1985

Molecular crosslinking of DNA by dichloro-n-methyl-azaadamantane isomers

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MOLECULAR CROSSLINKING OF DNA BY DICHLORO-N-METHYL-AZAADAMANTANE ISOMERS

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Thesis Submitted

In Partial Fulfillment of the

Requirements for the Degree of

Master of Science

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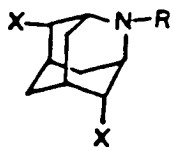
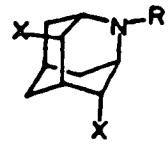
ABSTRACT

A series of disubstituted 2-azaadamantanes representing conformationally defined nitrogen mustards incorporated into an adamantyl framework with fixed separation distances between alkylating centers of 3.5 Å (*1*: 4,8-dichloro-2-azaadamantane) and 2.5 Å (*2*: 4,9-dichloro-2-azaadamantane; *3*: 4,10-dichloro-2-azaadamantane) have been synthesized (Henkel et al., (1981) J. Org. Chem., 46, 3483 and unpublished results) as rigid probes of DNA alkylation and crosslinking processes leading to cytotoxicity.



1

4,8 dichloro-2-aza adamantane



2

4,9 dichloro-2-aza adamantane

3

4,10 dichloro-2-aza adamantane

The extent of intermolecular DNA crosslinking by the N-methyl isomer series has been measured by an ethidium bromide fluorescence assay. Results indicate that isomers *1* and *2* form intermolecular DNA crosslinks, while *3* does not. Analysis of the kinetics of the reaction isotherms of *1* and *2* indicate significant differences in their rates and stability of crosslink formation. Crosslink reactions using DNAs of different base composition have established that the extent of crosslinking increases with increasing (G + C) content; however, the (G + C) dependence on crosslinking varies substantially between *1* and *2*, and between the isomers and nitrogen mustard itself. A relationship also appears to exist between the cytotoxic activity and stereochemistry of the isomers, which may indeed reflect differences in the crosslinking behavior at the molecular level, and which may lead to further elucidation of the molecular mechanism of action of both the chloroethylnitrosoureas and the nitrogen mustards.

INTRODUCTION

Relatively few agents have been discovered which have been useful in Central Nervous System (CNS) tumor chemotherapy (Henkel, 1983). Today, the most important class is the nitrosoureas, the principle members of which are 1-chloroethyl-3-cyclohexyl-1-nitrosourea (CCNU) and 1,3-Bis-chloroethyl-1-nitrosourea (BCNU) (Edwards et al., 1980; Walker, 1973). These compounds exhibit their antitumor activity in a complex manner, which involves both alkylation and carbamoylation activities (Wheeler, 1975). However, it is now well established that the major antitumor activity displayed by these compounds can be attributed to the alkylation of nuclear macromolecules (Colvin et al., 1976), followed by the formation of interstrand crosslinks in the DNA (Kohn, 1977; Erikson et al., 1980). The interstrand crosslinking of DNA is a lethal event to the cell compared with monoalkylation, since the latter is repaired more easily in vivo prior to replication (Joshua et al., 1980).

Previous studies have established correlations between antileukemic activity of mitomycin C and some aziridinoquinones and their efficiency of DNA crosslinking (Akhtar et al., 1975; Lown, 1979). These correlations strongly suggest that measurements of DNA crosslinking may be useful in the pre-screening of other potential antitumor agents, particularly those of the nitrogen mustard class. There is a substantial body of knowledge concerning the mechanisms of action of the N-mustards, and it is now well accepted that DNA crosslinking is the primary cytotoxic event (Connors, 1974). The

crosslinking mechanism for mechlorethamine, HN_2, a N-mustard prototype is illustrated in Figure 1.

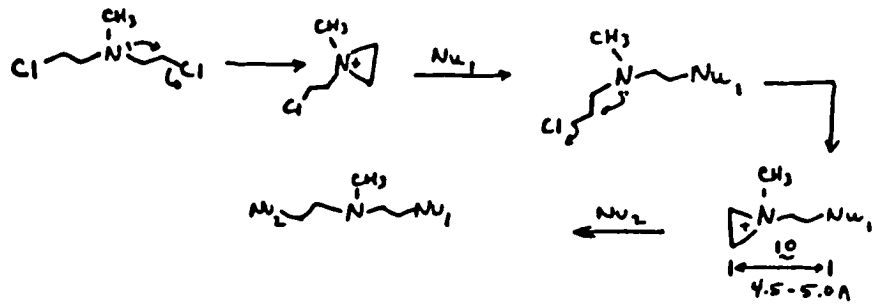


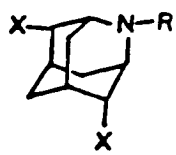
Figure 1. Crosslinking Mechanism for Mechlorethamine

This mechanism proceeds via a two-step process, requiring an initial activation of the substrate through an anchimeric effect of the nitrogen to yield an aziridium ion intermediate, *a*, alkylation of *a* by cellular nucleophiles form the covalent link, *b*, after which a second activation and alkylation step establishes the five atom crosslink, *c*, to a neighboring nucleophilic center. If alkylation occurs at nucleic acid base pairs located on opposite DNA strands, then an interstrand covalent crosslink is produced. The potential distance over which this crosslink occurs will be a function of (1) the molecular distance between the second alkylating center and the first alkylated nucleophile (approximately 4-5 Å) and (2) the degrees of conformational freedom available at the aziridium end of the second intermediate over a range of directions.

Presumably, a portion of the observed antitumor non-selectively and relatively high toxicity of the N-mustards (Thomas et al., 1978) is due to the mobility of the aziridium end of the second intermedi-

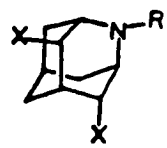
ate. Such conformational mobility could account for both the cytotoxic non-selective alkylation of other macromolecules and the more rapid DNA crosslinking by these agents, which appears to be enzymatically repaired in vivo at later times during the cell cycle. Consequently, there appears to be a requirement for both stereospecific DNA crosslinking and a delayed second alkylation step in order to minimize cytotoxicity while enhancing crosslinking antitumor activity. In addition, the alkylating agent must possess a high degree of lipophilicity for transport across the cell walls (Levin et al., 1974; Henkel, 1983).

A series of potential CNS antitumor agents have been recently synthesized which may exhibit DNA crosslinking activity as part of their antitumor activity (Henkel, 1983). In this series of compounds, a nitrogen mustard moiety has been incorporated into the hydrophobic framework of various azaadamantane isomers, *1*, *2*, and *3*. (See Figure 2.)



1

4,8 dichloro-2-aza adamantane



2

4,9 dichloro-2-aza adamantane

3

4,10 dichloro-2-aza adamantane

where, X = Cl; R = CH_3

Figure 2. Dichloro-N-Methyl-Azaadamantane Isomers

Although biological assays are currently being performed on these isomers to determine their CNS antitumor effectiveness and membrane permeability, in vivo, no experimental data has been reported on the mechanisms of their action with DNA with regard to molecular crosslinking. It is the purpose of this study to quantitate the extent of molecular crosslinking of these isomers with duplex DNA. Molecular crosslinking was measured using a sensitive fluorometric assay involving Ethidium Bromide with alkaline DNA denaturation. We have also monitored, under a variety of reaction conditions, the molecular crosslinking kinetics for these isomers in addition to their potential DNA base content specificity. From the results of this study, a correlation is made of the cytotoxic activity of these compounds with their DNA crosslinking properties.

EXPERIMENTAL

The synthesis and toxicological properties of compounds *1*, *2*, and *3* have been previously described (Henkel, 1983). These compounds were kindly provided by Dr. James Henkel, Department of Medicinal Chemistry, University of Connecticut. The molecular weights of the isomers (compounds *1*, *2*, and *3*) and HN_2 are 256.5 and 192.5 grams per mole, respectively. Ethidium Bromide was purchased from Sigma and has a molecular weight of 394.3 grams per mole, and an extinction coefficient of 5450 l $moles^{-1}$ cm^{-1} at 480 nm (Sigma). The DNA's used in these experiments were Calf-Thymus (Type I, polymeric), E. Coli (M.W. = 1.64 x 10^7, (G + C) = 50.0%, ε(258) = 6500 l/moles-cm), Micrococcus Luteus (M.W. = 1.9 x 10^7, (G + C) = 72.0%, ε(258) = 7040

l/moles-cm), and Calf-Thymus (M.W. = 9.0 x 10^6, (G + C) = 40.0%, ε(258) = 6500 l/moles-cm) (Sigma). All DNAs were purchased from Sigma. All DNAs were prepared using a 20 m$\underline{M}$ potassium phosphate solution at pH = 7, containing 0.15 $\underline{M}$ NaCl.

The four most commonly employed methods for determining the frequencies of crosslinks in DNA is as follows: (1) visualization of the DNA using electron microscopy under totally denaturing conditions (Hansen et al., 1976; Cech et al., 1977); (2) sedimentation of DNA in alkaline sucrose gradients (Cole and Zusman, 1970); (3) alkaline gel electrophoresis (Cech, 1981); (4) alkaline denaturation/renaturation (Geiduschek, 1961; Cole, 1970; Dall'Acqua et al., 1970). Because one crosslink is sufficient to hold complementary DNA strands in register during the denaturation process and serve as a nucleation site for complementary DNA strands to renature spontaneously after a cooling period, the latter method cannot be used to differentiate between singly crosslinked and multiply crosslinked DNA molecules (Cech, 1981). A modification of the alkaline denaturation/renaturation procedure based on the fluorescent properties of the Ethidium dye (Lown et al., 1976) was used to measure the interstrand crosslinking which occurs between the azaadamantane isomers and duplex DNA.

Ethidium Bromide binds by intercalation into duplex DNA, which results in an enhancement of fluorescence (LePecq and Paoletti, 1967). Upon heat denaturation of duplex DNA in the presence of Ethidium Bromide, the fluorescence enhancement will fall to zero providing that one uses high pH in order to prevent the formation of regions of self-complementarity (Morgan and Paektau, 1972). If one

crosslink is introduced by the action of alkylating agents, such as HN_2, a return of Ethidium fluorescence is observed even under alkaline conditions after denaturation since this crosslink serves as a nucleation site for the spontaneous renaturation of the DNA after a heating and cooling cycle (Lown et al., 1976).

The protocol for the Ethidium fluorescence assays is shown in Figure 3 (Morgan and Pulleybank, 1974; Akhtar et al., 1975; Morgan and Paetkau, 1972).

