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A comparative analysis of DNA molecular crosslinking by dichloro-N-substituted azaadamantane isomers

Harry Robbins

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A COMPARATIVE ANALYSIS OF DNA MOLECULAR CROSSLINKING BY DICHLORO-N-SUBSTITUTED 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 anti, anti-dichloro-N-substituted azaadamantanes have been synthesized (Henkel et al, 1981, J. Org. Chem., 46, 3483) representing conformationally rigid nitrogen mustards incorporated into an adamantyl framework with well defined stereochemistry and fixed separation distances between alkylating centers of 3.6 Å (1) and 2.5 Å (2,3) (Henkel et al, 1981, J. Org. Chem., 46, 3483) to be used as structurally rigid probes of DNA alkylation and intermolecular crosslink formation leading to cytotoxicity.

\[
\begin{align*}
\text{4,8-dichloro} & \quad \text{Azaadamantane} \\
\text{4,9-dichloro} & \quad \text{Azaadamantane} \\
\text{4,10-dichloro} & \quad \text{Azaadamantane}
\end{align*}
\]

\[X = \text{Cl} \]

\[R = \text{CH}_3; \text{CH}_2\text{CH}_2\text{N(C}_5\text{H}_{10})\]

The aqueous solution stability and the extent of intermolecular DNA crosslink formation for both the N-methyl and N-piperdinoethyl series of isomers have been measured using novel modifications of an ethidium bromide fluorescence assay technique. Results indicate that isomers 1 and 2 of both series form intermolecular crosslinks to an appreciable extent, while little or no crosslinking is observed under
similar conditions for isomer 3 of both series. Analysis of the aqueous solution stability, time dependent crosslinking reaction isotherms and crosslink stability indicate significant differences both within and between series, which suggests stereospecific crosslinking behavior at the molecular level.
TABLE OF CONTENTS

GENERAL INTRODUCTION: REVIEW OF DNA CROSSLINKING, EXAMPLES OF CROSSLINKING AGENTS AND METHODS OF DNA CROSSLINKING ANALYSIS

Molecular Crosslinking of DNA .................................................. 1
Biological Effects of Crosslinking ......................................... 2
Examples of Crosslinking and Crosslinking Agents .................. 3
   A. Thymine Dimer Intrastrand Crosslinking ............................. 3
   B. Interstrand Crosslinking by Bifunctional
      Alkylation Agents ....................................................... 6
         1. Psoralens .......................................................... 6
         2. Mitomycin C ...................................................... 9
         3. Nitrosoureas .................................................. 11
         4. Nitrogen Mustards .............................................. 13
Methods of Detection ........................................................... 17
   A. Gel Electrophoresis .................................................. 17
   B. Electron Microscopy ................................................. 20
   C. Ethidium Bromide Fluorescence Assay .............................. 21
Introduction of the Dichloro-N-substituted Azaadamantanes ... 28
Specific Objectives of Research ........................................... 30

Chapter I: STABILITY OF AZAADAMANTANE ISOMERS IN AQUEOUS SOLUTION

Introduction ............................................................................. 32
Experimental .......................................................................... 34
CHAPTER II: AZAADAMANTANE CROSSLINKING REACTION ISOHERMS

Introduction.................................................................64
Experimental.................................................................65
    A. Materials and Methods.............................................65
    B. Modification of the General Assay Procedure to
       Determine the Time Dependent Crosslinking Reaction
       Profiles of the Azaadamantane Isomers......................66
Results and Discussion..................................................69
CHAPTER III: STABILITY OF AZAADAMANTANE INDUCED DNA CROSSLINKS

Introduction...........................................................................................................102
Experimental........................................................................................................107
   A. Materials and Methods.................................................................................107
   B. Determination of Alkaline Denaturation Conditions...............................107
   C. Determination of the Extent of DNA Degradation
       Due to Hydrolysis.........................................................................................108
   D. Modification of the General Assay Procedure
       to Determine Heat Liability of the Induced
       DNA Crosslinks in the Standard Ethidium Bromide
       Assay Solution...................................................................................................109
Results and Discussion.........................................................................................110
Summary and Conclusions.....................................................................................111

List of Tables.........................................................................................................114
References..............................................................................................................115
It is important to note that in the Watson and Crick model of DNA, there exists no covalent bonds between either bases on the same nucleic strand or between bases on opposing nucleic strands. There are, however, a large number of bifunctional alkylating agents capable of forming such covalent bonds. These introduced bonds, referred to as crosslinks, can take two different forms; intrastrand crosslinks and interstrand crosslinks as shown.

An intrastrand crosslink occurs by alkylation of two of the nucleic acid bases located on the same DNA strand. Intrastrand crosslinks, as well as monoalkylation of bases by monofunctional...
alkylating agents may have serious consequences on normal DNA replication and cell proliferation (Eastman, 1981). However, many of these types of crosslinks have also been shown to be easily repaired in vivo prior to DNA replication (Joshua et al, 1980).

Interstrand crosslinks occur by alkylation of two nucleic acid bases on opposite DNA strands. These bases may be a complementary base pair or unassociated bases within the reaction distance of the alkylation agent. This type of crosslink formation has a significant effect on the cycle of normal DNA replication and transcription, and has been shown to correlate well with cell cytotoxicity (Lown, 1979). Because of this correlation, the rest of the discussion will focus on the formation of interstrand crosslinks.

BIOLOGICAL EFFECTS OF CROSSLINKING

It has previously been shown that the introduction of just one crosslink is sufficient to prevent complete strand separation of the entire DNA molecule (Kohn et al, 1966; Geduschak, 1961). Such crosslinks would therefore be expected to inhibit DNA replication and transcription, in which strand separation is a necessary step.

