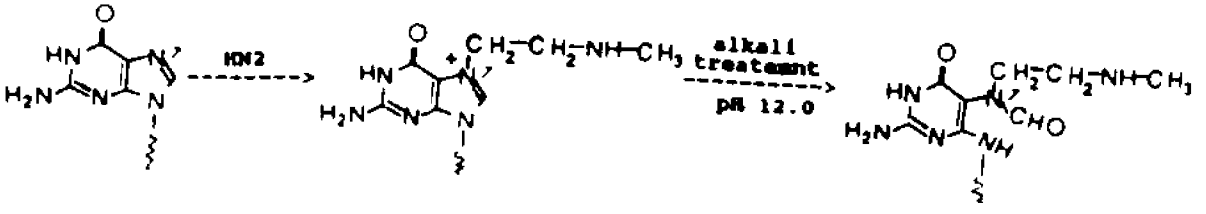
The low reactivity of poly(dG-dC) towards both mustards HN1 and HN2 is consistent with the recent findings of Mattes et al.[25], who studied the sequence-specificity of mustard binding to DNA. They found that 5'-GC-3' sites are the least reactive for almost all of the nitrogen mustards, with the possible exception of uracil mustard. Their data suggest that the formation of inter-strand crosslinks, by most nitrogen mustards may be limited by not only the low frequency of 5'-GC-3' sites but also by the weak alkylation reaction at these sites. Rather, nitrogen mustards preferentially attack regions of the genome rich in contiguous guanines. All nitrogen mustards show an enhanced reaction with guanines flanked by other guanines. Other studies[26] have also noted enhanced reactivity of HN2 and phosphoramide mustards with pairs of guanines in a segment of alpha DNA, a reiterated sequence in the human genome. What might explain the enhanced reactivity of such sequences ? One possibility is that the neighboring bases might alter the reactivity of the guanine N-7 position via electrostatic

interactions. A local increase in the electronegativity near the guanine N-7 position would be expected to increase the affinity for a positively charged species[27].

Although the poly(dG-dC)-mustard complexes prepared by us (Table 1) did not contain the desired crosslinks, they represented a class of modified polynucleotides containing monofunctional alkyl groups in the 7-position of the guanines, that is in the major groove of DNA. This notion was based on the known chemistry of interaction of the mustards with DNA and on our analysis of the modified polynucleotides for decrease of guanine content. It was of interest to study the effect of the G-7-alkyl groups on the B->Z transition of poly(dG-dC). This group confers a positive charge on the imidazole ring of guanines. Rich & coworkers reported that 7-methylated poly(dG-dC) facilitated the B->Z transition[28]. Our alkyl groups are more complex and larger, however, than a methyl group and therefore we may expect a different effect. The influences that base modifications exert on the B->Z transition have generally been rationalized in terms of the effects of the modification on the free energy levels of the B and the Z forms. The explanations for the changes in these levels usually involve steric or structural requirements for better van der Waals interactions in one form or the other, the energetics of syn/anti conversions, electrostatic factors and asymmertric distribution of diffusible ions in the grooves and/or hydration effects[29]. Recently, it has

been reported[30] that covalent attachment of an alkylamine at N^2 of guanine in the minor groove prevents the B->Z transition in poly(dG-dC). It has been demonstrated that this inhibition is dependent on the positive charge and not on the steric properties of the amine. It was concluded that the origin of the free energy decrease for the adduct in the B-form has to do with the electrostatic effects of placing the positive charge in the minor groove together with, possibly, some charge induced alterations of the hydration in that locus.

Before studying the B->Z transitions of the HN1- and HN2-modified poly(dG-dC), samples were divided into two portions. One of the two portions in each sample was further modified by treatment with alkali. The known chemistry involved in these steps is the following[31,32]:



("alkaline untreated samples")

("alkaline treated" samples)

Both sets of samples ("alkaline untreated" and "alkaline treated") were examined for ease of B->Z transition, relative to unmodified poly(dG-dC). The transition was induced by altering the aqueous buffer (10mM phosphate,pH 7.2) to 60% trifluoroethanol & 40% phosphate buffer[33]. As seen in Results, the "alkaline-untreated" samples exhibited partial, and the "alkaline-treated" ones exhibited total inhibition to conversion into Z-form. A clue to an explanation for this inhibition is given by the vacuum CD analysis in aqueous buffer of these samples: In each, the 187 nm positive peak is diminished in intensity, in exact proportion to the extent of alkylation, i.e. the "percent modification of guanine residues" (Table 1). From our work (see page 53 of this thesis) and of others[34], it is known, that the decrease of intensity of this peak is due to denaturation in a proportional manner. From the present results summarized in Table 1, we conclude that both the HN1- and HN2-modified poly(dG-dC) samples are denatured at the alkylated basepair. The observed partial inhibition of the B->Z transition may be due to interruption of the cooperative B->Z process and Z-structure at the local, denatured regions, since the extent of inhibition of the "alkaline untreated" samples in the HN2 series increases proportionally to their percent(%) modification and threrefore to their extent of denaturation. The "alkaline treated" samples were not assessed for extent of denaturation in the vacuum CD. Their complete resistance to the Z conformation may be due to more extensive disruption of the B-duplex

structure.

These results suggest a new type of inhibition of the natural conformational dynamics of DNA: 7-alkylation of guanines inhibits the B->Z transition because of the disruption of the basepairing at the alkylated site. It is also shown for the first time, that alternating GC sequences are not crosslinked by HN2 in poly(dG-dC). The inhibitory effects of the monofunctional alkylation on the basepaired structure of poly(dG-dC) and on its B->Z transition as observed in the present work may be further substantiated in the future by other probes of DNA structure such as NMR or X-ray diffraction methods.