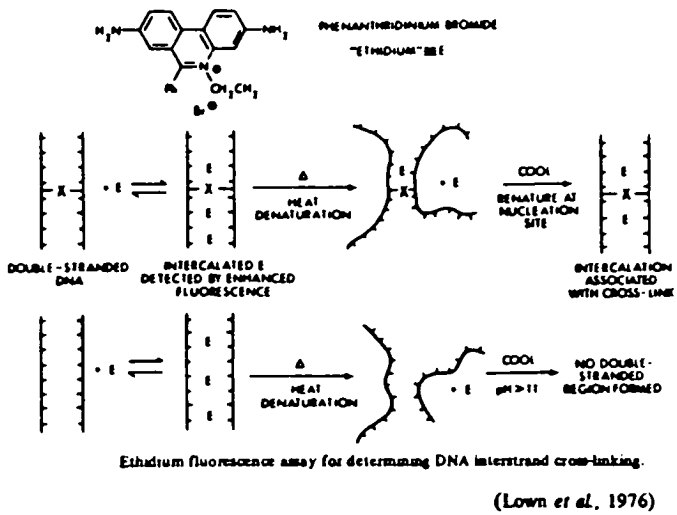


Figure 3. Protocol for Ethidium fluorescence assay

Assay Method

Calf-Thymus DNA (Type I, polymeric) was used in all direct pellet reactions. A 20 mM potassium phosphate solution at pH = 7, containing 0.15 M NaCl and 2×10^{-4} M DNA-phosphate was used in these experiments. All reactions were performed at ambient temperature. Reactions were initiated at $t = t_o$, by addition of a previously

weighed sample of the crosslinking agent contained in a micro-capsule dropped into the reaction vessel containing the DNA solution. Approximately one to one and half milligrams of the crosslinking agent was used for each reaction. All drugs were weighed on a Mettler Analytical Microbalance using a microcapsule. Forty microliter aliquots were removed from the reaction solution at different time intervals and added to one milliliter of Standard Ethidium Bromide assay solution. This solution contains 20 mM potassium phosphate, pH = 11.4, 0.2mM EDTA, and 0.5 µg/ml of Ethidium Bromide (Lown et al., 1978). All samples were mixed on a Vortex mixer and then centrifuged for one minute on a Fisher MicroCentrifuge. The fluorescence of all samples was measured on a Perkin-Elmer Fluorescence Spectrophotometer MPF 3L with excitation and emission wavelengths set at 520 and 590 nanometers respectively. All samples were subsequently heat denatured for three minutes at 97°C in a Fisher High Temp Bath Model 160 and cooled to 23°C for five minutes (Lown et al., 1978). All samples were then allowed to reach ambient temperature (approximately fifteen minutes). The samples were then re-centrifuged for one minute and the fluorescence re-measured under the conditions described earlier.

The pre-dilution method is a homogeneous reaction. Previously weighed crosslinking agents were pre-diluted in 20 mM phosphate buffer at pH = 7, containing 0.15 M NaCl solution for three minutes prior to initiating the crosslinking reaction. Reactions were initiated at $t = t_o$ by mixing equal volumes (200 µl) of the crosslinking agent and DNA stock solutions at twice the reaction concen-

trations. Reactions were performed using a 20 mM phosphate solution at pH = 7, containing 0.15 M NaCl and 1.5 x 10^{-4} M DNA-phosphate. Forty microliter aliquots were removed from the reaction mixture at specific time intervals and added to one milliliter of Standard Ethidium Bromide assay solution. This solution contains 20 mM potassium phosphate, pH = 11.4, 0.2 mM EDTA, and 0.5 µg/ml of Ethidium Bromide (Lown, et al., 1978). All samples were treated identically to those samples obtained in the direct pellet method and all data obtained from these experiments were treated in the same manner as in the direct pellet method, however, the percent cross-linked was converted to the number of links per nucleotide for those DNAs of known molecular weight.

Under the conditions described in the preceding paragraphs, only the covalently crosslinked complementary DNA shows a return of Ethidium fluorescence, since the covalent crosslink provides a nucleation site for the spontaneous renaturation of the DNA (Lown et al., 1976). Thus, the ratio of Ethidium fluorescence after heating to the Ethidium fluorescence before heating, after correction for the assay control blank, is proportional to the extent of the covalent crosslinking of the DNA (Lown et al., 1978).

Fluorescence Measurements

The fluorescence of all samples was measured on a Perkin-Elmer Fluorescence Spectrophotometer MPF 3L using 0.2 cm fluorescence cells. The one centimeter pathlength was placed in the excitation beam. Excitation and emission wavelengths were set at 520 and 590

nm, respectively. The sensitivity was usually set at 10 and the scan rate at 3 nm/second. The instrument was in the ratio mode during all fluorescence measurements. All fluorescence measurements were obtained at ambient temperature.

With the excitation wavelength set at 520 nm, the emission spectra was scanned from 540 to 640 nm. In addition, fluorescence measurements were obtained at the maximum emission wavelength, that is, at 590 nm and blanked to the standard Ethidium Bromide assay solution alone. A standard solution of Ethidium Bromide served as an internal instrument reference.

Analysis of Fluorescence Data

As previously described, the ratio of Ethidium fluorescence after heat denaturation to Ethidium fluorescence before heat denaturation (when corrected for the control blank) is proportional to the extent of crosslinked DNA. For polymeric, Type I Calf-Thymus DNA, of unknown molecular weight, this ratio is reported as the fluorescence percent crosslinked DNA. However, this ratio does not equal the actual number of crosslinks per DNA molecule since only one crosslink per DNA molecule is sufficient to produce rapid renaturation after cooling and more than one crosslink per DNA may be present and not detected by this technique. Furthermore, the DNA segments may have an unequal distribution of molecular weights rendering a calculation of the number of DNA molecules impossible.

For DNA's of specific (G + C) content and known molecular weight, crosslinking data is reported as the average number of

crosslinks per nucleotide, as calculated from the corrected number of crosslinks per DNA molecule. Allowance was made for difference in the average molecular weight of the different DNAs, DNAs of lower molecular weight require more crosslinking events per nucleotide residue to produce the same percentage of crosslinked DNA. Assuming a Poisson distribution of links, and further that one crosslink is sufficient to permit spontaneous renaturation of the molecule, the corrected average number of crosslinks per DNA molecule, M (M = ln $(1/P_o)$ where P_o is the proportion of unlinked molecules) was computed (Hatchard, 1956), and correlated to the actual number of crosslinks per nucleotide residue using the duplex concentration and the average molecular weight.

RESULTS AND DISCUSSION

Heterogeneous (Direct Pellet) Reactions with Polymeric, Type I, Calf-Thymus DNA

The intermolecular crosslinking studies of *1*, *2*, and *3* with high molecular weight calf-thymus DNA have clearly established that both *1* and *2* form interstrand DNA crosslinks (in vitro) under physiological conditions of temperature, pH and salt concentration, whereas no interstrand crosslinking activity can be detected for *3* under similar reaction conditions. The reaction profiles shown in Figures 4 and 5, were from heterogeneous reactions in which the crystalline form of the crosslinking agent was added directly to the DNA in aqueous buffered solution.

As shown in Figures 4 and 5, the extent of interstrand crosslinking associated with isomers *1* and *2* is both time-dependent and

Figure 4. Time Dependent Crosslinking of Mechlorethamine, 4,9-dichloro-2-methyl-2-azaadamantane and 4,8-dichloro-2-methyl-2-azaadamantane with Calf-Thymus DNA (Polymeric Type I); Direct Pellet Method. D/P = 10.

Reactions were performed at 37°C in a total volume of 10 ml buffered with 20 mM potassium phosphate at pH = 7, 0.15 M NaCl, containing 2×10^{-4} DNA-phosphate. Reactions were initiated at $t = t_o$, by the direct addition of a previously weighed sample of drug contained in a microcapsule dropped into the reaction vessel containing the DNA solution. Aliquots of 40 µl were withdrawn from the reaction solution at intervals, and analyzed for the extent of crosslinking by the addition of one milliliter of standard pH = 11.4 ethidium bromide assay solution. Samples were subsequently heat denatured for 3 minutes at 97°C and cooled to 23°C. Under these conditions, only covalently linked complementary DNA shows a return of fluorescence (Ethidium Fluorescence) since the covalent links provide a nucleation site for renaturation. The ratio of ethidium fluorescence after heating to the fluorescence before heating is proportional to the extent of covalent crosslinking of the DNA.

Figure 4

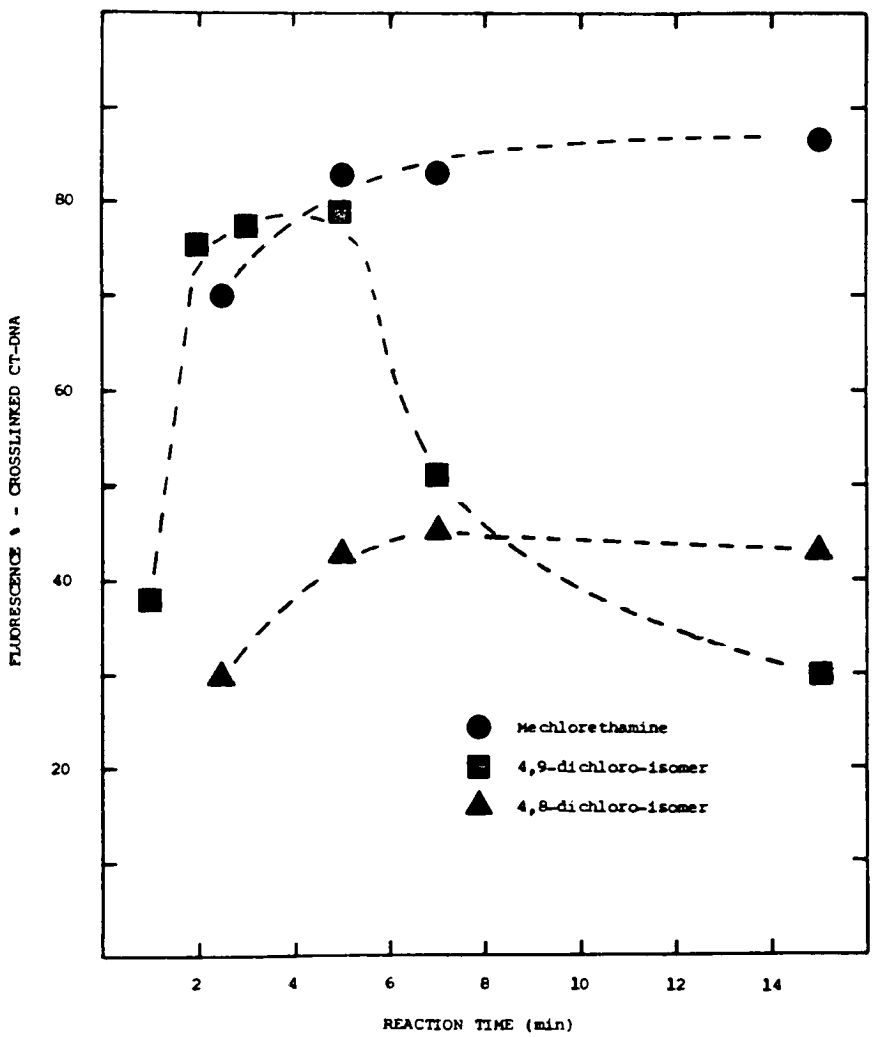
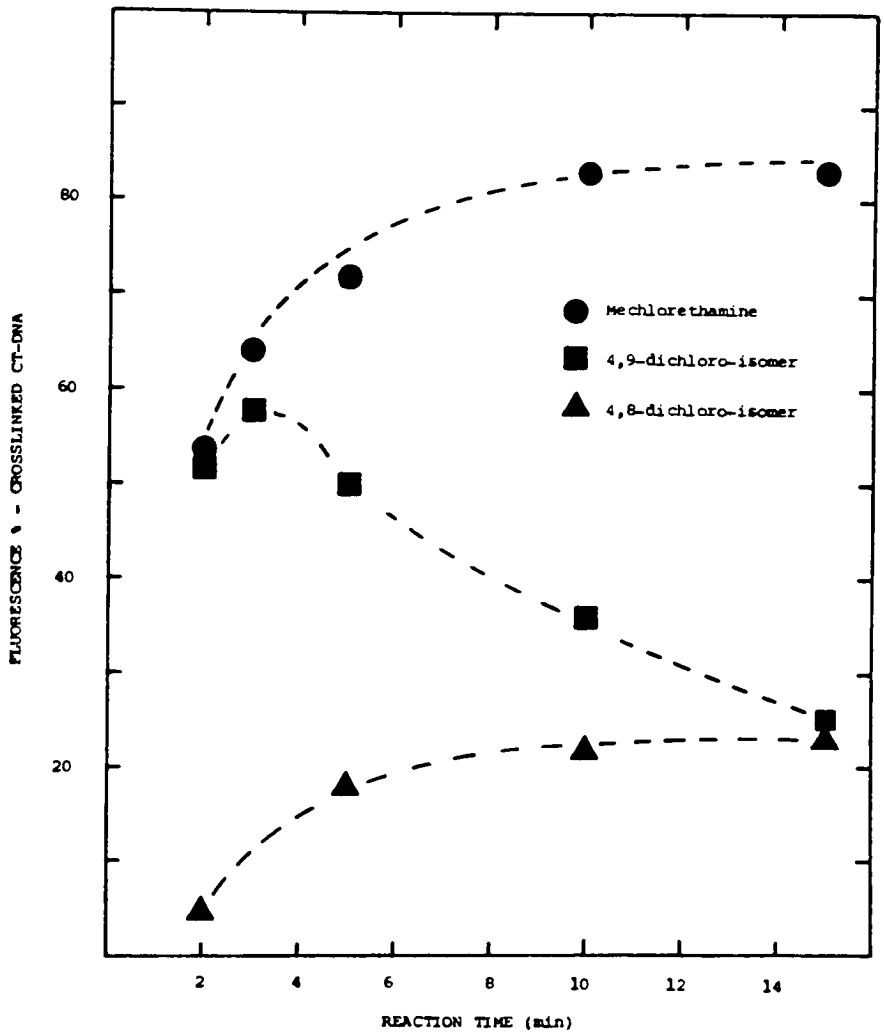