Previous studies have established direct correlations between the efficiency of DNA crosslinking by various alkylation agents, such as mitomycin C and some aziridinoquinones, and anti-leukemic activity (Akthar et al, 1975; Lown, 1979). These helped to explain earlier observations that potent tumor inhibition of alkylation agents occurred only with compounds having at least two functional groups (Haddow et al, 1948; Loveless, 1951). It is now well accepted that
the formation of DNA interstrand crosslinks is the primary cytotoxic event associated with the observed anti-leukemic activity, and that the measurement of DNA crosslinking may be a useful method for the pre-screening of potential anti-leukemic drugs (Conners, 1974).

Generally, the crosslinks formed may be repaired by nuclear repair enzymes if enough time is available before the S phase of DNA replication is reached. If insufficient time is allowed for repair, lethality results due to the inhibition of DNA replication. Therefore, it can be seen that the crosslinking event is not immediately a lethal event itself, but causes cell death only if the timing of the cycle is right. Hence, due to the rapid replication cycle of cancerous cells, interstrand crosslink formation exhibits good anti-leukemic activity (Skipper, 1971). Because of this, many of the anti-cancer chemotherapeutic drugs used today are crosslinking agents.

EXAMPLES OF CROSSLINKING AND CROSSLINKING AGENTS

A. Thymine Dimer Intrastrand Crosslinking:

The most common example of crosslinking is the formation of thymine dimers. These dimers are natural, ultraviolet induced, intrastrand crosslinks between adjacent pyrimidine bases requiring no alkylating agent. The dimers generally result in the formation of cyclobutane type dimers between adjacent thymines, although this type of dimer also forms between adjacent cytosines or between a thymine and an adjacent cytosine, but to a much lesser extent. The dimers themselves are extremely stable and could block normal replication and transcription.
The formation of thymine dimers is not such a problem today as in the early development of life on earth. Today, the ozone in the earth's atmosphere screens out essentially all sunlight wavelengths less than 300 nm., and short wavelengths are the most damaging as the absorption of pyrimidine bases peaks near 260 nm. However, even sunlight in the 300 to 350 nm. range can be damaging, so cells have developed two types of repair mechanisms for the lesions caused by ultraviolet light. The first of these is an excision repair mechanism involving the removal of the mutated bases by an exonuclease, the replacement of these bases by DNA polymerase activity and the backbone of the DNA completed by DNA ligase.

The second, and more common, repair mechanism is that by an enzymatic photoreactivation by a photoreacting enzyme that uses the energy of light to decompose the dimers. The enzyme responsible for this activity in E. Coli. has been purified and has been found to bind specifically to DNA containing cyclobutane type dimers, forming a complex that absorbs maximally near 350 nm. When the complex is irradiated in this wavelength region, the dimers separate into their
corresponding monomers repairing the DNA (Zubay, 1983).

\[ \text{T T} \]
\[ \text{A A} \]

\[ \text{Incision} \]

\[ \text{T T} \]
\[ \text{A A} \]

\[ \text{Excision} \]
\[ (10-100 \text{ base pairs}) \]

\[ \text{A A} \]

\[ \text{DNA repair synthesis} \]

\[ \text{T T} \]

\[ \text{Ligase action} \]

\[ \text{T T} \]

**Figure 3.** Excision repair mechanism for thymine dimers.

\[ \text{T T} \]
\[ \text{A A} \]

\[ \text{Photoreactivating enzyme} \]
\[ + \text{visible light} \]

\[ \text{T T} \]
\[ \text{A A} \]

**Figure 4.** Enzymatic photoreactivation of thymine dimers.

Unrepaired cyclobutane pyrimidine dimers induced in DNA by ultraviolet light are known to have lethal, mutagenic and tumourigenic effects (Setlow, 1966; 1968; Meistrich, 1972; Hart et al, 1977).
It is also likely that these dimers make a significant contribution to the incidence of skin cancer, which results largely from overexposure to solar ultraviolet light (Blum, 1959; Urbach, 1975; Black and Chan, 1977). Most of the carcinogenic effects of solar ultraviolet radiation probably arise from exposure to wavelengths of 295 to 315 nm., since, as previously stated, shorter wavelengths are filtered out by atmospheric ozone and longer wavelength (>310 nm.), despite their higher flux, have less biological effect (Setlow, 1974).

B. Interstrand Crosslinking by Bifunctional Alkylating Agents:

With respect to alkylation by bifunctional alkylating agents, the compounds can be divided into two main categories; those called "direct" because they are reactive with DNA without enzymatic activation, and those that require metabolic activation (Singer and Grunberger, 1983).

1. Psoralens:

A well known group of naturally occurring substances which act as direct alkylating agents are the psoralens, also known as furocoumarins, which exhibit strong photosensitizing properties by irradiation with near ultraviolet light (320 to 380 nm). Many psoralen derivatives have also been synthesized, for example the 4,5',8-trimethylpsoralen, and some of these are the most potent photosensitizing substances in the group (Musajo and Rodighiero, 1970).

The psoralens are used clinically to treat skin pigmentation disorders and to increase tolerance of sensitive skin to light (Cole,
1970). These compounds photoreact directly with biological macromo-
lecules, primarily DNA and to a lesser extent RNA and proteins

Although the mechanism of action of these compounds is not known
for sure, the best evidence to date points to the formation of two
cycloadditions between neighboring pyrimidines on opposite strands.
Reactions carried out with psoralen and several of its derivatives
with unsaturated ketones show photodimerizations and photochemical
adducts of two types. Some of the reactions produced cyclodimers and
cycloadducts via the C3-C4 double bond of the psoralens, whereas
other photoaddition reactions resulted in cycloadducts involving the
C4'-C5' double bonds (Wessely and Kotlan, 1955; Krauch and Farid,
1967). Reactions between pyrimidine bases and psoralens indicated
the formation of cycloadducts of the two with the pyrimidine bases
reacting with their 5,6 double bond while the psoralens react either
with their 3,4 double bond or with their 4',5' double bond (Musajo
and Rodighiero, 1970).