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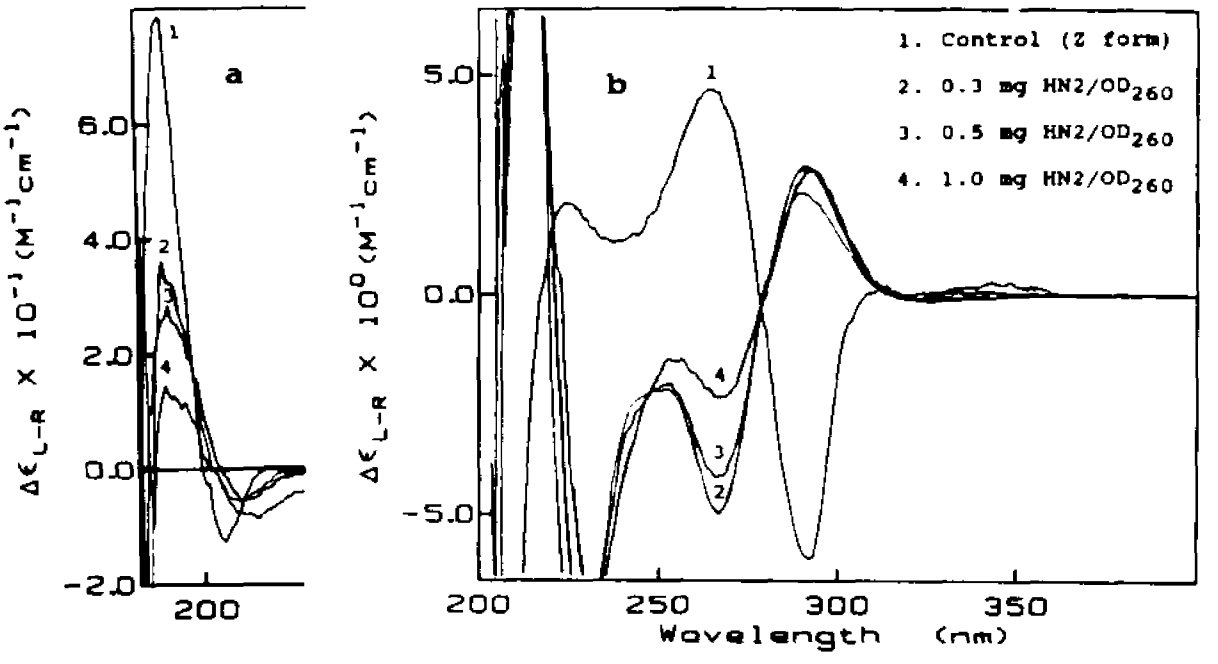


Figure 4: CD of alkaline treated HN2-poly(dG-dC) complex in 10 mM phosphate buffer, pH 7.2. (a). Decrease in the intensity of 187 nm peak of vacuum CD of different complexes (different level of modification of guanine residues). (b). CD under conditions for B->Z transition of same three complexes (60% trifluoroethanol (TFE), 40% 10 mM sodium phosphate buffer, pH 7.2).

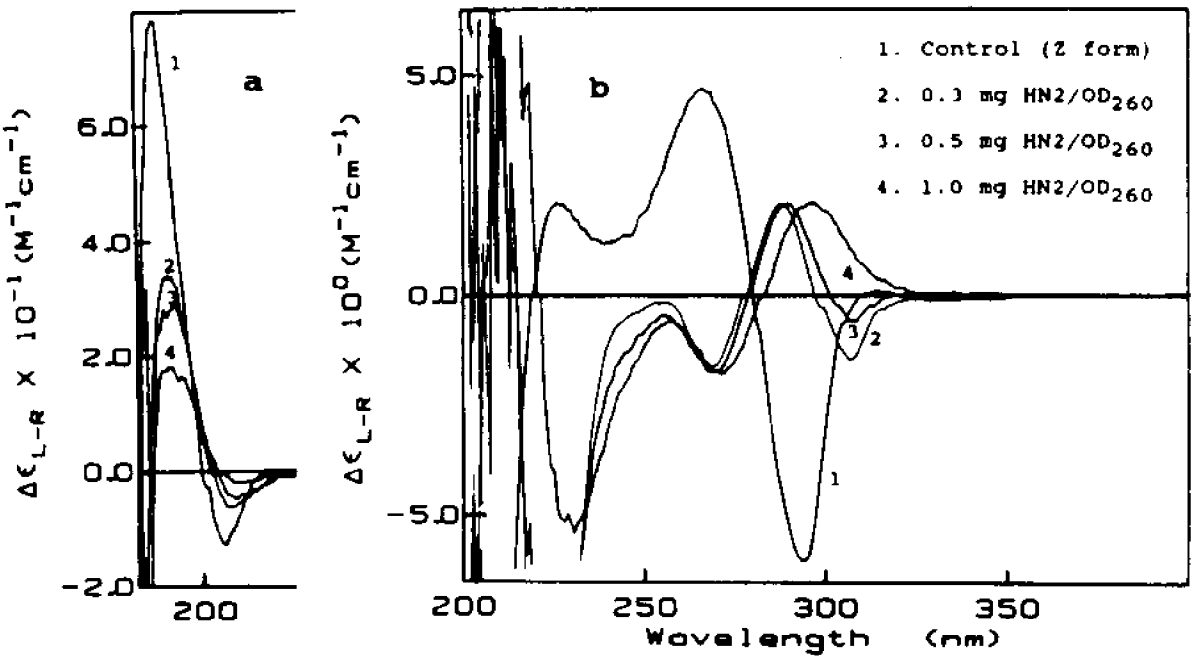


Figure 5: CD of alkaline untreated HN2-poly(dG-dC) complexes in 10 mM phosphate buffer, pH 7.2. (a). Decrease in the intensity of 187 nm peak of vacuum CD of different complexes (different level of modification of guanine residues). (b). CD under conditions of B->Z transition of same three complexes (60% trifluoroethanol (TFE), 40% 10 mM sodium phosphate buffer, pH 7.2).