FLUORESCENCE % - CROSSLINKED CT-DNA
80
60
40
20
2
4
6
8
10
12
14
REACTION TIME (min)
Mechlorethamine
4,9-dichloro-isomer
4,8-dichloro-isomer

Figure 5. Time Dependent Crosslinking of Mechlorethamine, 4,9-dichloro-2-methyl-2-azaadamantane and 4,8-dichloro-2-methyl-2-azaadamantane with Calf-Thymus DNA (Polymeric Type I); Direct Pellet Method. D/P = 5.

As per Figure 4, at drug/nucleotide-P ratio of 5:1.

Figure 5


FLUORESCENCE % - CROSSLINKED CT-DNA
80
60
40
20
2
4
6
8
10
12
14
REACTION TIME (min)
Mechlorethamine
4,9-dichloro-isomer
4,8-dichloro-isomer

sensitive to the drug to nucleotide reaction stoichiometry. The kinetics of the reaction profile of the 4,8-dichloro-N-methyl isomer (*1*) are similar to the kinetics associated with the mechlorethamine (HN_2) control, forming crosslinks that are complete after approximately ten minutes. However, the maximal extent of crosslinking for *1* at saturation appears to be approximately 40% of that for HN_2. In contrast, both the reaction kinetics and the extent of crosslinking of *2* differ significantly from *1*. Specifically, the initial kinetics of the crosslinking reaction for *2* are considerably more rapid than the kinetics associated with *1*. Also, at sufficiently high D/P ratios the maximal extent of crosslinking for *2* parallels that of HN_2 (Figure 4). In addition, an unusual time-dependent <u>decrease</u> in crosslinking is observed for *2* at reaction intervals greater than three minutes. Both the extent of initial crosslinking and the associated time-dependent decrease in crosslinking of *2* appear to be sensitive to the reaction stoichiometry (Figures 4 and 5).

The extent of intermolecular crosslinking for HN_2 does not appear to be directly proportional to reaction stoichiometry under the conditions of Figures 4 and 5. However, at drug to phosphate ratios of 2:1 or lower, the extent of intermolecular crosslinking does become proportional to the reaction stoichiometry (data not shown). This is due to the elimination of the saturation effects which are evident at higher D/P ratios. Saturation for HN_2 appears to be approximately fifteen minutes (data not shown) at D/P less than or equal to 2. The extent of intermolecular crosslinking for the 4,8 isomer appears to be directly proportional to the reaction stoichio-

metry at D/P ratios of 10:1 or lower. Saturation for the 4,8 isomer appears to be approximately six minutes. The maximal extent of intermolecular crosslinking of the 4,9 isomer is also found to decrease with decreasing D/P ratios, however, not proportionally.

Homogeneous (Pre-Dilution) Reactions with Polymeric Type I, Calf-Thymus DNA

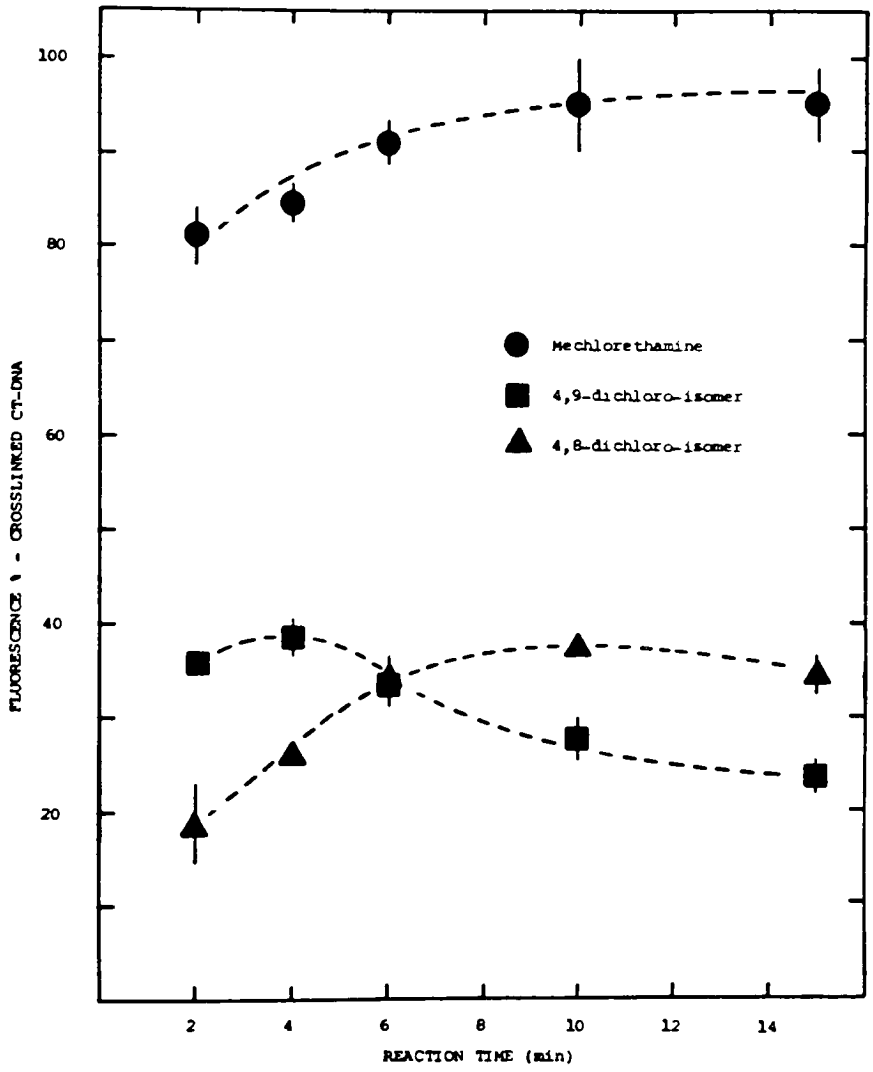
Due to the observed time-dependent solubilization of HN_2 and the various isomers in aqueous solution, and to facilitate more quantitative measurements of DNA crosslinking studies with highly purified DNAs of different (G + C) content, considerable time was devoted to the development of an assay protocol to measure crosslinking under homogeneous reaction conditions. A method was developed to initiate the crosslinking reaction under conditions in which both DNA and the crosslinking agent were in aqueous solution. Reactions would then be homogeneous, eliminating the kinetics associated with the solubilization of the different crosslinking compounds. For all reactions subsequent to Figures 4 and 5, the crosslinking agent was predissolved and pre-equilibrated in reaction buffer for exactly three minutes before initiating the crosslink reaction by mixing equal volumes of aqueous DNA with aqueous crosslink agent.

As illustrated by comparison of Figures 4 and 6, the reaction kinetics and the extent of crosslinking of HN_2 and *1* do not appear to be significantly changed by introducing these compounds as aqueous components into the DNA reaction solution. These results clearly indicate that the solubilization rate of HN_2 and *1* are considerably shorter than that of the crosslinking kinetics. Compound *2* does,

Figure 6. Time Dependent Crosslinking of Mechlorethamine, 4,9-dichloro-2-methyl-2-azaadamantane and 4,8-dichloro-2-methyl-2-azaadamantane with Calf-Thymus DNA (Polymeric Type I): Pre-Dilution Method. D/P = 10, [Nucleotide-P] = 0.15 mM, time dissolved = three minutes.

Reactions were performed at 37°C, pH = 7 (20 mM phosphate buffer), 0.15 M NaCl containing 1.5×10^{-4} M DNA-phosphate. Reactions were initiated at $t = t_o$ by mixing equal volumes of drug and DNA stock solutions at twice the reaction concentrations. Drugs were predissolved in buffer solution prior to initiating the crosslinking reaction for three minutes.

Figure 6


100
80
60
40
20
FLUORESCENCE % - CROSSLINKED CT-DNA
Mechlorethamine
4,9-dichloro-isomer
4,8-dichloro-isomer
2
4
6
8
10
12
14
REACTION TIME (min)

however, show a reduction in the extent of crosslinking when introduced in aqueous form (at identical D/P ratios), but the reaction kinetics appear to be similar to those observed for *2* in the heterogeneous reaction. These results would suggest that the rate of solubilization and/or the life-time associated with the reactive intermediate form of *2* does influence the overall extent of observed crosslinking for this compound. As shown in Figures 6 and 7, when *1* and *2* are introduced to the reaction in aqueous form after dissolving for a three-minute period, the maximal extent of crosslinking is similar (approximately 40% that of HN_2) for both isomers, while the reaction kinetics of *2* remains enhanced 3-4 fold as compared to the crosslinking kinetics of *1* or HN_2.

Comparison of the HN_2 profiles in Figures 6 and 7 illustrates that at sufficiently low D/P ratios, the maximal extent of crosslinking occurs at longer reaction times and becomes proportional to the HN_2/DNA reaction stoichiometry at D/P ratios below 2:1 (data not shown). Subsequent crosslinking experiments were therefore performed at HN_2/DNA ratios of 1:1. Comparison of the 4,8 and 4,9 isomers' reaction profiles in Figures 6 and 7, indicate that the extent of crosslinking for these isomers also decrease with decreasing ratios of drug to DNA and become proportional to the reaction stoichiometry at D/P ratios of 4:1 or lower (see Tables 1 and 2). DNA content dependence experiments were therefore performed at drug to DNA ratios of 4:1.

Figure 7. Time Dependent Crosslinking of Mechlorethamine, 4,9-dichloro-2-methyl-2-azaadamantane and 4,8-dichloro-2-methyl-2-azaadamantane with Calf-Thymus DNA (Polymeric Type I): Pre-Dilution Method. D/P = 2, [Nucleotide-P] = 0.15 mM, time dissolved = three minutes.

As per Figure 6, at drug/nucleotide ratio of 2:1.