The photochemical reaction of psoralens with DNA involves the
formation of covalent interstrand crosslinks (Lown and Sim, 1978).
In DNA, however, stereochemical considerations and the known distance
of the reaction sites of the psoralens limit the number of geometric configurations which could easily lead to a crosslink. Ronald S. Cole assembled a number of relative configurations, examining between probable reactive sites and found that a psoralen intercalated in the stacked bases of a DNA duplex could overlap two bases to form the observed crosslinks. There were at least two configurations where a psoralen that is intercalated between the stacked bases has its two photochemically reactive positions aligned with the 5,6 double bond of two neighboring pyrimidines on opposite strands.

The base pairs containing the pyrimidines are rotated 24° because of the intercalation rather than the normal 36°. The psoralen reaction sites and the 5,6 pyrimidine double bonds are adjacent and have relative orientations which could lead to the formation of a
crosslinking adduct (Cole, 1970).

The major advantage of these compounds is their ability to penetrate cells and viruses in vivo with no apparent disruption of cellular processes in the absence of photoexitation (Wiesehahn et al, 1977). They show a high specificity for DNA compared with proteins and form stable interstrand crosslinks with a high reaction efficiency without the accompanying degradation of DNA that is found with other crosslinking agents such as mitomycin C (Lown, 1979; Cech and Pardue, 1976; Hatchard and Parker, 1956).

2. Mitomycin C:

The mitomycin antibiotics were first isolated by Hata in Japan from *Streptomyces caespitosus* (Hata et al, 1956). Of these, mitomycin C has been the most thoroughly studied of the group and has been found to be effective against a range of neoplasms. Therefore, mitomycin C is clinically used today for the treatment of carcinomas of the breast, lungs, colon and stomach (Lown, 1979; Szybalski and Iyer, 1967).

![Figure 8. Structure of mitomycin C.](image)

Earlier studies by Szybalski and Iyer (1967) on the mechanism of action established that DNA is the principle target of mitomycin C and predicted three possible reactive sites by which it exhibited its anti-leukemic activity by forming interstrand crosslinks in DNA.
An enzymatic reduction activation produces the hydroquinone which in turn loses methanol to give the active aziridinomitosene.

Mitomycin C has been shown to crosslink DNA (Kirsch, 1967). Although the exact mechanism of action has not, as of yet, been thoroughly worked out, studies by Lown (1979) and Tomasz (1974) have shown that the drug does not alkylate the N7 of guanine, a common reactive site for a variety of alkylating agents (Lawley, 1966). Lown has therefore postulated that the alkylation probably takes place at the O6 of guanine (Lown, 1979).

Besides the formation of interstrand crosslinks with DNA, mitomycin C has also been observed to degrade DNA in vivo (Reich et al, 1960; Wakaki, 1961) which had previously been attributed to the stimulation of exonucleases in the repair cycle (Kersten, 1962; Nakata et al, 1961). However, it has since been demonstrated that the mitomycins actually inhibit nuclease activity (Goodman et al, 1974). Lown (1979), using covalently closed circular DNA and selective enzymatic and chemical inhibitors, proposed another mechanism of
DNA strand scission by mitomycin C in vivo. Through a series of metabolic steps, a hydroxyl radical is formed which has been shown to degrade DNA through a series of reactions involving initial hydrogen extraction from C4 of the ribose (Dizdaroglu et al, 1975). This DNA strand scission is a very important feature, as it is an irreversible event and should prove even more lethal to the cell than interstrand crosslinking.

3. Nitrosoureas:

During the routine screening of compounds for activity against murine leukemia L1210 for the Cancer Chemotherapy National Science Center in 1959, it was found that N-methyl-N'-nitro-N-nitrosoguanidine (MNNg) was somewhat active. This began an investigation of nitrosoureas at the Southern Research Institute and of nitrosoguanidines at the Stanford Research Center (Skinner et al, 1960; Hyde et al, 1962). Soon it was discovered that replacement of the methyl group on the nitrosated N atom for each series of compounds with a 2-haloethyl group gave increased anti-leukemic activity and that the nitrosoureas showed greater activity than the nitrosoguanidines (Wheeler, 1976). Further studies on nitrosoureas led to the conclusion that even greater activity was obtained when the substituent on N1 was a 2-chloroethyl or a 2-fluoroethyl and the substituent on N3 was a 2-chloroethyl, 2-fluoroethyl, cycloaliphatic or a heteroalicyclic group (Johnston et al, 1966, 1971).

Unlike most of the previous anti-cancer agents, it was found that intraperitoneally administered N-methyl-N-nitrosourea (MNU) was active against intracerebrally inocculated L1210 cells (Skipper et
This aspect of the nitrosoureas is important and will be discussed in further detail in a later section.

The three disubstituted nitrosoureas shown (Figure 10) are currently undergoing extensive clinical trials.

Nitrosoureas like BCNU decompose under physiological conditions without enzymatic activation to give rise to isocyanates and 2-halo-ethyl cationic species and the latter alkylate and crosslink DNA to give the observed biological effects (Ludlum and Tong, 1981).

The crosslink occurring in halonitrosourea-treated mammalian cells has not, as yet, been isolated or characterized. It has, however, been suggested to consist of an ethylene bridged crosslink between the O6 of guanine and the N4 of cytosine as shown (Ton et al, 1982).

The proposed mechanism involves a chloroethyl carbonium ion attack on the O6 of guanine to form the O6-chloroethyl substituted
Figure 11. Mode of decomposition of nitrosoureas.

deoxyguanosine. This derivative then rearranges and subsequently alkylates the opposite nucleotide to form the interstrand crosslink.

4. Nitrogen Mustards:

One of the earliest bifunctional alkylating agents studied, of which a substantial body of knowledge is known, is the nitrogen mustards, the prototype of which is mechlorethamine (HN2).