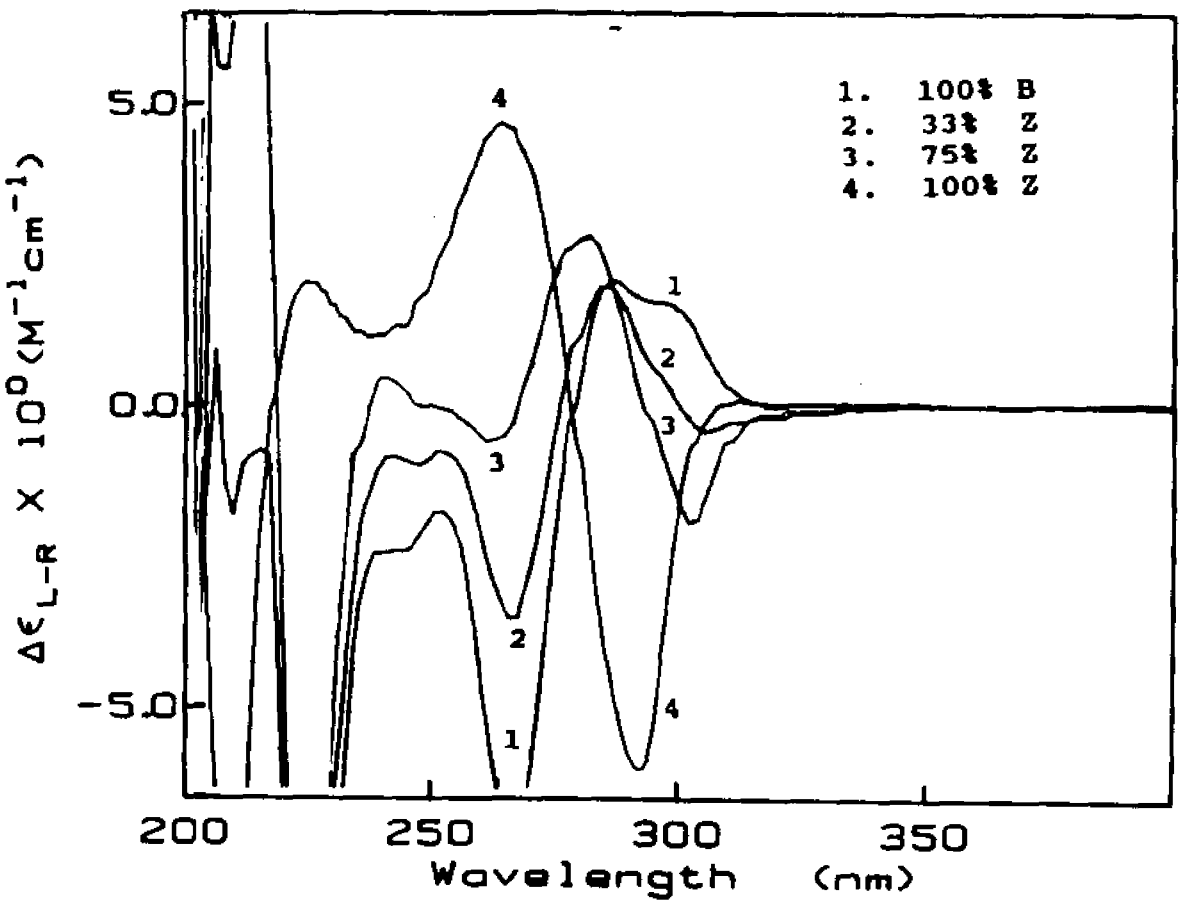


Figure 6: Computer generated mixture of B & Z forms CD of poly(dG-dC).poly(dG-dC). Composite of B- and Z- form CD was made by mixing CD of pure B- and Z- form DNA in different proportions.

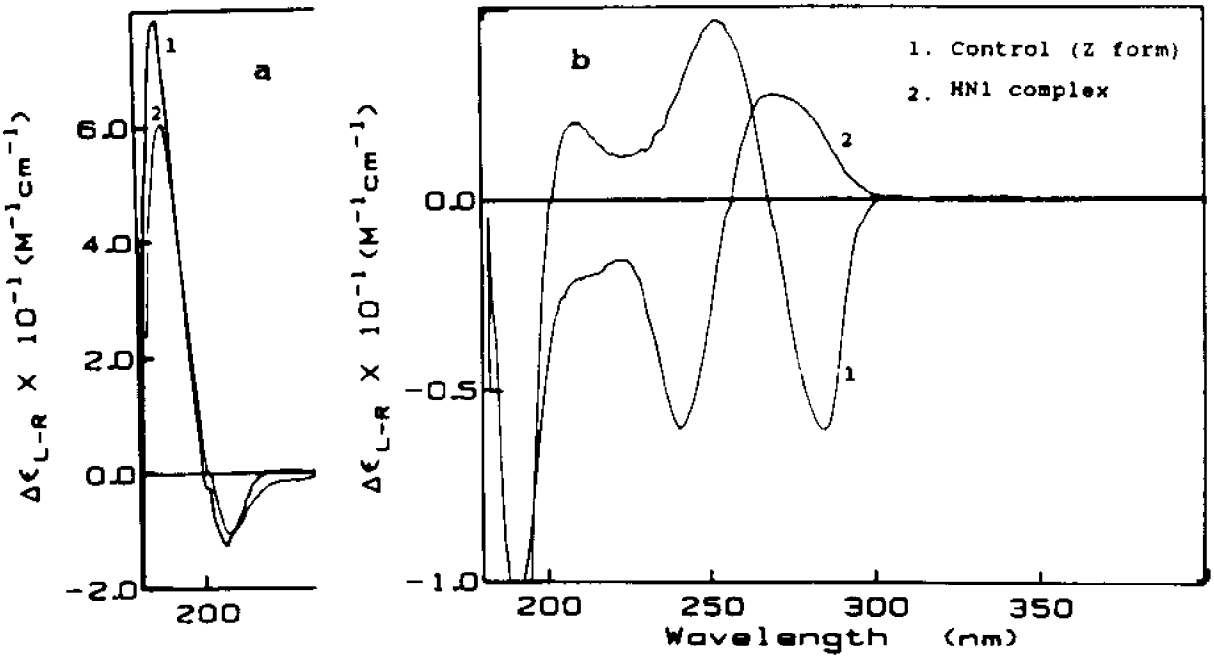


Figure 7: CD of alkaline treated HN1-poly(dG-dC) complex in 10 mM phosphate buffer, pH 7.2. (a). Vacuum CD of the complex showing decrease in the intensity of 187 nm peak (as seen by comparison with control poly(dG-dC). (b). CD under conditions of B->Z transition (60% TFE, 40% 10 mM sodium phosphate buffer, pH 7.2).