Figure 7

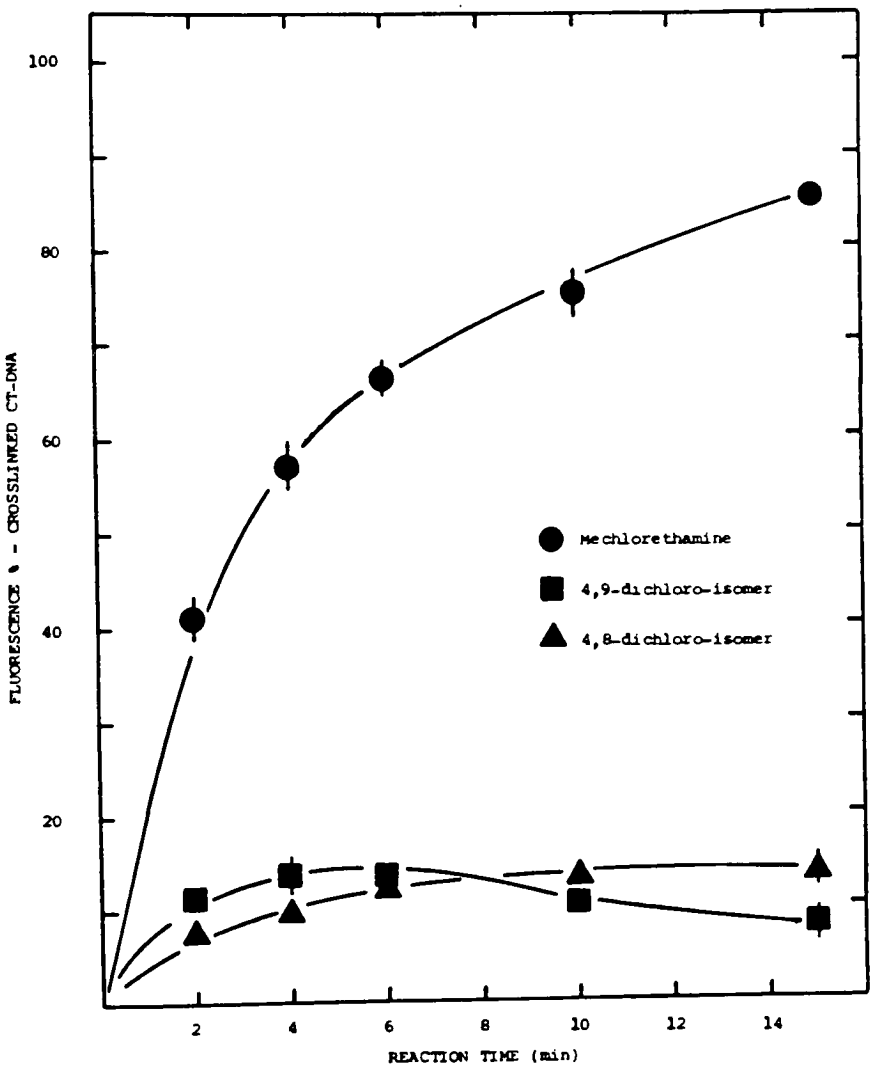

100
80
60
40
20
FLUORESCENCE % - CROSSLINKED CT-DNA
2
4
6
8
10
12
14
REACTION TIME (min)
Mechlorethamine
4,9-dichloro-isomer
4,8-dichloro-isomer

Table 1

Crosslinking Data for 4,8-Dichloro-2-Methyl-2-Azaadamantane and Calf-Thymus DNA (Type 1, Polymeric)

Time (minutes)	Fraction Crosslinked	Fraction Un-Crosslinked	Corrected Number of Crosslinks/DNA
2.0	0.076	0.924	0.079
4.0	0.098	0.902	0.103
6.0	0.123	0.874	0.132
10.0	0.130	0.870	0.139
15.0	0.137	0.863	0.147

Homogeneous reactions were performed at 37° C in a total volume of 800 µl buffered with 20 mM potassium phosphate solution at pH equal to 7, 0.15 M NaCl, containing 1.5×10^{-4} M DNA. Drug: Phosphate ratio was 2.

Table 2

Crosslinking Data for 4,8-Dichloro-2-Methyl-2-Azaadamantane and Calf-Thymus DNA (Type 1, Polymeric)

Time (minutes)	Fraction Crosslinked	Fraction Un-Crosslinked	Corrected Number of Crosslinks/DNA
1.0	0.135	0.865	0.145
2.0	0.205	0.795	0.229
3.0	0.265	0.735	0.308
4.0	0.308	0.692	0.368
6.0	0.327	0.673	0.396
10.0	0.306	0.694	0.365
14.0	0.296	0.704	0.351

Homogeneous reactions were performed at 37° C in a total volume of 800 µl buffered with 20 mM potassium phosphate solution at pH equal to 7, 0.15 M NaCl, containing 1.5×10^{-4} M DNA. Drug: Phosphate ratio was 4.

The "apparent" decrease in the extent of crosslinking of the 4,9 isomer at longer reaction times (4 minutes or longer) as observed in Figures 4-7 deserves special comment. At longer reaction times, the extent of crosslinking of the 4,9 isomer appears to decrease even

though the DNA prior to denaturation, under alkaline conditions, remains double-stranded as indicated by Ethidium fluorescence. It is possible that the 4,9 isomer crosslinked DNA may be more susceptible to heat induced depurination under alkaline conditions which may account for this unusual decrease in "measurable" crosslinks. Alternatively, it is possible that the 4,9 crosslink is either unstable or in some manner produces single strand breaks in the DNA rendering the crosslink undetectable by this technique. The nature of this decreased crosslinking is currently under investigation.

Dependence of (G + C) DNA Base Content Crosslinking Efficiency

Using highly purified DNAs of known (G + C) base content and with established molecular weights (as measured by sedimentation analyses), we can now report the extent of interstrand crosslinking by HN_2, *1*, and *2* in terms of the average number of crosslinks per nucleotide, in addition to the base-sequence dependence of this crosslinking as a function of DNA (G + C) content. For example, HN_2 at 1.5×10^{-4} M gives 2.9×10^{-5}, 5.1×10^{-5}, 6.5×10^{-5}, and 5.0×10^{-5} interstrand crosslinks per nucleotide at saturation with DNAs of (G + C) content of 26.5, 40, 50, and 72%, respectively (Figures 8 and 9). The extent of HN_2 crosslinking (at saturation) and its (G + C) dependence are comparable to the interstrand crosslinking activity of Mitomycin C (Lown et al., 1976) and to the photo-induced crosslinking activity of psoralen and other furocoumarins with nucleic acids (Lown et al., 1978). This (G + C) dependent behavior is indicative of preferential crossslinking at guanine and/or cytosine residues. At

Figure 8. Crosslinking Reaction Profile of Mechlorethamine (HN_2) with Defined Molecular Weight DNAs of Different (G + C) Content.

Pre-Dilution Method: D/P = 0.82, [Nucleotide-P] = 0.18 mM, time dissolved = three minutes.

As per Figure 6, at drug/nucleotide ratio of 0.82:1 (all ratios scaled to constant D/P = 1.0). DNA molecular weights were determined by sedimentation velocity studies, which allow for the direct calculations of the average number of crosslinks per nucleotide residue. The DNAs used were Clostridium Perfringens (M.W. = 2.0×10^7, (G + C) = 26.5%, $\varepsilon(258)$ = 6300 1/moles-cm), Calf-Thymus (M.W. = 9.0×10^6, (G + C) = 40%, $\varepsilon(258)$ = 6420 1/moles-cm), E. Coli (M.W. = 1.64×10^7, G + C) = 50%, $\varepsilon(258)$ = 6500 1/moles-cm).

Figure 8

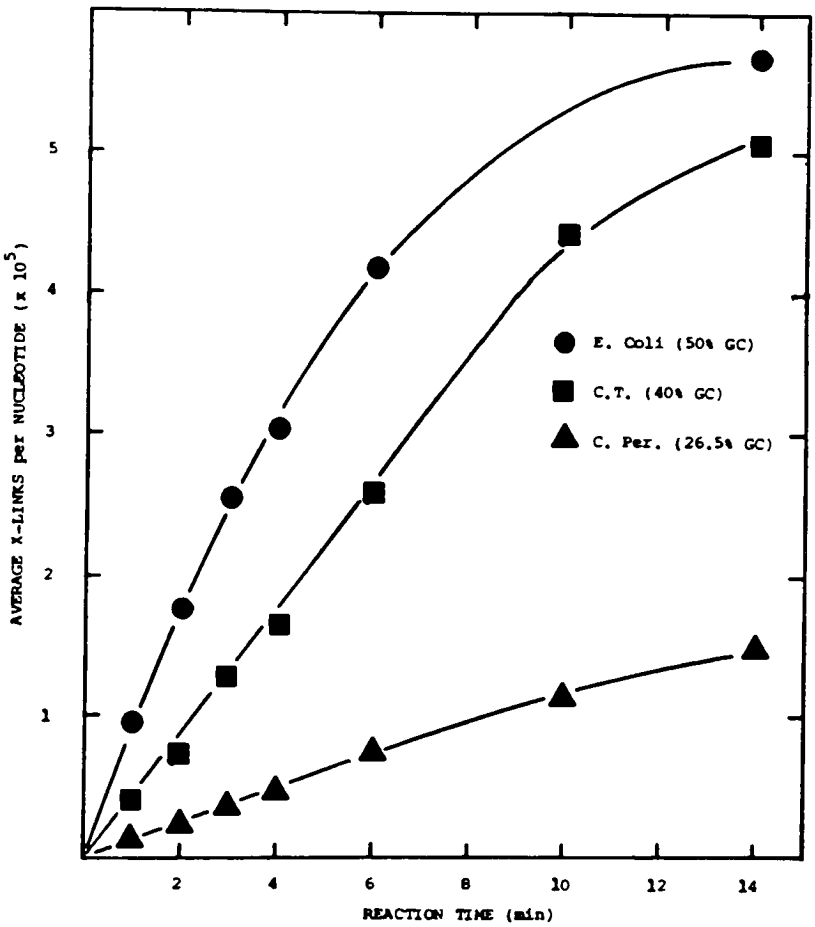
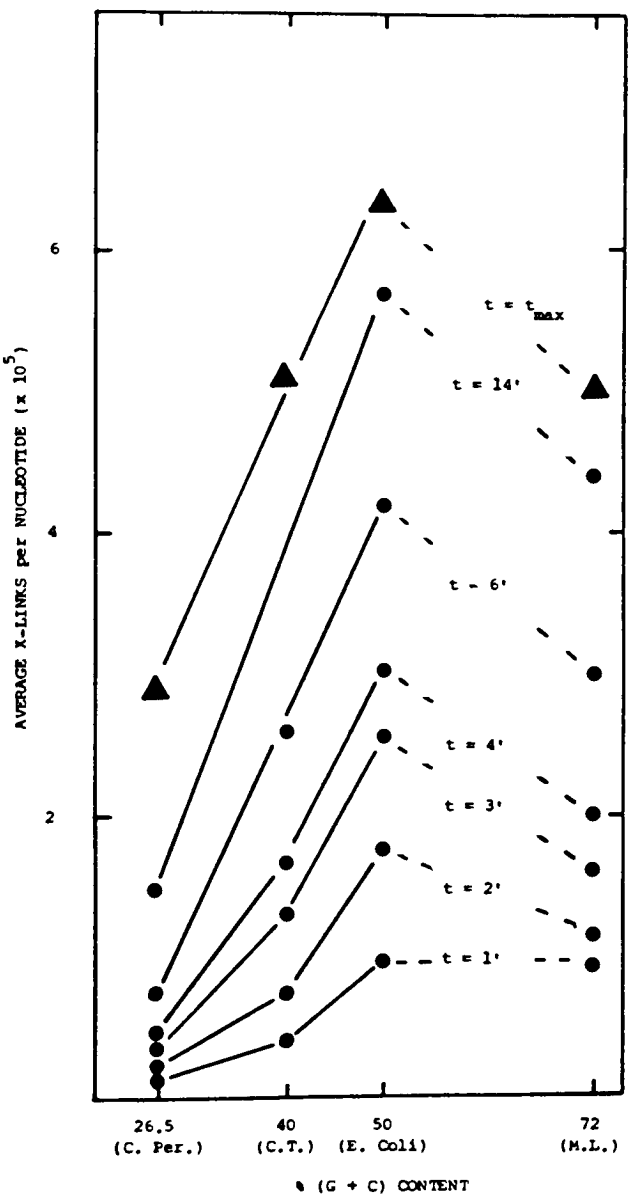

AVERAGE X-LINKS per NUCLEOTIDE (x 10^5)
1
2
3
4
5
2
4
6
8
10
12
14
REACTION TIME (min)
E. Coli (50% GC)
C.T. (40% GC)
C. Per. (26.5% GC)

Figure 9. (G + C) Dependence of the Efficiency of Crosslinking of DNA by HN_2.