HN2 has been found to produce covalent crosslinks between the paired strands of a DNA duplex (Geiduschek, 1961; Brookes and Lawley, 1961; Kohn et al, 1966), and reacts via an Sn2 type of reaction by
Figure 12. Possible route to formation of crosslink.

\[
\text{CH}_3 \\
\text{Cl-CH}_2\text{-CH}_2\text{-N-CH}_2\text{-CH}_2\text{-Cl}
\]

Figure 13. Structure of mechlorethamine.
forming a transition complex with the nucleophile, with the kinetics of the alkylation therefore being bimolecular (Lawley, 1966).

The two step process involves an initial activation through an anchimeric effect of the nitrogen to yield an aziridinium intermediate. Alkylation by a nucleophile forms the initial covalent link, after which a second activation and alkylation step establishes a 5-atom crosslink to one of the neighboring nucleophilic centers (Henkel, 1983).

The distance over which the crosslink occurs has been estimated (by the use of Dreiding models) using the second activated intermediate, and has been found to be ca. 4.5-5.0 Å away from the alkylated nucleophile on the other arm. Although it is possible for the molecule to assume other conformations in which the reaction distances are less (2.5-3.0 Å), these conformations would be highly disfavored due to the presence of severe gauche interactions (Henkel, 1983).

Brookes and Lawley identified a reaction product of HN2 with DNA consisting of an HN2 moiety bound to two guanine residues and suggested that the interstrand crosslink occurs between two guanines. They later identified the major site of alkylation on the bases on bihelical DNA as being the N7 of guanine (Brookes and Lawley, 1961; Kohn et al, 1966).

Although the exact site of DNA alkylation by HN2 has not been unequivically determined, examinations of space filling models by Kohn (1966) have indicated that an HN2 crosslink could possibly occur between two guanine N7 positions on opposite strands, requiring a slight distortion of the double helix. The resulting crosslink would
(Mechlorethamine)

\[
\begin{align*}
HN_2 \\
(\text{Mechlorethamine}) \\
CH_3
\end{align*}
\]

Figure 14. Two step mechanism of mechlorethamine.

then lie in the major groove of the DNA duplex connecting guanines of adjacent base pairs. The base sequence required for this type of crosslink must therefore be G(3'-5')C, as the sequence C(3'-5')G does
not allow this type of crosslink formation (Kohn et al, 1966). Kohn also found that a large majority of DNA alkylations by HN2 do not result in interstrand crosslink formation, and that only one crosslink was produced per approximately 25 HN2 alkylations. Alkylation without crosslink formation may occur when the initial binding is at a site from which no second alkylation site on the opposite DNA strand can be reached, or when hydrolysis or reaction with other available nucleophiles in the solution intervenes (Kohn et al, 1966).

Reactions of HN2 with DNA have shown a large decrease in the apparent molecular weight of the DNA at in vitro physiological conditions of 37°C and pH=7 (Butler et al, 1952), with the kinetics of this apparent decrease increasing at acidic conditions (Lawley, 1957). It has also been previously shown that HN2 crosslinked DNA is stable in alkaline conditions over a very wide temperature range (Kohn et al, 1966). DNA methylation experiments indicated that N7 methylated guanines on DNA result in the loss of 7-methyl guanine by hydrolysis of the 7-alkyl guanine deoxyriboside linkage leading to guanine ring cleavage at acidic or in vitro physiological conditions (Lawley, 1957). At alkaline pH, N7 methylated guanines undergo base catalyzed ring opening of the guanines with the 7-alkyl guanine deoxyriboside linkage remaining intact (Townsend and Robbins, 1962). These findings account for the acidic and neutral instability and alkaline stability of the HN2 induced crosslinks.

METHODS OF DETECTION

A. Gel Electrophoresis:
Gel electrophoresis of DNAs generally involves the cutting of the DNA with restriction endonucleases and analyzing the fragments to establish an internally consistent set of molecular weights. A horizontal gel system with agarose or polyacrylamide gels is usually used, with the gels at very low concentrations so they do not distort during electrophoresis and, therefore accurate relative mobilities of large DNA's are obtained. Excellent resolution can be gained for DNA's of molecular weights up to at least $26.5 \times 10^5$, a difference of less than 10% being readily resolved even for molecules of this size. In both neutral and alkaline gels, the relative mobilities of DNA's shorter than about 1000 base pairs (or bases) are essentially insensitive to voltage gradients commonly employed (1-5 V/cm.). The larger the DNA, however, the more sensitive the relative mobilities become to the voltage gradient, with DNAs longer than about 20,000 base pairs (or bases) being severely affected. This is most likely due to the distortion of the DNA equilibrium conformations allowing the molecule to enter and penetrate channels in the gel that would normally exclude them in their undisturbed conformations. Thus, low voltage gradients are necessary for the separations of large DNAs (McDonnel et al, 1976).

In the analysis of psoralen crosslinked DNA in neutral and agarose gels (Cech, 1981), it is noted that the electrophoretic mobility of the various extents of crosslinked DNA differ for each gel. In the example shown, electrophoresis of 4,5',8-trimethylpsoralen crosslinked ϕX174 Hae III fragments was conducted on neutral and alkaline agarose gels. The electrophoresis was carried out for 12-14 hours at 100 mA constant current (50 V) and the gels were then
stained with ethidium bromide and illuminated by a 300 nm. radiation source. The alkaline gels were at a sufficient pH (12.4) to ensure denaturation of the DNA.

Figure 15. Electrophoresis of crosslinked DNA in neutral and alkaline agarose gels. Capital letters indicate Hae III fragments. (CO)DNA exposed neither to drug nor radiation. The samples were electrophoresed on (a) a neutral agarose 1.4% agarose gel and (b) an alkaline 1.4% agarose gel. (OR) Origin. Numbers on top indicate time of irradiation in presence of drug (min.).