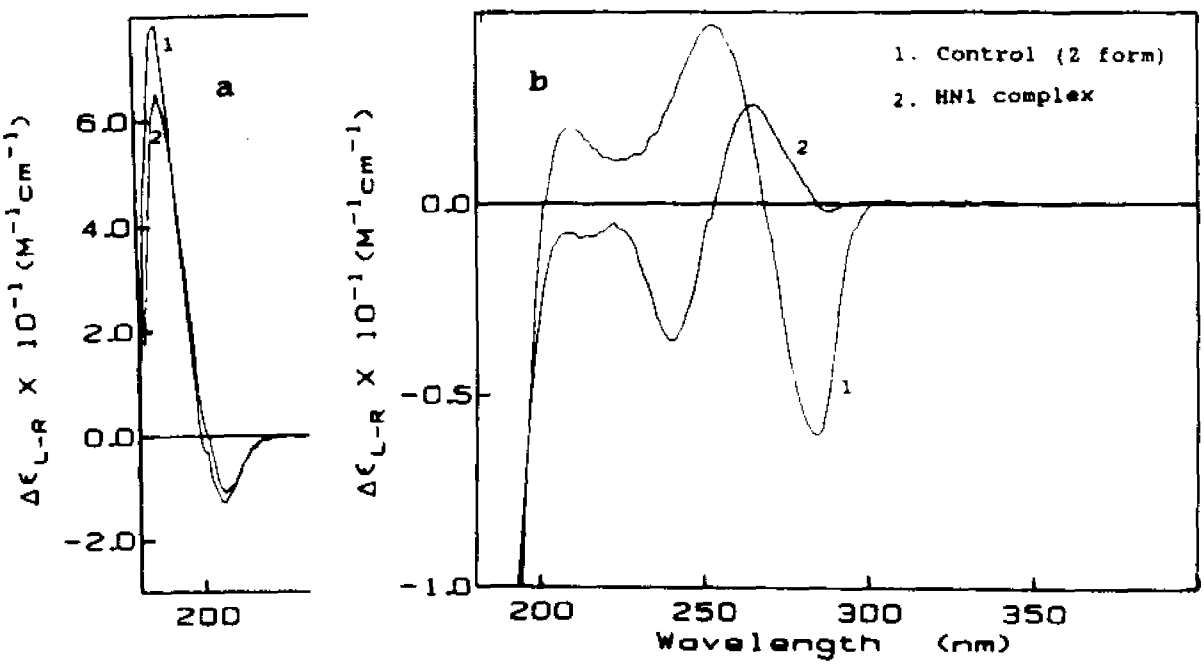


Figure 8: CD of alkaline untreated HN1-poly(dG-dC) complex in 10 mM phosphate buffer, pH 7.2. (a). Vacuum CD of yhe complex showing decrease in the intensity of 187 nm peak (as seen by comparison with control poly(dG-dC). (b). CD under conditions of B->Z transition (60% TFE, 40% 10 mM sodium phosphate buffer, pH 7.2).

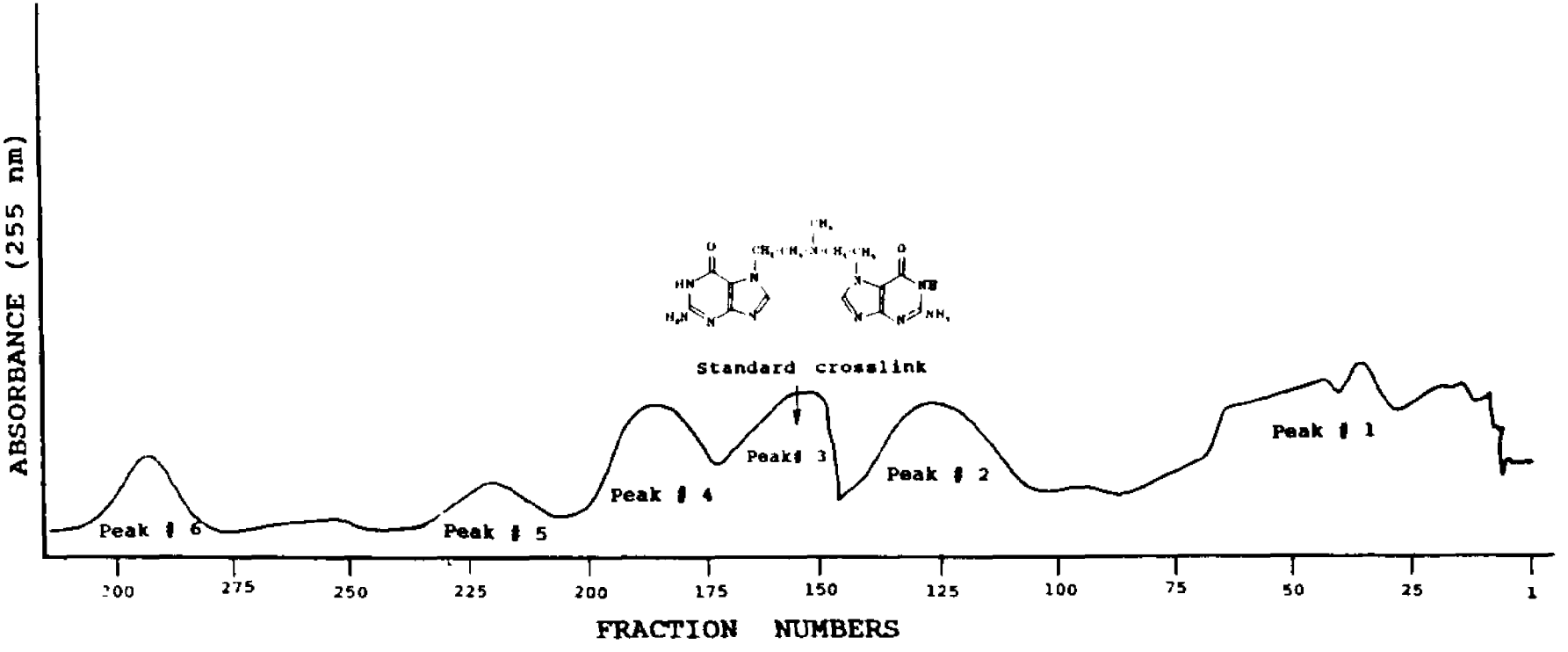
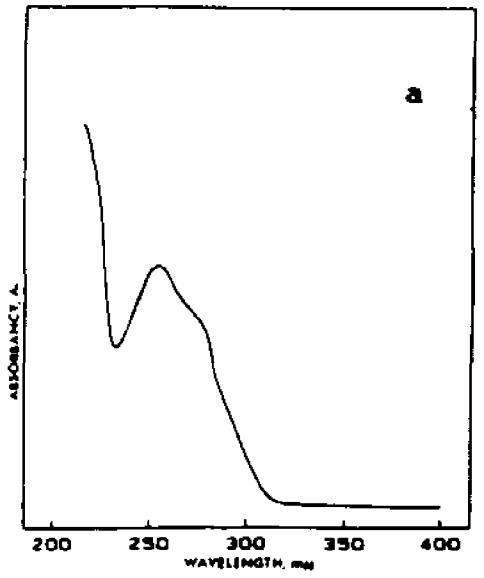