Pre-Dilution Method: D/P = 0.82, [Nucleotide-P] = 0.18 m$\underline{M}$, time dissolved = three minutes (all ratios scaled to constant (D/P = 1:1).

Data from Figure 8 representing (G + C) dependence of DNA crosslinking by HN_2 at various reaction time intervals.

Figure 9


AVERAGE X-LINKS per NUCLEOTIDE (x 10^5)
6
4
2
t = t max
t = 14'
t = 6'
t = 4'
t = 3'
t = 2'
t = 1'
26,5
(C. Per.)
40
(C.T.)
50
(E. Coli)
72
(M.L.)
% (G + C) CONTENT

saturation, the extent of crosslinking for HN_2 appears to be directly proportional to the (G + C) content from 25 to 50%, and levels off or decreases to some extent beyond 50% (G + C) content. The initial rate of crosslinking increases from 1.25×10^{-6} crosslinks/nucleotide-min to 8.8×10^{-6} crosslinks/nucleotide-min from 26.5 to 50% (G + C) DNA content, respectively. This represents a 7-fold enhancement in crosslinking kinetic efficiency for HN_2 with increasing (G + C) content from 26.5 to 50%.

Although both *1* and *2* clearly show (G + C) DNA content dependence in their crosslinking efficiencies, these isomers differ significantly with regard to both the characteristics of their base-sequence dependence and the kinetics of their (G + C) dependent crosslinking efficiency (Figures 10-13). The crosslinking reaction profiles of HN_2, *2*, and *1* with DNAs of different (G + C) content are shown in Figures 8, 10, and 12, respectively. The (G + C) dependence of the efficiency of crosslinking for these compounds are shown in Figures 9, 11, 13, as a function of various reaction time intervals.

The 4,9-dichloro-N-methyl isomer, *2*, at 6.6×10^{-4} M gives 1.4×10^{-6}, 6.0×10^{-6}, $> 9.0 \times 10^{-6}$ interstrand crosslinks per nucleotide at maximum measurable crosslinking (time approximately 3 minutes) with DNAs of (G + C) content of 26.5, 50, and 72%, respectively (Figures 10 and 11), while under similar reaction conditions the 4,8-dichloro-N-methyl isomer, *1*, gives 0.44×10^{-5}, 1.2×10^{-5}, and 0.7×10^{-5} interstrand crosslinks per nucleotide at saturation (time approximately six minutes) for 26.5, 50, and 72% (G + C) content.

Figure 10. Crosslinking Reaction Profile of 4,9-dichloro-2-methyl-2-azaadamantane with defined Molecular Weight DNAs of Different (G + C) content.

Pre-Dilution Method: D/P = 3.28, [Nucleotide-P] = 0.2 mM, time dissolved = three minutes.

As per Figure 8, at a drug/nucleotide ratio of 3.28:1 (all ratios scaled to constant D/P ratio = 4).

Figure 10

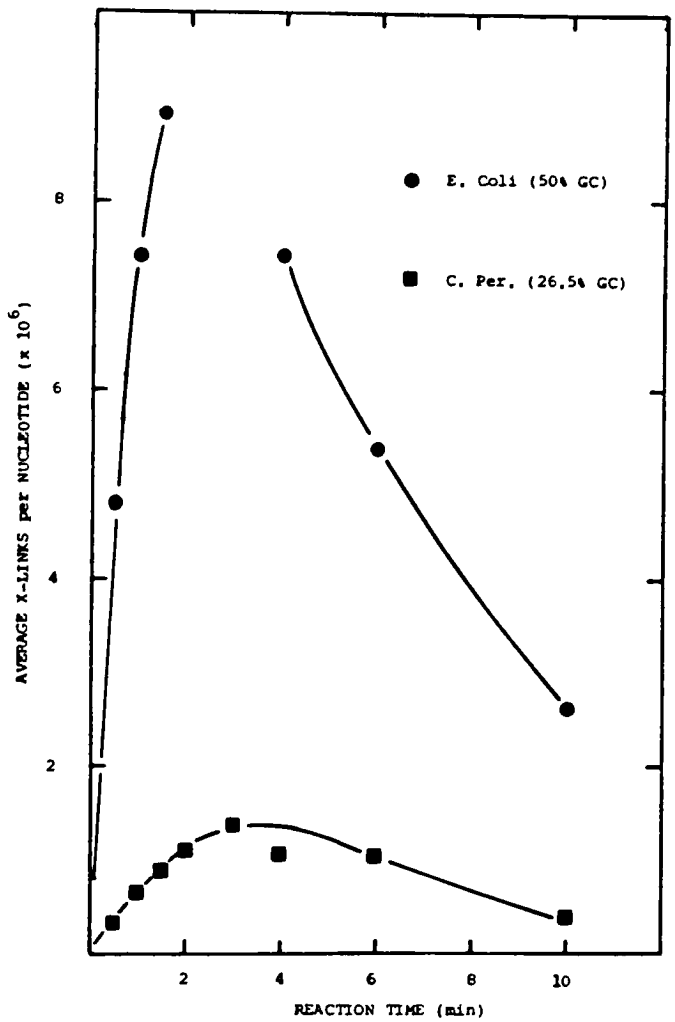

E. Coli (50% GC)
C. Per. (26.5% GC)
AVERAGE X-LINKS per NUCLEOTIDE (x 10^6)
8
6
4
2
2
4
6
8
10
REACTION TIME (min)

Figure 11. (G + C) Dependence of the Efficiency of Crosslinking of DNA by 4,9-dichloro-2-methyl-2-azaadamantane.

Data from Figure 10 representing (G + C) dependence of DNA crosslinking by the 4,9 isomer at various time intervals during the reaction, up to the onset of the time-dependent decrease in measured crosslinking.

Figure 11

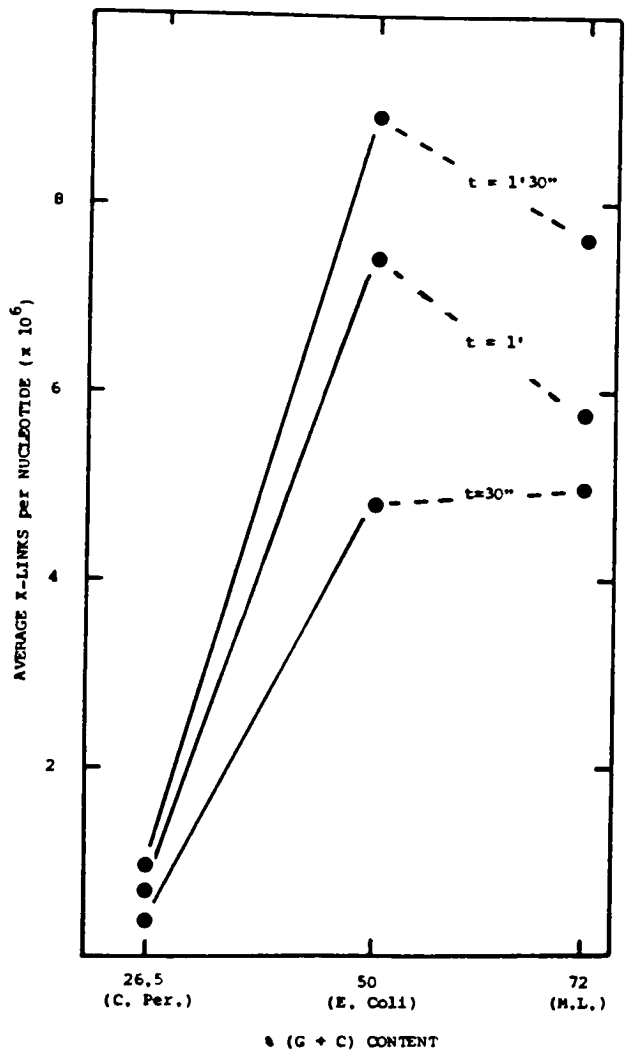

AVERAGE X-LINKS per NUCLEOTIDE (x 10^6)
8
6
4
2
t = 1'30"
t = 1'
t=30"
26,5
(C. Per.)
50
(E. Coli)
72
(M.L.)
% (G + C) CONTENT

Figure 12. Crosslinking Reaction Profile of 4,8-dichloro-2-methyl-2-azaadamantane with defined Molecular Weight DNAs of Different (G + C) content.

Pre-Dilution Method: D/P = 3.28, [Nucleotide-P] = 0.2 mM, time dissolved = three minutes.

As per Figure 8, at a drug/nucleotide ratio of 3.28:1 (all ratios scaled to constant D/P ratio = 4).

Figure 12

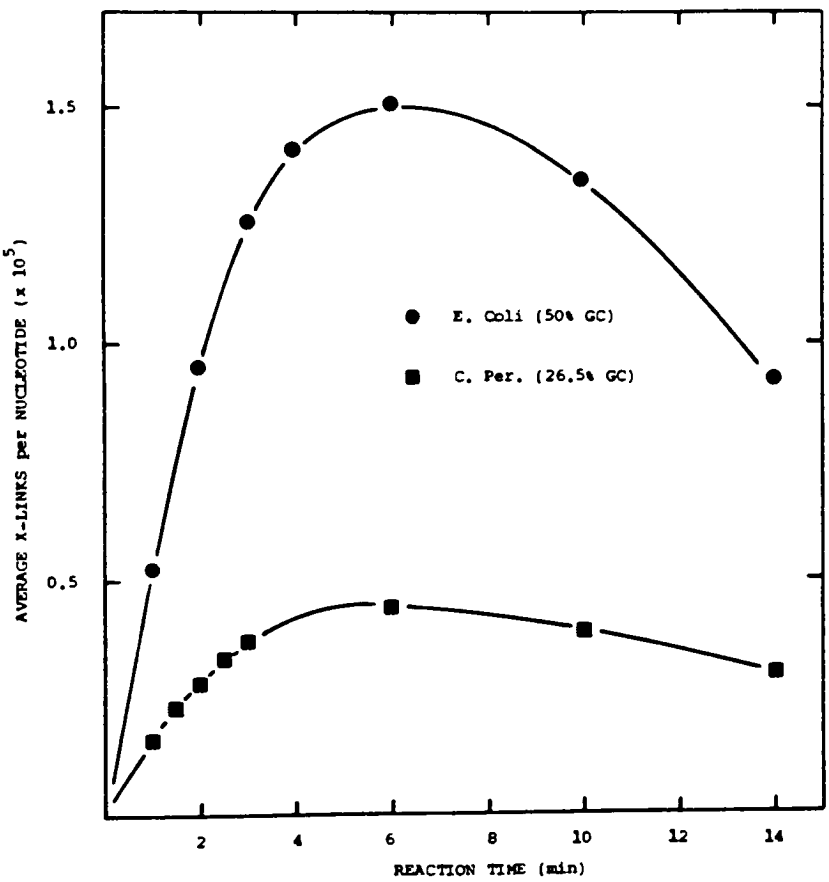
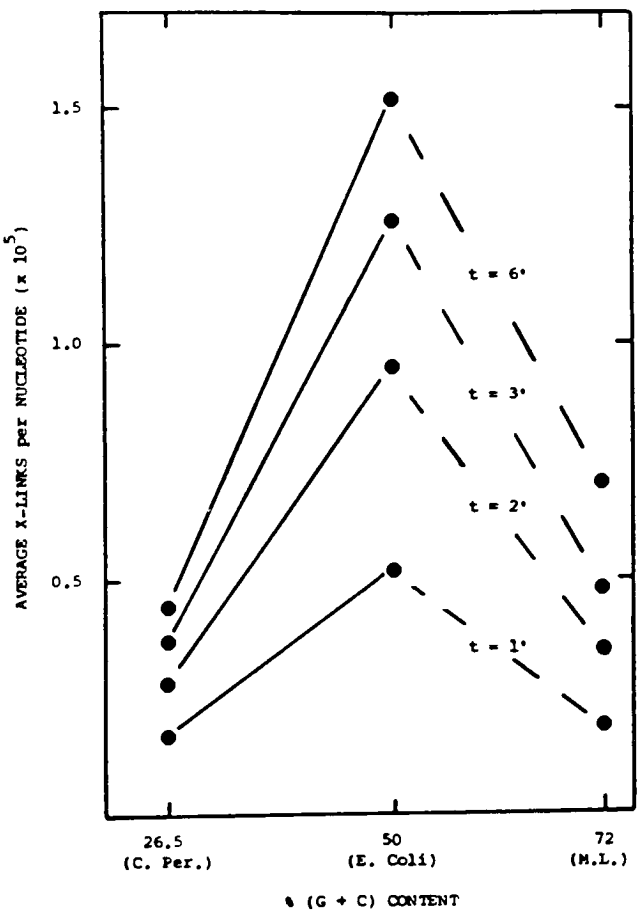