Electrophoresis, in both neutral and alkaline gels, of DNA mixtures irradiated in the absence of drug and DNA with drug that
were kept in the dark had the same electrophoretic mobilities.

Analysis of the fragments in neutral agarose gel (Figure 15a) show that the electrophoretic mobility of the DNA fragments decreased slowly with increased photoreaction, with the only substantial shift occurring in the sample irradiated for 24 minutes containing about 22 crosslinks per kilobase pair, as determined from radioactivity measurement using $^3$H-trimethylpsoralen. This separation in neutral gel, in which the DNA is maintained in its normal conformation, appears to be based solely on molecular weight, with a sufficient increase needed for a reasonable separation.

Analysis by alkaline gel electrophoresis showed that even the samples that had been photoreacted for a short time in the presence of trimethylpsoralen showed a substantial decrease in mobility. As opposed to the results obtained using neutral agarose gel, under alkaline conditions, the largest shift occurred with the most lightly photoreacted DNA. The difference appears to be attributed to the denaturing conditions of the alkaline gel, in which the increased surface area affects of molecules containing only 1 or 2 crosslinks per molecule, outweigh the molecular weight affects. In this manner, Cech has provided a method of analysis, unlike previous methods, which is more sensitive to lightly crosslinked DNA, specifically to molecules containing 0 to 1 crosslink per molecule (Cech, 1981).

B. Electron Microscopy:

In electron microscopy analysis of crosslinked DNA, the reacted DNA is spread under totally denaturing conditions and the crosslinks are visualized as points of contact of the single strands of DNA. In
this manner, it is important that the DNA concentration is kept very low so that any contact of the single strands can, with probable certainty, be attributed to a crosslinked pair and not a coincidental overlap of unassociated DNA strands.

In the example shown, Hae III fragments of ØX174 RFII DNA were photoreacted with 4,5',8-trimethylpsoralen for various reaction times (Cech, 1981).

The control DNA sample (a) which contained DNA fragments irradiated for 1.5 minutes in the absence of drug, shows only single stranded DNA. The sample photoreacted with drug for 0.5 minutes (b) contained single strands of DNA as well as two strands connected by a crosslink. The sample irradiated for 1.5 minutes in the presence of drug (c) contained denatured regions of varying size, as well as regions having the appearance of normal double-stranded DNA. According to Cech (Cech et al, 1977), the apparent double-stranded regions actually contained denatured regions too small to be visualized. The DNA sample photoreacted in the presence of drug for 24 minutes (d) was almost entirely double-stranded in appearance with visible denatured regions rare (Cech, 1981).

The method is useful in that it gives the location of the crosslinks as well as the number of crosslinks. However, because one is looking at a unit area, it is not a reliable method for quantifying very low extents of crosslinking (i.e. <1 crosslink per molecule) because, in this case, the unit area observed may not be representative of the sample.

C. Ethidium Bromide Fluorescence Assay:
Figure 16. Hae III restriction fragments of ØX174 RFII DNA photoreacted with trimethylpsoralen and spread for electron microscopy under totally denaturing conditions.

Ethidium bromide (3,8-diamino 6-phenyl-5-ethyl phenathridinium) is a trypanocidal dye (Hawking, 1963; Newton, 1964).

It has been shown to interfere with nucleic acid synthesis in
vivo (Newton, 1957; Henderson, 1963), inhibit both DNA polymerase and
the DNA dependent RNA polymerase by binding to the DNA template
(Elliot, 1963; Waring, 1964), as well as showing antiviral properties
(Dickinson et al, 1953; Vilagines and Atanasiu, 1967; Content, 1969).
Previous studies (LePecq et al, 1964; Waring, 1965) have also shown
an interaction of ethidium bromide with double-stranded nucleotides
which is characterized by a marked increase in the fluorescence of
the dye which has been attributed to the removal of the quenching by
the polar solvent as the molecule enters the hydrophobic interior of
the DNA or RNA duplex. This phenomenon will be examined in further
detail, as well as its usefulness as a basis for an analytical
technique for the detection of crosslinked DNA.

Ethidium bromide binds to two different kinds of sites on
polynucleotides, the first of which is known as the intercalative
binding site, or the fluorescent site, and is the most important. It
is this site for which the ethidium bromide has the strongest affini-
ity for double-stranded polynucleotides. The binding of ethidium
bromide to the second site has a higher affinity for single-stranded
rather than double-stranded polynucleotides, especially at very low ionic strengths or in the presence of a large excess of ethidium bromide. The affinity constant for this site drops dramatically for increased ionic strength, and in 0.1 M NaCl, the binding to this site is considered negligible. Similarly, the quantum efficiency of the binding to this second site is negligible compared to the quantum efficiency of ethidium bromide bound to the first site (LePecq, 1971). Because of this and the use of proper controls as well as maintaining solutions at in vitro physiological ionic strengths of 0.15 M NaCl, the binding to the second site can be considered insignificant and we will concentrate on the properties of ethidium bromide bound to the fluorescent site.

When ethidium bromide intercalates, the quantum efficiency of fluorescence is increased 21-fold upon binding to double-stranded DNA and 25-fold upon binding to double-stranded RNA, and the maximum of the absorption spectrum is shifted to longer wavelengths. Therefore, because the fluorescence measured for a given excitation wavelength is proportional to the quantum efficiency and to the extinction coefficient of the compound for the same wavelength, the fluorescence intensity emitted by a bound ethidium bromide molecule can be almost 100 times larger than the fluorescence intensity measured for a free molecule under the same conditions (LePecq, 1971). By exploiting these properties, increased ethidium bromide fluorescence can be used as a delicate probe of duplex DNA concentration.