Figure 9: Dowex 50 H^+ chromatography of reaction mixture of HN2 with disodium guanylate. Reaction mixture was loaded to column (23 x 2.5 cm) which was previously equilibriated with 2N HCl. After obtaining uv-absorbing two peaks (140 fractions), HCl conc. was raised to 5N.



Standard crosslink (G-HN2-G)

Figure 10: UV spectrum of standard crosslink at low & neutral pH. (a). Standard crosslink of pH=0 (b). Standard crosslink at pH=7.0

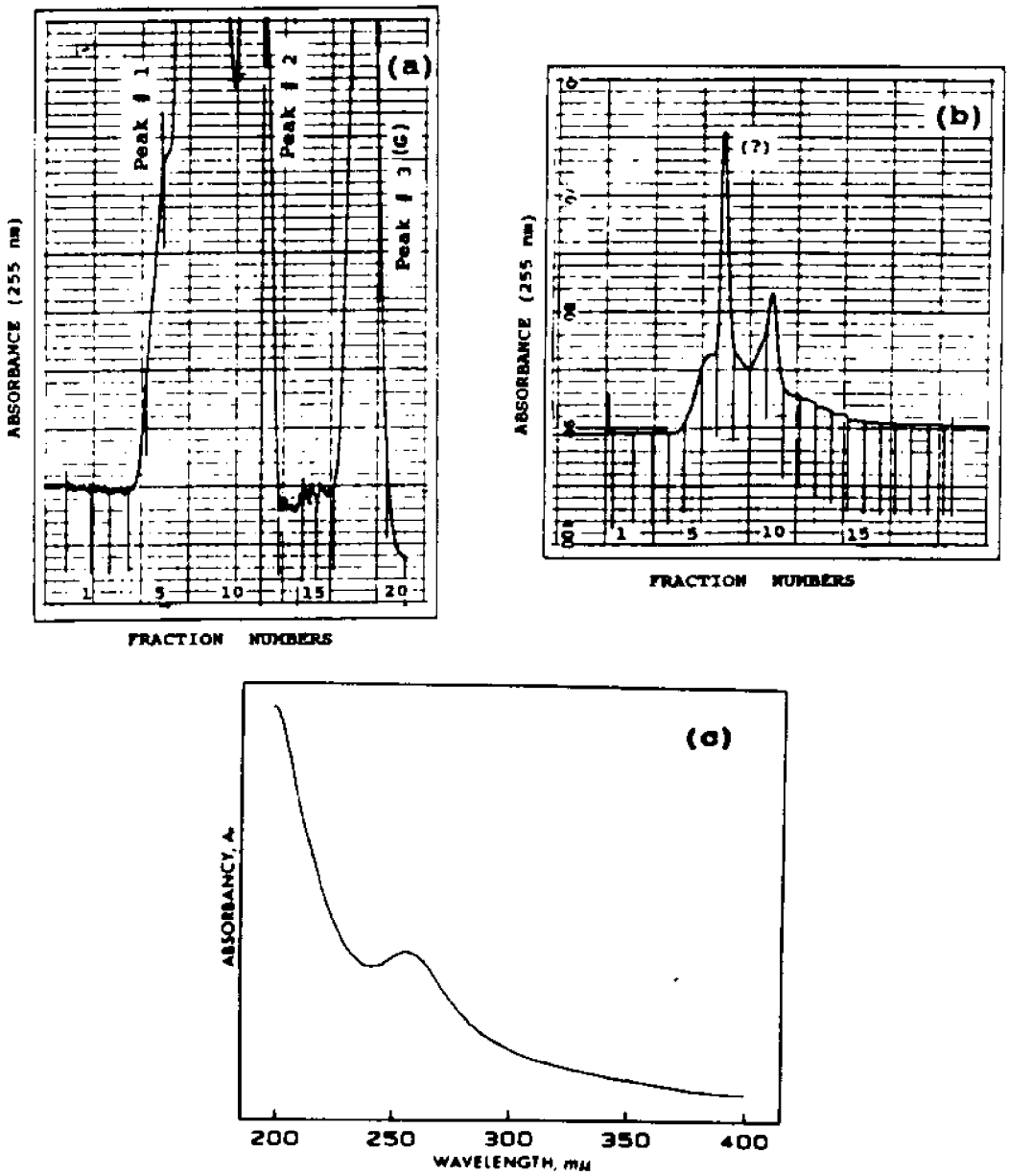
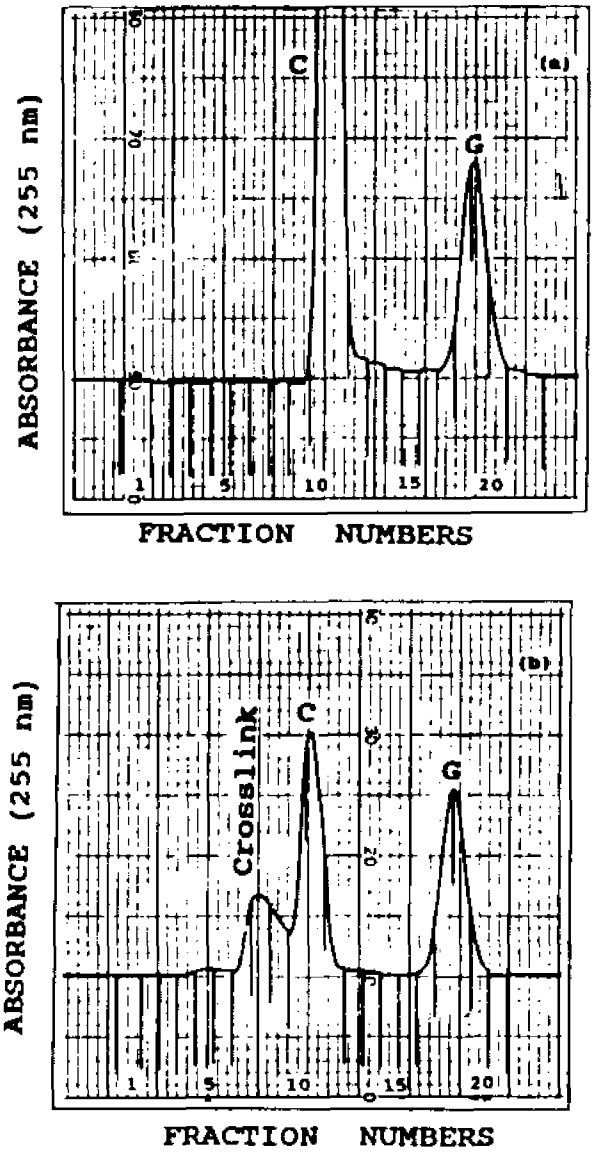