AVERAGE X-LINKS per NUCLEOTIDE (x 10^5)
1.5
1.0
0.5
E. Coli (50% GC)
C. Per. (26.5% GC)
2
4
6
8
10
12
14
REACTION TIME (min)

Figure 13. (G + C) Dependence of the Efficiency of Crosslinking of DNA by 4,8-dichloro-2-methyl-2-azaadamantane.

Data from Figure 12 representing (G + C) dependence of DNA crosslinking by the 4,8 isomer at various reaction time intervals.

Figure 13


1.5
1.0
0.5
AVERAGE X-LINKS per NUCLEOTIDE (x 10^5)
t = 6'
t = 3'
t = 2'
t = 1'
26.5
(C. Per.)
50
(E. Coli)
72
(M.L.)
% (G + C) CONTENT

Due to the time-dependent decrease observed in the crosslinking reaction for *2*, it is important to note that the absolute extent of crosslinking at saturation cannot be measured for this isomer. As a result, direct comparisons of the overall extent of crosslinking between *1* and *2* can only be made for identical reaction time intervals and/or from initial rates of crosslinking for time intervals preceding the observed decrease in crosslinking. Analysis of the initial crosslinking rates of *1* and *2* for Clostridium Perfringens DNA (26.5% G + C) and E. Coli DNA (50% G + C) from Figures 12 and 10, respectively, indicate a 14-fold enhancement in the initial crosslinking rate for *1*. Furthermore, *2* appears to be considerably more specific with regard to its (G + C) dependence showing increased crosslinking with increased (G + C) content. In contrast, *1* shows both a (G + C) dependence and (A + T) dependence of approximately equal magnitudes. This is evident from Figure 13, in that the decrease in (G + C) dependent crosslinking for (G + C) contents greater than 50% are equally indicative of an (A + T) dependent increase in crosslinking for (A + T) up to 50%. One could therefore conclude that the 4,8 isomer may have increasing (G + C) and (A + T) dependencies up to 50% base pair content.

The (G + C) content dependent crosslinking of the 4,8 and 4,9 isomers may be associated with alkylation at specific sites on the pyrimidine or purine bases as is observed with the preferential crosslinking of HN_2 at the N-7 guanine position (Brookes and Lawley, 1961). However, at the present time, it is impossible to distinguish if alkylation is occurring uniquely at purine or pyrimidine residues

or across purine/pyrimidine base pairs. The differences observed in the (G + C) content dependent crosslinking of the 4,8 and 4,9 isomers are most probably associated with the stereochemistry of these isomers and may indeed reflect stereospecific crosslinking selectivity.

Correlation of Cytotoxic Activity with Molecular Crosslinking

The cytotoxic activity of these compounds was determined by investigating the effect of these compounds on the viability of a neoplastic cell line in vitro. The KB 9 cell line, derived from a human carcinoma of the nasopharynx was chosen for the initial screening of the compounds. The test compounds were added to previously plated KB 9 cells. Controls consisted of the addition of saline in the place of the drug solution to the cell cultures. The increase in the cell colony number of the controls and test cultures were determined following 7-8 days of growth. Table 3 contains estimated ID_{50} values based on probit analysis of the dose-response data resulting from the percent inhibition in the drug-treated cell cultures relative to the controls.

Table 3
Cytotoxic Activity of the 4,8 and 4,9-disubstituted Series Expressed as ID_{50} (M)

R	4,8-isomer	4,9-isomer	4,9/4,8 ratio
Hydrogen	13 (9-24)	----	----
Methyl	275 (234-354)	87 (49-113)	3.2
Isopropyl	208 (115-479)	62 (55-72)	3.4
Isoamyl	160 (141-186)	57	2.8
Benzyl	86 (71-102)	----	----
Mechlorethamine	0.066		

(Henkel, et al., 1983)

All of the compounds tested in both the 4,8 and 4,9 series have significant cytotoxic activity. Those compounds tested in the 4,10 series do not crosslink DNA and do not exhibit any cytotoxic activity. Within the methyl series, the 4,9 isomer appears to exhibit significantly more cytotoxic activity than the 4,8 isomer. It should be noted that the 4,9 isomer produces crosslinks much more rapidly than the 4,8 isomer and demonstrates a stronger (G + C) dependence than the 4,8 isomer. A relationship appears to exist between the potency of the cytotoxic activity and the stereochemistry of the isomer series. Since the 4,8 and 4,9 isomers possess similar chemical and physiochemical properties, the observed differences may indeed reflect differences in the crosslinking behavior at the molecular level. These results suggest that both the chemical nature of the crosslink produced by bifunctional alkylating agents and the stereochemical constraints of that crosslink on the DNA may greatly influence the overall pharmacological behavior of the drug.

ACKNOWLEDGMENTS

This project is part of an ongoing project currently funded by the National Cancer Institute through a consortium grant between the University of Connecticut and Rochester Institute of Technology (P.S. #3789) to Dr. Christian G. Reinhardt, Department of Chemistry, Rochester Institute of Technology.

I would like to thank my thesis advisor, Dr. Christian G. Reinhardt, for his guidance and support in the course of my graduate work.

I would also like to thank my family for all their love and support.

I would especially like to thank my husband, David Denison, for his love and understanding.

Table 1

Crosslinking Data for Mechlorethamine and Calf-Thymus DNA (Type 1, Polymeric)

Time (minutes)	Fraction Crosslinked	Fraction Un-Crosslinked	Corrected Number of Crosslinks/DNA
0.0	0.000	1.000	0.000
2.5	0.700	0.300	1.204
5.0	0.830	0.170	1.309
7.5	0.830	0.170	1.309
15.0	0.870	0.130	1.470

Table 2

Crosslinking Data for 4,8-Dichloro-2-Methyl-2-Azaadamantane and Calf-Thymus DNA (Type 1, Polymeric)

Time (minutes)	Fraction Crosslinked	Fraction Un-Crosslinked	Corrected Number of Crosslinks/DNA
0.0	0.000	1.000	0.000
2.5	0.300	0.700	0.357
5.0	0.430	0.570	0.562
7.5	0.450	0.550	0.598
15.0	0.430	0.570	0.562

Table 3

Crosslinking Data for 4,9-Dichloro-2-Methyl-2-Azaadamantane and Calf-Thymus DNA (Type 1, Polymeric)

Time (minutes)	Fraction Crosslinked	Fraction Un-Crosslinked	Corrected Number of Crosslinks/DNA
0.0	0.000	1.000	0.000
1.0	0.380	0.620	0.478
2.0	0.770	0.230	0.109
3.0	0.770	0.230	0.109
5.0	0.790	0.210	0.561
7.0	0.510	0.490	0.713
15.0	0.300	0.700	0.357

Heterogeneous reactions were performed at 37° C in a total volume of 10 ml buffered with 20 mM potassium phosphate solution at pH equal to 7, 0.15 M NaCl, containing 2×10^{-4} M DNA. Drug: Phosphate ratio was 10.

Table 4

Crosslinking Data for Mechlorethamine and Calf-Thymus DNA (Type 1, Polymeric)

Time (minutes)	Fraction Crosslinked	Fraction Un-Crosslinked	Corrected Number of Crosslinks/DNA
2.0	0.529	0.471	0.753
3.0	0.638	0.362	1.016
5.0	0.720	0.280	1.273
10.5	0.827	0.173	1.754
15.0	0.827	0.173	1.754
30.0	0.827	0.173	1.754

Table 5

Crosslinking Data for 4,8-Dichloro-2-Methyl-2-Azaadamantane and Calf-Thymus DNA (Type 1, Polymeric)

Time (minutes)	Fraction Crosslinked	Fraction Un-Crosslinked	Corrected Number of Crosslinks/DNA
2.0	0.035	0.965	0.036
3.0	0.200	0.800	0.223
5.0	0.168	0.832	0.184
10.0	0.207	0.793	0.232
15.0	0.224	0.776	0.254
30.0	0.136	0.864	0.146

Table 6

Crosslinking Data for 4,9-Dichloro-2-Methyl-2-Azaadamantane and Calf-Thymus DNA (Type 1, Polymeric)

Time (minutes)	Fraction Crosslinked	Fraction Un-Crosslinked	Corrected Number of Crosslinks/DNA
2.0	0.509	0.491	0.711
3.0	0.573	0.427	0.851
5.0	0.490	0.510	0.673
10.0	0.352	0.648	0.434
15.0	0.433	0.567	0.567
30.0	0.228	0.772	0.259

Heterogeneous reactions were performed at 37° C in a total volume of 10 ml buffered with 20 mM potassium phosphate solution at pH equal to 7, 0.15 M NaCl, containing 2×10^{-4} M DNA. Drug: Phosphate ratio was 5.

Table 7

Crosslinking Data for Mechlorethamine and Calf-Thymus DNA (Type 1, Polymeric)

Time (minutes)	Fraction Crosslinked	Fraction Un-Crosslinked	Corrected Number of Crosslinks/DNA
2.0	0.813	0.187	1.677
4.0	0.842	0.158	1.845
6.0	0.912	0.088	2.430
10.0	0.957	0.043	3.146
15.0	0.952	0.048	3.036
30.0	0.931	0.069	2.674

Table 8

Crosslinking Data for 4,8-Dichloro-2-Methyl-2-Azaadamantane and Calf-Thymus DNA (Type 1, Polymeric)

Time (minutes)	Fraction Crosslinked	Fraction Un-Crosslinked	Corrected Number of Crosslinks/DNA
2.0	0.186	0.814	0.206
4.0	0.266	0.734	0.309
6.0	0.330	0.670	0.400
10.0	0.378	0.622	0.475
15.0	0.341	0.659	0.417
30.0	0.229	0.771	0.260

Table 9

Crosslinking Data for 4,9-Dichloro-2-Methyl-2-Azaadamantane and Calf-Thymus DNA (Type 1, Polymeric)

Time (minutes)	Fraction Crosslinked	Fraction Un-Crosslinked	Corrected Number of Crosslinks/DNA
2.0	0.359	0.641	0.445
4.0	0.385	0.615	0.486
6.0	0.331	0.669	0.402
10.0	0.277	0.723	0.324
15.0	0.218	0.782	0.246
30.0	0.192	0.808	0.213

Homogeneous reactions were performed at 37° C in a total volume of 800 µl buffered with 20 mM potassium phosphate solution at pH equal to 7, 0.15 M NaCl, containing 1.5×10^{-4} M DNA. Drug: Phosphate ratio was 10.