There is also a preference for ethidium bromide for double-stranded nucleic acids at higher salt concentrations as evidenced by the strong binding of ethidium bromide to poly (A-U) and poly
(I-C) but not to poly U, poly A (neutral pH), poly I, or poly C and only weakly to poly A-2U) and poly (A2I) (LePecq and Paoletti, 1965). Because poly A is believed to consist of considerable local secondary structure due to stacking interactions (Leng and Felsenfeld, 1966), these results indicate that a double-stranded hydrogen bonded structure is required for strong binding and not simply a stacked structure. It is important to note that single-stranded DNA or RNA is able to fold in on itself to form short double-stranded regions, and ethidium bromide is able to bind these structures as well (Studier, 1969; Thomas and Spencer, 1969).

The binding to the double-stranded polynucleotides itself is believed to be by intercalation between the base planes of the double helix. The preferential binding of ethidium bromide to double-stranded nucleic acids, the occurrence of energy transfer from DNA to ethidium bromide (LePecq and Paoletti, 1967), x-ray diffraction studies (Fuller and Waring, 1964), flow dichroism experiments (LePecq and Paoletti, 1967) and the effect on the supercoiling of circular DNA's (Crawford and Waring, 1967; Bauer and Vinograd, 1968; Bujard, 1968) all support this proposal. Further studies using Scatchard plots and fluorometric measurements show one fluorescent binding site per five nucleotides for DNA and one fluorescent binding site per ten nucleotides for RNA (LePecq and Paoletti, 1967).

The intercalation of one ethidium bromide molecule is essentially the same as the addition of one base pair to the double helix, which results in a slight unwinding of the DNA duplex to allow for this addition. Therefore, for covalently closed circular DNA, which does not allow the rotation of one strand around the other, the
unwinding of the DNA to accommodate the ethidium bromide molecule results in a modification of the tertiary turn number because of the imposed topological constraint. This topological constraint therefore limits the amount of ethidium bromide that the covalently closed circular DNA can bind if saturated with ethidium bromide. However, since only one nick on this type of DNA is sufficient to release all the topological constraints, the introduction of one break in such DNA will result in the binding of additional ethidium bromide followed by a characteristic increase in fluorescence (LePecq, 1971).

Having discussed the binding of ethidium bromide to double-stranded nucleotides, it is important to note the effect of various conditions on this binding. As evidenced by the breakage without denaturation of DNA by hydrodynamic shearing, the binding equilibria for ethidium bromide is not affected by nucleic acid molecular weight (LePecq, 1965). Identical Scatchard plots for binding of ethidium bromide were observed for DNAs of varying base composition (LePecq and Paoletti, 1966) indicating no base preference. There are abrupt changes in fluorescence measured at pH values resulting in either acid or alkaline denaturation of DNA (LePecq and Paoletti, 1967), as would be expected, at higher salt concentrations, and increased temperature results in a lowering of the ethidium bromide binding constant (LePecq and Paoletti, 1967).

The fluorescence properties of ethidium bromide have since been applied towards an analytical technique for the quantitative measure of duplex DNA, and have further been expanded to measure the extent of DNA interstrand crosslinking by the following fluorescence assay (Morgan and Pulleyblank, 1974; Lown et al, 1976).
Figure 18. Ethidium bromide fluorescence assay for the detection of crosslinked DNA.

Native, unreacted DNA is exposed to an excess of ethidium bromide to measure the maximum increase in fluorescence. Upon heat denaturation of the duplex DNA at high pH (>11), the fluorescence enhancement falls to zero due to the prevention of renaturation by the high pH (Morgan and Paetkau, 1972). However, if a crosslink is induced by the action of a crosslinking agent, a return of fluorescence is observed after cooling, even under alkaline conditions, since the crosslink serves as a nucleation site for the spontaneous renaturation of the DNA. The observed return of fluorescence is then proportional to the percent of crosslinked DNA (Lown et al, 1976).

Although the procedure is relatively easy and rapid, there are precautions which must be considered. First, the DNA must be saturated with ethidium bromide to achieve maximum fluorescence enhancement to ensure that the total amount of duplex DNA is being measured. Second, one must be careful to not exclude ethidium bromide binding sites by the introduction of extensive crosslinking to again ensure...
that the total amount of duplex DNA is being measured. Third, it must be certain that the fluorescence enhancement from the intercalation of the ethidium bromide falls on the linear region of the spectra to accurately determine the correct amount of duplex DNA (LePecq, 1971).

**INTRODUCTION OF THE DICHLORO-N-SUBSTITUTED AZAADAMANTANES**

Of the bifunctional alkylating agents previously described, only the nitrosoureas show any activity against neoplasia of the central nervous system (i.e. brain tumors). There are a number of factors which make central nervous system tumors one of the most difficult to treat. These include the surgical inaccessibility of the tumors, the critical nature of the host tissue and the existence of the blood brain barrier which restricts the facile penetration of polar species into the brain tissue. The nitrosoureas themselves, although somewhat effective, have inherent problems associated with them. Some of these include cytotoxicity due to nonspecificity of alkylation and a need for increased lipophilicity for increased membrane permeability.

Until recently, no consideration was given to the concept of stereoselective alkylation as a means of increasing antitumor selectivity. Of the crosslinking agents previously studied, after the initial alkylation, the intermediate species was essentially free to react with any nucleophile within a defined radius. It has now been considered that a direct correlation of crosslinking distance with cell cytotoxicity could be obtained if the reaction radius was restricted to a fixed distance, and if rigid alkylating agents of
highly defined stereochemistry and variable physiological properties could be synthesized to not only show a potential increase for antitumor selectivity, but which could also possibly be used as a series of "molecular rulers" to provide an insight into the relationship of crosslinking distance and strict stereochemistry to DNA intermolecular crosslinking and cell cytotoxicity (Henkel, 1983).

With these ideas in mind, Dr. James G. Henkel of the University of Connecticut has synthesized a series of compounds that meet these stringent requirements, to be used as probes to examine the mechanism of action leading to the formation of DNA interstrand crosslinks (Henkel, Faith and Reinhardt, 1983).