Figure 11: Sephadex G-25 chromatography of perchloric acid digested complex of HN2-poly(dG-dC). (a). Perchloric acid digested samples of HN2-poly(dG-dC) complex loaded to column (26 x 1.5 cm) of Sephadex G-25. (b). Fractions 7-9 (corresponding to elution volume of standard crosslink) were digested further with perchloric acid and rechromatographed to same column. (c). UV spectrum of pooled fractions 7 & 8 of (b).



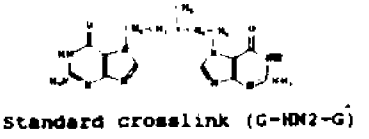


Figure 12: Sephadex G-25 chromatography of standard cytosine, guanine and crosslink. (a). Standard cytosine (C) and guanine (G). (b). Standard crosslink (G-HN2-G), cytosine and guanine.

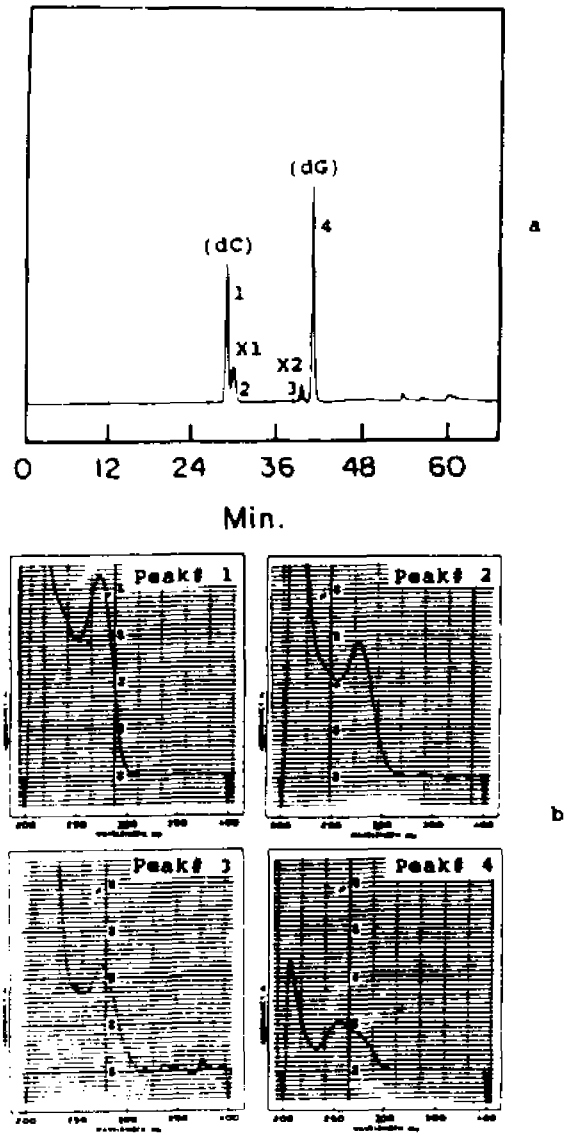


Figure 13: HPLC and UV of mixed enzyme digested sample of HN2-poly(dG-dC). (a). HPLC pattern in 93% 0.03M phosphate-7% acetonitrile buffer, pH 5.0. from DNAse I/ snake venom diesterase/ alkaline phosphatase digest of complex of HN2-poly(dG-dC). (b). UV specta corresponding to eluted peaks (1-4) of HPLC above (a) HPLC.