Table 10

Crosslinking Data for 4,10-Dichloro-2-Methyl-2-Azaadamantane and Calf-Thymus DNA (Type 1, Polymeric) - Drug: Phosphate = 10

Time (minutes)	Fraction Crosslinked	Fraction Un-Crosslinked	Corrected Number of Crosslinks/DNA
2.0	-0.014	1.000	0.000
4.0	-0.014	1.000	0.000
6.0	0.000	1.000	0.000
10.0	-0.015	1.000	0.000
15.0	0.025	0.975	0.025
30.0	0.037	0.963	0.037

Table 11

Crosslinking Data for 4,10-Dichloro-2-Methyl-2-Azaadamantane and Calf-Thymus DNA (Type 1, Polymeric) - Drug: Phosphate = 2

Time (minutes)	Fraction Crosslinked	Fraction Un-Crosslinked	Corrected Number* of Crosslinks/DNA
2.0	0.131	0.868	0.141
4.0	0.079	0.921	0.083
6.0	0.099	0.901	0.104
10.0	0.073	0.927	0.076
15.0	0.078	0.922	0.081
30.0	0.079	0.921	0.082

Table 12

Crosslinking Data for 4,10-Dichloro-2-Methyl-2-Azaadamantane and Calf-Thymus DNA (Type 1, Polymeric) - Drug: Phosphate = 0.5:1

Time (minutes)	Fraction Crosslinked	Fraction Un-Crosslinked	Corrected Number of Crosslinks/DNA
2.0	0.032	0.968	0.032
4.0	0.065	0.935	0.067
6.0	0.060	0.940	0.062
10.0	0.062	0.938	0.064
15.0	0.056	0.944	0.058
30.0	0.089	0.911	0.093

Homogeneous reactions were performed at 37° C in a total volume of 800 µl buffered with 20 mM potassium phosphate solution at pH equal to 7, 0.15 M NaCl, containing 1.5×10^{-4} M DNA.

* This data is suspect because the control did not denature properly.

Table 13

Crosslinking Data for Mechlorethamine and Calf-Thymus DNA (Type 1, Polymeric)

Time (minutes)	Fraction Crosslinked	Fraction Un-Crosslinked	Corrected Number of Crosslinks/DNA
2.0	0.411	0.589	0.529
4.0	0.569	0.431	0.842
6.0	0.663	0.337	1.088
10.0	0.753	0.247	1.398
15.0	0.856	0.144	1.938

Table 14

Crosslinking Data for 4,8-Dichloro-2-Methyl-2-Azaadamantane and Calf-Thymus DNA (Type 2, Polymeric)

Time (minutes)	Fraction Crosslinked	Fraction Un-Crosslinked	Corrected Number of Crosslinks/DNA
2.0	0.076	0.923	0.079
4.0	0.098	0.901	0.103
6.0	0.123	0.876	0.132
10.0	0.130	0.870	0.139
15.0	0.137	0.863	0.147

Table 15

Crosslinking Data for 4,9-Dichloro-2-Methyl-2-Azaadamantane and Calf-Thymus DNA (Type 1, Polymeric)

Time (minutes)	Fraction Crosslinked	Fraction Un-Crosslinked	Corrected Number of Crosslinks/DNA
2.0	0.101	0.899	0.106
4.0	0.124	0.876	0.132
6.0	0.124	0.876	0.132
10.0	0.094	0.906	0.099
15.0	0.068	0.932	0.070
30.0	0.059	0.941	0.061

Homogeneous reactions were performed at 37° C in a total volume of 800 µl buffered with 20 mM potassium phosphate solution at pH equal to 7, 0.15 M NaCl, containing 1.5×10^{-4} M DNA. Drug: Phosphate ratio was 2.

Table 16

Crosslinking Data for Mechlorethamine
and Calf-Thymus DNA (Type 1, Polymeric)

Time (minutes)	Fraction Crosslinked	Fraction Un-Crosslinked	Corrected Number of Crosslinks/DNA
1.0	0.786	0.214	1.542
2.0	0.837	0.163	1.814
3.0	0.947	0.053	2.937
4.0	0.920	0.080	2.625
6.0	0.982	0.018	4.017
10.0	0.043	0.957	3.146
15.0	0.962	0.038	3.290

Table 17

Crosslinking Data for 4,8-Dichloro-2-Methyl-2-Azaadamantane
and Calf-Thymus DNA (Type 1, Polymeric)

Time (minutes)	Fraction Crosslinked	Fraction Un-Crosslinked	Corrected Number of Crosslinks/DNA
1.0	0.135	0.865	0.145
2.0	0.205	0.795	0.229
3.0	0.265	0.735	0.308
4.0	0.308	0.692	0.368
6.0	0.327	0.673	0.396
10.0	0.306	0.694	0.365
14.0	0.296	0.704	0.351

Homogeneous reactions were performed at 37° C in a total volume of 800 µl buffered with 20 mM potassium phosphate solution at pH equal to 7, 0.15 M NaCl, containing 1.5×10^{-4} M DNA. Drug: Phosphate ratio was 4.

Table 18

Crosslinking Data for Mechlorethamine and Clostridium Perfringens DNA

Time (minutes)	Fraction Crosslinked	Fraction Un-Crosslinked	Corrected Number of Crosslinks/DNA	Number of Crosslinks/ nucleotide ($\times 10^{-6}$)
1.0	0.0738	0.9262	0.0766	1.345
2.0	0.1329	0.8671	0.1426	2.318
3.0	0.1946	0.8054	0.2164	3.518
4.0	0.2550	0.7450	0.2944	4.786
6.0	0.3691	0.6309	0.4606	7.487
10.0	0.5168	0.4832	0.7273	11.820
14.0	0.6026	0.3974	0.9228	15.000
120.0	0.8309	0.1691	1.7770	28.890

Lot #86C-6850; DNA m.w. 2.0×10^{7}; DNA - P 1.984×10^{-4} M; DNA - dup 3.23×10^{-9} M; drug/DNA 0.82; drug/DNA - dup 5.04×10^{4}.

Table 19

Crosslinking Data for Mechlorethamine and Calf-Thymus DNA

Time (minutes)	Fraction Crosslinked	Fraction Un-Crosslinked	Corrected Number of Crosslinks/DNA	Number of Crosslinks/ nucleotide ($\times 10^{-6}$)
2.0	0.0870	0.9130	0.0910	3.288
3.0	0.1250	0.8750	0.1335	4.826
4.0	0.1913	0.8087	0.2123	7.668
6.0	0.2719	0.7281	0.3173	11.460
10.0	0.4210	0.5790	0.5464	19.740
14.0	0.4786	0.5214	0.6512	23.520

Lot #91F-6833; DNA m.w. 9.0×10^{6}; DNA - P 8.576×10^{-4} M; DNA - dup 3.09×10^{-9} M; drug/DNA 1.82; drug/DNA - dup 5.04×10^{-4}.

Homogeneous reactions were performed at 37° C in a total volume of 800 µl buffered with 20 mM potassium phosphate solution at pH equal to 7, 0.15 M NaCl.

Table 20

Crosslinking Data for Mechlorethamine and Calf-Thymus DNA

Time (minutes)	Fraction Crosslinked	Fraction Un-Crosslinked	Corrected Number of Crosslinks/DNA	Number of Crosslinks/ nucleotide ($\times 10^{-6}$)
1.0	0.0942	0.9058	0.0989	3.573
2.0	0.1259	0.8741	0.1346	4.861
3.0	0.1655	0.8345	0.1809	6.535
4.0	0.2206	0.7794	0.2492	9.002
6.0	0.3094	0.6906	0.3719	13.370
10.0	0.4638	0.5362	0.6232	22.510
14.0	0.5357	0.4663	0.7672	27.710
60.0	0.8217	0.1786	0.7230	62.220
90.0	0.8222	0.1778	1.7270	62.380
120.0	0.8370	0.1630	1.8140	65.520
150.0	0.8188	1.1812	1.7080	61.700

Lot #91F-6833; DNA m.w. 9.0×10^{6}; DNA - P 1.577×10^{-4} M; DNA - dup 5.69×10^{-9} M; drug/DNA 1; drug/DNA - dup 2.77×10^{4}.

Table 21

Crosslinking Data for Mechlorethamine and E. Coli DNA

Time (minutes)	Fraction Crosslinked	Fraction Un-Crosslinked	Corrected Number of Crosslinks/DNA	Number of Crosslinks/ nucleotide ($\times 10^{-6}$)
1.0	0.3364	0.6634	0.4104	8.134
2.0	0.5093	0.4907	0.7119	14.110
3.0	0.6551	0.3449	1.0640	21.090
4.0	0.7167	0.2833	1.2610	34.990
6.0	0.8246	0.1754	1.7410	34.510
10.0	0.9500	0.0500	2.9960	59.380
14.0	0.9083	0.0917	2.3890	47.350

Lot #101F-6815; DNA m.w. 16.4×10^{6}; DNA - P 1.573×10^{-4} M; DNA - dup 3.12×10^{-4} M; drug/DNA 1; drug/DNA - dup 5.04×10^{4}.

Homogeneous reactions were performed at 37° C in a total volume of 800 µl buffered with 20 mM potassium phosphate solution at pH equal to 7 and 0.15 M NaCl.

Table 22

Crosslinking Data for Mechlorethamine and E. Coli DNA (Trial 1)

Time (minutes)	Fraction Crosslinked	Fraction Un-Crosslinked	Corrected Number of Crosslinks/DNA	Number of Crosslinks/ nucleotide ($\times 10^{-6}$)
1.0	0.2074	0.7926	0.2324	4.606
2.0	0.3125	0.6875	0.3747	7.427
3.0	0.3606	0.6394	0.4472	8.864
4.0	0.4682	0.5218	0.6315	1.252
6.0	0.6136	0.3864	0.9509	18.850
10.0	0.7109	0.2891	1.2400	24.600
14.0	0.8110	0.1890	1.6660	33.020

Lot #101F-6815; DNA m.w. 16.4×10^{6}; DNA - P 1.573×10^{-4} M; DNA - dup 3.12×10^{-9} M; drug/DNA 1; drug/DNA - dup 5.04×10^{4}.

Table 23

Crosslinking Data for Mechlorethamine and E. Coli DNA (Trial 2)

Time (minutes)	Fraction Crosslinked	Fraction Un-Crosslinked	Corrected Number of Crosslinks/DNA	Number of Crosslinks/ nucleotide ($\times 10^{-6}$)
1.0	0.0465	0.9535	0.0476	1.160
2.0	0.0938	0.9062	0.0985	2.389
3.0	0.1231	0.8769	0.1314	3.187
4.0	0.1970	0.8030	0.2194	5.337
6.0	0.2923	0.7077	0.3457	8.388
10.0	0.3939	0.6061	0.5007	12.150
14.0	0.4463	0.5537	0.5911	14.340

Lot #72F-6836; DNA m.w. 13.4×10^{6}; DNA - P 1.600×10^{-4} M; DNA - dup 3.88×10^{-9} M; drug/DNA 1; drug/DNA - dup 4.12×10^{4}.

Homogeneous reactions were performed at 37° C in a total volume of 800 µl buffered with 20 mM potassium phosphate solution at pH equal to 7 and 0.15 M NaCl.