![Structures of the N-substituted azaadamantane isomers.](image)

<table>
<thead>
<tr>
<th>Structure</th>
<th>Isomer</th>
<th>Formula</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>4,8-dichloro Azaadamantane</td>
<td>X = Cl; R = CH₃; CH₂CH₂N(C₅H₁₀)</td>
</tr>
<tr>
<td>2</td>
<td>4,9-dichloro Azaadamantane</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>4,10-dichloro Azaadamantane</td>
<td></td>
</tr>
</tbody>
</table>

Figure 19. Structures of the N-substituted azaadamantane isomers.

The N-substituted azaadamantanes have within their structures specific properties which make them theoretically good candidates to overcome the problems previously discussed. A nitrogen mustard moiety is incorporated into the framework of each species giving the
compounds crosslinking potential. The stereochemistry of the alkyla-
ing centers for each is highly defined due to the rigid azaadamant-
tane framework, and after initial alkylation at one of the centers, the activation and subsequent alkylation of the remaining reaction site for generating a crosslink must occur stereoselectively and at a fixed distance. In the case of 1, its reaction sites lie on opposite sides of the molecular framework with a reaction distance of 4.5-5.0 Å, similar to the second activated species of nitrogen mustard. Compounds 2 and 3 have reactions sites separated by about 2.5 Å, similar to the ethylene-like crosslinks of nitrosoureas. For compound 2, the active sites lie on opposite sides of the structure requiring that the stereochemistry of the formation of a crosslink will be "anti". For compound 3, the reaction sites are on the same side of the molecule so that alkylation must occur in a "syn" manner. Finally, due to the incorporation of the large three ring system, an increase in lipophilicity should be observed thereby leading to possible increased membrane permeability for enhancement of central nervous system antitumor activity (Henkel, 1983).

SPECIFIC OBJECTIVES OF RESEARCH

While the biological studies of these compounds with respect to cytotoxicity are currently under investigation at the University of Connecticut, it has been the task of this laboratory to study the in vitro interactions of these compounds with isolated DNA at the molecular level.

The specific objectives for this work were to (1) determine the
physiochemical properties of these compounds in aqueous solution, (2) determine and compare the crosslinking profiles both within and between the two different substituted isomer series and (3) examine the relative stability of the induced intermolecular crosslinks. The results obtained will be used to examine the relationship between crosslinking of duplex DNA at the molecular level with the results of the biological studies being conducted at the University of Connecticut.
CHAPTER I

STABILITY OF AZAADAMANTANE ISOMERS IN AQUEOUS SOLUTION

INTRODUCTION

Time dependent crosslinking studies of the various azaadamantane isomers with CT-DNA (Cabral, 1984; Doty, 1984) were previously performed at constant DNA concentrations, and the percent of crosslinked DNA was measured as a function of reaction time at varying drug concentrations. The results indicated that the 4,8 isomers and the 4,9 isomers of both series of the azaadamantanes do indeed crosslink DNA, whereas the 4,10 isomers of both series do not appear to crosslink DNA to any appreciable extent under the same experimental conditions. Furthermore, the results indicated that the maximum percent of crosslinked CT-DNA was directly related to the initial concentration of crosslinking agent at the time of initiation of the crosslinking reaction.

The reactions were first performed by adding the crystalline form of the crosslinking agent directly into reaction vessels containing the aqueous DNA solutions. However, due to observed time dependent solubilization of the various isomers in solution, an alternative method was developed to initiate the crosslinking reactions under more homogeneous conditions. Subsequently, the crosslinking agents were separately dissolved in aqueous solution prior to initiation of the crosslinking reactions, thereby elimina-
ting the kinetics associated with the solubilization of the various crosslinking compounds. However, although the kinetics of the reactions appeared to be similar for both methods, a noticeable reduction in the overall extent of crosslinking was observed when the drugs were introduced in aqueous form. It was also observed that the reaction profiles further varied for the same crosslinking agent depending upon the amount of time that the agent was allowed to spend in the aqueous solution prior to initiating the crosslinking reactions. Both of these observations would suggest a reduction in the initial concentration of active crosslinking species due to exposure of the drugs to aqueous solution prior to initiation of the crosslinking reactions.

Further experiments performed at the University of Connecticut (Henkel, 1984) have shown a time dependent increase in the electrochemical measurement of chloride ion concentration upon addition of the azaadamantane isomers to aqueous solution. This would suggest a time dependent loss of the azaadamantane chloride substituents to form the expected adamantyl aziridinium ion intermediates which could possibly lead to the formation of inactive crosslinking products due to hydrolysis.

Based on these results, it was deemed necessary to determine the extent to which possible hydrolysis of the intermediates may decrease crosslinking efficiency of the azaadamantane isomers by lowering the effective concentration of active crosslinking agent. Therefore, a novel modification of the ethidium bromide assay procedure was used to quantitatively measure the rate of loss of active crosslinking species as a function of the amount of time that the drugs were
dissolved in aqueous solution prior to exposure to the CT-DNA and initiation of the crosslinking reactions. This measured rate of loss of active crosslinking agent will then be taken into account to more accurately compare the crosslinking efficiencies of the 4,8 and 4,9 azaadamantane isomers both within and between the two N-substituted series.

EXPERIMENTAL

A. Crosslinking agents:

The azaadamantane crosslinking agents were kindly provided by Dr. James Henkel, Department of Medicinal Chemistry, University of Connecticut. The molecular weights of the dichloro-N-methyl azaadamantanes \((C_{10}H_{15}NCl_2:HCl)\) are 256.6 grams per mole. The molecular weight of the 4,9-dichloro-N-piperdinoethyl azaadamantane \((C_{16}H_{26}N_2Cl_2:2HCl:H_2O)\) is 399 grams per mole and the molecular weight of the 4,8-dichloro-N-piperdinoethyl azaadamantane \((C_{16}H_{26}N_2Cl_2:HCl)\) is 390 grams per mole. The molecular weight of mechlorethamine hydrochloride (N-methyl-bis(2-chloroethyl)amine), or HN2, a well established interstrand crosslinking agent, is 192.5 grams per mole. The crosslinking agents were weighed on a Mettler Analytical Microbalance using a microcapsule, and 0.25 to 1 mg. samples of each were used for all reactions.