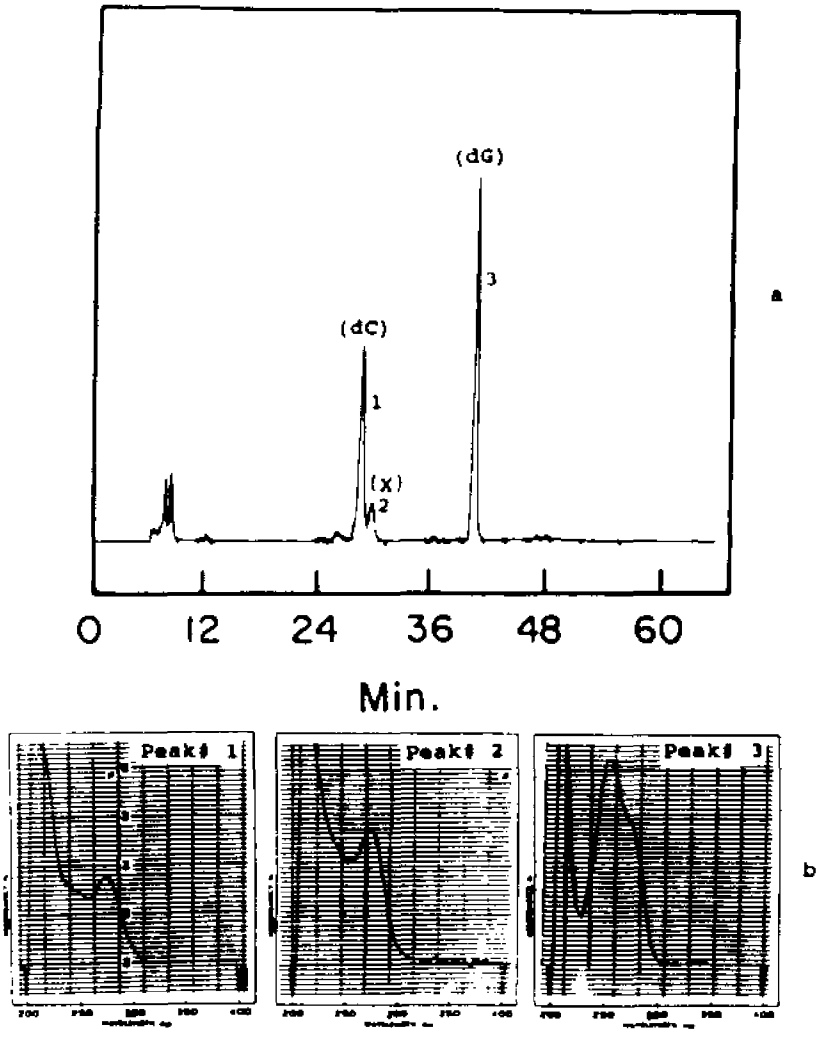


Figure 14: HPLC and UV of mixed enzyme digested sample of HN1-poly(dG-dC). (a). HPLC pattern in 93% 0.03M phosphate-7% acetonitrile buffer, pH 5.0 from DNAse I/ snake diesterase/ alkaline phosphatase digest of HN1-poly(dG-dC) complex. (b). UV spectra corresponding to eluted peaks (1-3) of above (a) HPLC.

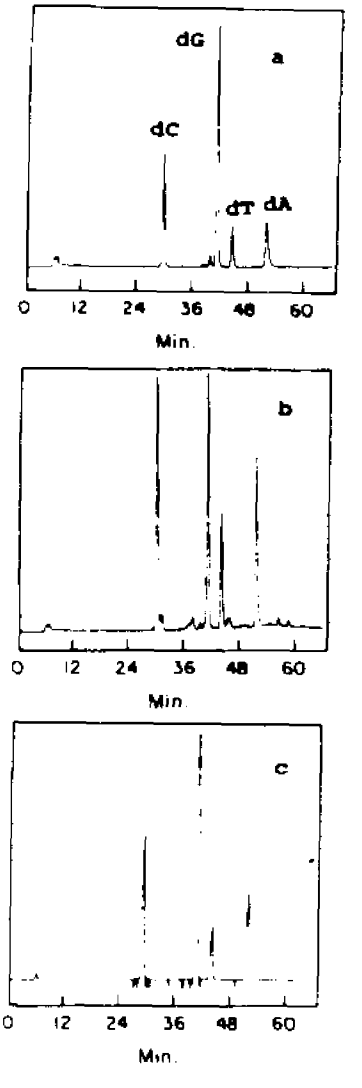


Figure 15: HPLC patterns from DNAse I/snake venom diesterase/alkaline phosphatase digests of HN1 & HN2 M.luteus DNA complexes in 93% 0.03M phosphate, 7% acetonitrile buffer, pH 5.0. (a). Control M.luteus DNA. (b). HN2-M.luteus DNA complex. (c). HN1-M.luteus DNA complex.

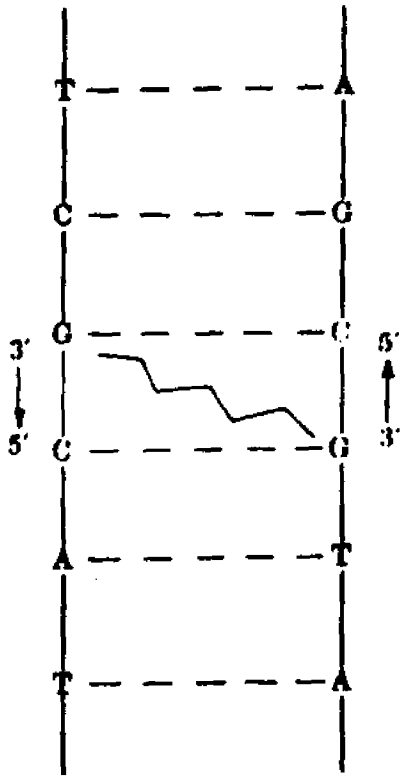


Figure 16: Diagramatic representation of crosslinking of DNA by bifunctional alkylating agent (HN2)

COMPLEXES	Sample #	Conc. of HN2 or HN1 in reaction mixture (mg/OD_{260nm})	% G modified (HPLC)	CD, 187 nm peak (% decrease)	CD under B->Z transition conditions (alkaline untreated samples)	CD under B->Z transition conditions (alkaline treated samples)
HN2 COMPLEXES	1	0.3 mg	29	33	40% B, 60% Z	100% B
	2	0.5 mg	44	48	60% B, 40% Z	100% B
	3	1.0 mg	62	66	100% B	100% B
HN1 COMPLEXES	4	3.0 mg	11	13	70% B, 30% Z	100% B

Table 1: Correlation of the percent modification of guanine, decrease in 187 nm peak of vacuum CD and B->Z transitions of HN1 & HN2-poly(dG-dC) complexes.

that of unmodified Z-DNA (Figure 25a & 25b). The fit of the mitosene unit in Z-DNA was also demonstrated by examining of van der Waals radii as shown in the figure 27. It is evident from the view of van der Waals radii that the indoloquinone ring of monofunctionally bound MC molecule almost projects outside the helix thus causing least distortion to the overall geometry of Z-DNA. In contrast the computer model showed complete disruption of the both backbone geometry & base pairing when MC was