Table 24

Crosslinking Data for Mechlorethamine and M. Luteus DNA (Trial 1)

Time (minutes)	Fraction Crosslinked	Fraction Un-Crosslinked	Corrected Number of Crosslinks/DNA	Number of Crosslinks/ nucleotide ($\times 10^{-6}$)
1.0	0.4000	0.6000	0.5108	8.741
2.0	0.4710	0.5290	0.6368	10.890
3.0	0.5857	0.4142	0.8812	15.080
4.0	0.6712	0.3288	1.1120	19.032
6.0	0.8138	0.8620	1.6810	28.670
10.0	0.8919	0.1081	2.2250	38.070
14.0	0.9137	0.0863	2.4500	41.920

Lot #89C-6820; DNA m.w. 1.9×10^{7}; DNA - P 1.854×10^{-4} M; DNA - dup 3.17×10^{-9} M drug/DNA 0.86; drug/DNA - dup 5.04×10^{-4}.

Table 25

Crosslinking Data for Mechlorethamine and M. Luteus DNA (Trial 2)

Time (minutes)	Fraction Crosslinked	Fraction Un-Crosslinked	Corrected Number of Crosslinks/DNA	Number of Crosslinks/ nucleotide ($\times 10^{-6}$)
1.0	0.1780	0.8220	0.1960	3.350
2.0	0.3162	0.6838	0.3801	6.504
3.0	0.4250	0.5750	0.5534	9.469
4.0	0.4915	0.5085	0.6763	11.570
6.0	0.6271	0.3729	0.9864	16.880
10.0	0.7458	0.2542	1.3690	23.440
14.0	0.8136	0.1864	1.6790	28.740
60.0	0.8361	0.1639	1.8080	30.940
90.0	0.8702	0.1298	2.0420	34.940
120.0	0.9115	0.0885	2.4250	41.490
150.0	0.9174	0.0826	2.4940	42.610

Lot #89C-6820; DNA m.w. 1.9×10^{7}; DNA - P 1.562×10^{-4} M; DNA - dup 2.67×10^{-9} M; drug/DNA 1; drug/DNA - dup 5.84×10^{-4}.

Homogeneous reactions were performed at 37° C in a total volume of 800 µl buffered with 20 mM potassium phosphate solution at pH equal to 7 and 0.15 M NaCl.

Table 26

Crosslinking Data for 4,8-Dichloro-2-Methyl-2-Azaadamantane and Clostridium Perfringens DNA

Time (minutes)	Fraction Crosslinked	Fraction Un-Crosslinked	Corrected Number of Crosslinks/DNA	Number of Crosslinks/ nucleotide ($\times 10^{-6}$)
0.50	0.0559	0.9441	0.0575	0.934
1.00	0.0963	0.9037	0.1012	1.646
1.30	0.1293	0.8707	0.1384	2.251
2.00	0.1575	0.8424	0.1714	2.787
2.30	0.1862	0.8138	0.2060	3.350
3.00	0.2013	0.7987	0.2248	3.655
4.00	0.2053	0.7947	0.2298	3.736
6.00	0.2381	0.7619	0.2719	4.422
10.00	0.2148	0.7852	0.2418	3.932
14.00	0.1678	0.8322	0.1837	2.987
20.00	0.1409	0.8591	0.1619	2.469
60.00	0.0604	0.9396	0.0623	1.013

Lot #86C-6850; $_{9}$DNA m.w. 2.0×10^{7}; DNA - P 1.984×10^{-4} M; $_{4}$DNA - dup 3.23×10^{-9} M; drug/DNA 3.28; drug/DNA - dup 2.01×10^{4}.

Table 27

Crosslinking Data for 4,8-Dichloro-2-Methyl-2-Azaadamantane and Calf-Thymus DNA

Time (minutes)	Fraction Crosslinked	Fraction Un-Crosslinked	Corrected Number of Crosslinks/DNA	Number of Crosslinks/ nucleotide ($\times 10^{-6}$)
0.50	0.0511	0.9489	0.0524	1.893
1.00	0.0652	0.9348	0.0674	2.435
1.30	0.0797	0.9203	0.0831	3.000
2.00	0.0942	0.9058	0.0989	3.574
2.30	0.1094	0.8906	0.1159	4.185
3.00	0.1143	0.8857	0.1214	4.390
4.00	0.1181	0.8819	0.1257	4.534
6.00	0.1888	0.8112	0.2092	7.558
10.00	0.2000	0.8000	0.2231	8.060
14.00	0.1857	0.8143	0.2054	7.420

Lot #91F-6833; $_{9}$DNA m.w. 9.0×10^{6}; DNA - P 1.577×10^{-4} M; DNA - dup 5.69×10^{-9} M; drug/DNA 4; drug/DNA - dup 1.10×10^{5}.

Homogeneous reactions were performed at 37° C in a total volume of 800 µl buffered with 20 mM potassium phosphate solution at pH equal to 7 and 0.15 M NaCl.

Table 28

Crosslinking Data for 4,8-Dichloro-2-Methyl-2-Azaadamantane and E. Coli DNA

Time (minutes)	Fraction Crosslinked	Fraction Un-Crosslinked	Corrected Number of Crosslinks/DNA	Number of Crosslinks/ nucleotide ($\times 10^{-6}$)
1.00	0.1938	0.8062	0.2154	4.267
2.00	0.3258	0.6742	0.3942	7.814
3.00	0.4060	0.5940	0.5209	10.320
4.00	0.4436	0.5564	0.5863	11.620
6.00	0.4662	0.5338	0.6277	12.440
10.00	0.4286	0.5714	0.5597	11.090
14.00	0.3158	0.6842	0.3795	7.522

Lot #101F-6815; DNA m.w. 16.4×10^{6}; DNA - P 1.573×10^{-4} M; DNA - dup 3.12×10^{-9} M; drug/DNA 4; drug/DNA - dup 2.01×10^{5}.

Table 29

Crosslinking Data for 4,8-Dichloro-2-Methyl-2-Azaadamantane and M. Luteus DNA

Time (minutes)	Fraction Crosslinked	Fraction Un-Crosslinked	Corrected Number of Crosslinks/DNA	Number of Crosslinks/ nucleotide ($\times 10^{-6}$)
0.50	0.0970	0.9030	0.1020	1.746
1.00	0.0930	0.9070	0.0976	1.670
1.30	0.1504	0.8946	0.1630	2.789
2.00	0.1765	0.8235	0.1942	3.323
2.30	0.2353	0.7647	0.2683	4.590
3.00	0.2353	0.7647	0.2683	4.590
4.00	0.2797	0.7206	0.3277	5.607
6.00	0.3235	0.6765	0.3908	6.687
10.00	0.3433	0.6567	0.4205	7.195
14.00	0.3134	0.6866	0.3760	6.434

Lot #89C-6820; DNA m.w. 1.9×10^{7}; DNA - P 1.854×10^{-4} M; DNA - dup 3.17×10^{-9} M; drug/DNA 3.45; drug/DNA - dup 2.01×10^{5}.

Homogeneous reactions were performed at 37° C in a total volume of 800 µl buffered with 20 mM potassium phosphate solution at pH equal to 7 and 0.15 M NaCl.

Table 30

Crosslinking Data for 4,9-Dichloro-2-Methyl-2-Azaadamantane and Clostridium Perfringens DNA

Time (minutes)	Fraction Crosslinked	Fraction Un-Crosslinked	Corrected Number of Crosslinks/DNA	Number of Crosslinks/ nucleotide ($\times 10^{-6}$)
0.50	0.0199	0.9801	0.0201	0.326
1.00	0.0399	0.9601	0.0407	0.662
1.30	0.0519	0.9481	0.0533	0.866
2.00	0.0649	0.9351	0.0671	1.091
2.30	0.0633	0.9367	0.0654	1.063
3.00	0.0828	0.9172	0.0864	1.405
4.00	0.0633	0.9367	0.0654	1.063
6.00	0.0637	0.9363	0.0658	1.069
10.00	0.0253	0.9747	0.0256	0.417
14.00	0.0255	0.9745	0.0258	0.419
20.00	0.0127	0.9873	0.0128	0.208

Lot #86C-6850; $_9$DNA m.w. 2.0×10^7; DNA - P 1.984×10^{-4} M; $_5$DNA - dup 3.23×10^{-9} M; drug/DNA 3.28; drug/DNA - dup 2.01×10^5.

Table 31

Crosslinking Data for 4,9-Dichloro-2-Methyl-2-Azaadamantane and Calf-Thymus DNA

Time (minutes)	Fraction Crosslinked	Fraction Un-Crosslinked	Corrected Number of Crosslinks/DNA	Number of Crosslinks/ nucleotide ($\times 10^{-6}$)
0.50	0.0511	0.9489	0.0324	1.893
1.00	0.0652	0.9348	0.0764	2.445
1.30	0.0797	0.9203	0.0831	3.000
2.00	0.0942	0.9058	0.0989	3.574
2.30	0.1094	0.8906	0.1159	4.185
3.00	0.1143	0.8857	0.1214	4.390
4.00	0.1181	0.8819	0.1257	4.539
6.00	0.1888	0.8112	0.2192	7.558
10.00	0.2000	0.8000	0.2231	8.060
14.00	0.1857	0.8153	0.2054	7.420

Lot #91F-6833; $_9$DNA m.w. 9.0×10^6; DNA - P 1.577×10^{-4} M; DNA - dup 5.69×10^{-9} M; drug/DNA 4; drug/DNA - dup 1.10×10^5.

Homogeneous reactions were performed at 37° C in a total volume of 800 µl buffered with 20 mM potassium phosphate solution at pH equal to 7 and 0.15 M NaCl.

Table 32

Crosslinking Data for 4,9-Dichloro-2-Methyl-2-Azaadamantane and E. Coli DNA

Time (minutes)	Fraction Crosslinked	Fraction Un-Crosslinked	Corrected Number of Crosslinks/DNA	Number of Crosslinks/ nucleotide ($\times 10^{-6}$)
0.50	0.1804	0.8196	0.1989	3.943
1.00	0.2647	0.7353	0.3075	6.090
1.30	0.3088	0.6912	0.3693	7.320
2.00	0.2941	0.7059	0.3483	6.900
4.00	0.2647	0.7353	0.3074	6.094
6.00	0.1985	0.8015	0.2213	4.385
10.00	0.1029	0.8971	0.1086	2.152

Lot #101F-6815; DNA m.w. 16.4×10^{6}; DNA - P 1.573×10^{-4} M; DNA - dup 3.12×10^{-9} M; drug/DNA 4; drug/DNA - dup 2.01×10^{5}.

Table 33

Crosslinking Data for 4,9-Dichloro-2-Methyl-2-Azaadamantane and M. Luteus DNA

Time (minutes)	Fraction Crosslinked	Fraction Un-Crosslinked	Corrected Number of Crosslinks/DNA	Number of Crosslinks/ nucleotide ($\times 10^{-6}$)
0.50	0.2374	0.7626	0.2710	4.637
1.00	0.2700	0.7300	0.3147	5.385
1.30	0.3451	0.6549	0.4233	7.242
2.00	0.3819	0.6181	0.4811	8.232
2.30	0.4045	0.5944	0.5202	8,901
3.00	0.4126	0.5874	0.5320	9.104
4.00	0.4113	0.5887	0.5298	9.066
6.00	0.3759	0.6241	0.4714	8.067
10.00	0.2411	0.7589	0.2759	4.721
14.00	0.2128	0.7872	0.2393	4.094

Lot #89C-6820; DNA m.w. 1.9×10^{7}; DNA - P 1.854×10^{-4} M; DNA - dup 3.17×10^{-9} M; drug/DNA 3.45; drug/DNA - dup 2.01×10^{5}.

Homogeneous reactions were performed at 37° C in a total volume of 800 µl buffered with 20 mM potassium phosphate solution at pH equal to 7 and 0.15 M NaCl.

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