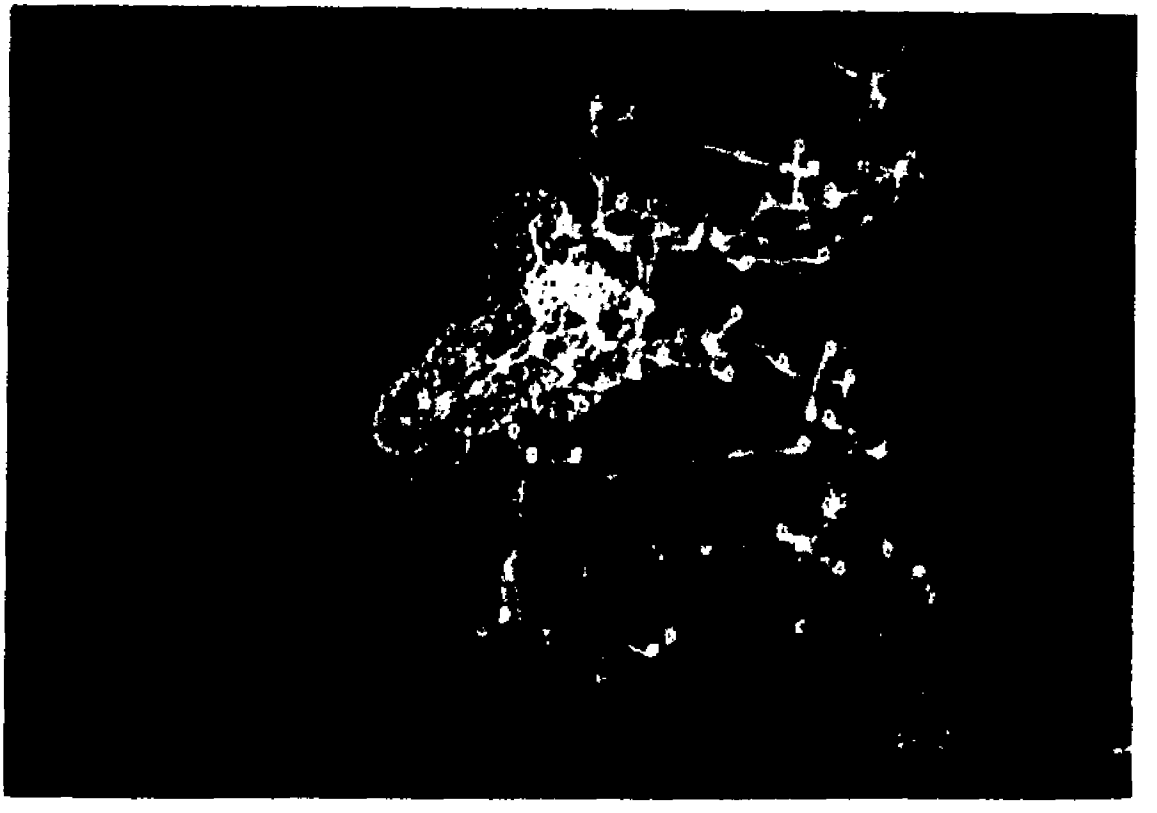


Figure 27: Computer generated molecular model showing van der Waals radii of monofunctionally bound MC-Z-DNA.

crosslinked into the central CpG sequence (Figure 25c). It is evident from the figure 28 that the closest distances for the second linkage of MC to nearest guanines is too large (9.2 A & 6 A) (Figure 28) to make the crosslink. Thus it was concluded that Z-DNA will bind MC monofunctionally but will not be crosslinked. All such conclusions were found to be fully consistent with experimental data.

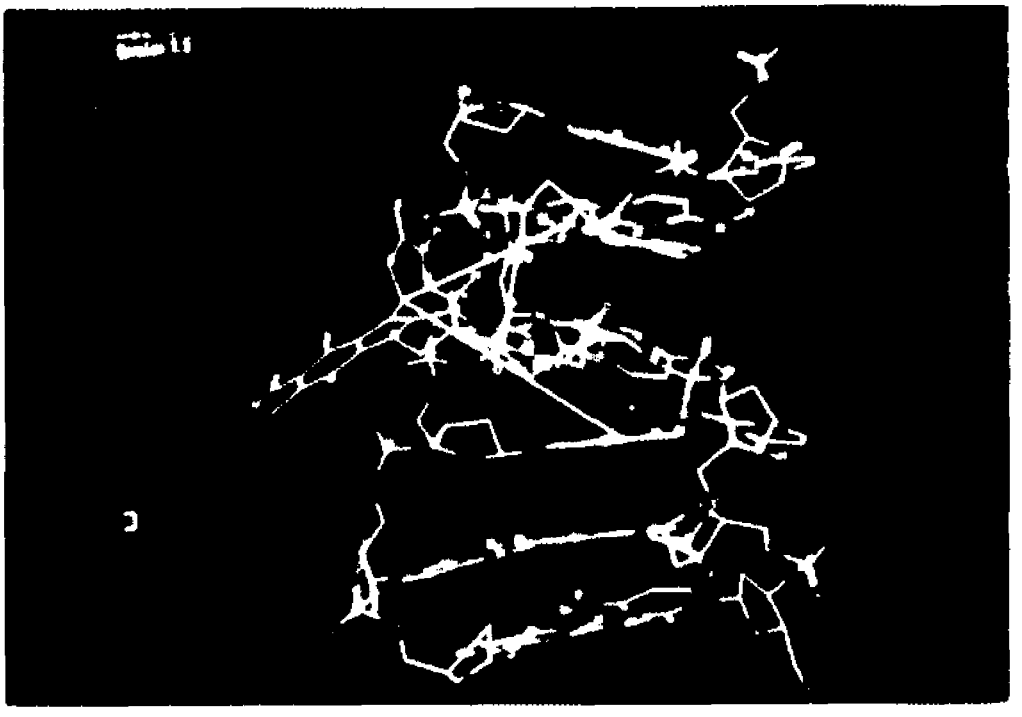


Figure 28: Computer generated molecular model of monofunctionally bound MC-Z-DNA, showing the distances for the second linkage of MC to the nearest guanines of opposite strand.