B. Material Safety Data and Precautions:

The following data on mechlorethamine was taken from the Material Safety Data Sheet obtained from Sigma Chemical Company:
Product Name: Mechlorethamine Hydrochloride

Hazard Data:

Poison

1980 RTECS #IA2100000

Suspected carcinogen

TLV and Source: LD50 = 10 mg/kg oral-rat

Acute Effects of Exposure:

Swallowing - Poison. May be fatal if swallowed.

Skin Absorption - Poison. May be fatal if absorbed through skin. Vesicant.

SCU-Rat LD50 - 1900 ug/kg

Inhalation - Poison. May be fatal if inhaled. Do not breathe dust, vapor, mist or gas.

Eye Contact - Do not get in eyes.

Emergency and First Aid Procedures:

Swallowing - Call a physician immediately.

Exposure to Skin - Wash skin with copious amount of water for at least 15 minutes, followed by 2% sodium thiosulfate. Remove contaminated clothing and shoes. Call a physician.

Inhalation - Move to fresh air. If breathing becomes difficult, call a physician.

Exposure to Eyes - Flush eyes with copious amount of water for at least 15 minutes. Call a physician.
Safety Precautions Taken in Laboratory:

Protective gloves worn.

Eye protection worn.

Long-sleeve laboratory coats worn.

Chemical used in microgram quantities.

Chemical used in mechanically exhausted area.

Chemical stored in tightly sealed containers below 0° C.

C. DNA Preparation:

The DNA used for all experiments was Calf-Thymus DNA (Type 1, polymeric) which was purchased from Sigma (Lot: 525-9585) and has a nucleotide-phosphate extinction coefficient of 6600 1 mole⁻¹ cm at 260 nm. (Sigma). A stock solution was prepared by adding 69.8 mg. of CT-DNA to 66 ml. of 20 mM sodium phosphate at pH=7 containing 0.15 M NaCl and allowing the mixture to stir overnight to dissolve. The absorbance at 260 nm. of the resulting solution was measured on a Perkin-Elmer 552A UV/Vis Spectrophotometer in a 0.2 cm. cell. The stock DNA solution was found to be 2.49 mM in nucleotide-phosphate \( (\ell_{260} = 16.4) \). Aliquots of the solution were then placed in 1.9 ml. polypropylene microcentrifuge tubes and frozen. As needed, samples were allowed to reach ambient temperature, and subsequent dilutions were made with 20 mM sodium phosphate at pH=7 containing 0.15 M NaCl to result in 10 ml. of 0.30 mM nucleotide-phosphate \( (\ell_{260} = 2) \) CT-DNA solutions. Aliquots of the 0.30 mM nucleotide-phosphate CT-DNA were then placed in 500 μl. polypropylene microcentrifuge tubes and frozen. All experiments were performed using the 0.30 mM nucleotide-phosphate \( (\ell_{260} = 2) \) CT-DNA solutions which were thawed as needed.
D. Standard Ethidium Bromide Assay Solution Preparation:

The ethidium bromide (3,8-diamino-6-phenyl-5-ethyl phenanthridinium) was purchased from Sigma (Lot# E-8751) and has a molecular weight of 394.3 grams per mole and an extinction coefficient of 5450 1 mole\(^{-1}\) cm at 480 nm. (Sigma). The standard ethidium bromide assay solution was prepared according to previous literature (Lown et al, 1976) with the modification of 0.2 mM EDTA instead of 0.4 mM EDTA. This solution contained 0.2 mM EDTA and 0.5 \(\mu\)g/ml ethidium bromide in 20 mM sodium phosphate at pH=11.4, resulting in a solution that was 1.27 \(\mu\)M in ethidium bromide. A stock solution was prepared by dissolving 10.5 mg ethidium bromide to a total volume of 100 ml with 20 mM sodium phosphate at pH=11.4 containing 0.2 mM EDTA. The absorbance of this stock solution was measured on a Perkin-Elmer 552A UV/VIS Spectrophotometer in a 0.2 cm cell. The resulting solution was found to have an \(A_{480}=1.48\), corresponding to 0.272 mM ethidium bromide. Appropriate dilutions were made with 20 mM sodium phosphate at pH=11.4 containing 0.2 mM EDTA to result in 1 liter of standard ethidium bromide assay solution at 0.5 \(\mu\)g/ml ethidium bromide. Due to the photosensitivity of ethidium bromide, both solutions were placed in opaque containers and stored at 5°C.

E. Buffer Solutions:

The sodium phosphates used for the buffer solutions were \(\text{Na}_2\text{HPO}_4:12\text{H}_2\text{O}\), molecular weight equals 380.12 grams per mole, purchased from Andor Labs (Lot# 2582) and \(\text{Na}_3\text{PO}_4:12\text{H}_2\text{O}\), molecular weight equals 380.12 grams per mole, pur-
chased from J.T. Baker (Lot# 41-3836). The NaCl used was purchased from J.T. Baker (Lot# 3624-1) and has a molecular weight of 58.4 grams per mole.

F. Measure of DNA Interstrand Crosslinking:

For all experiments, the measure of the extent of DNA interstrand crosslink formation was based on variations of an ethidium bromide fluorescence assay (LePecq, 1971; Morgan and Pulleyblank, 1974; Lown et al, 1976; Lown, 1979). Ethidium bromide binds by intercalation into duplex DNA resulting in an enhancement of fluorescence once it enters the hydrophobic interior of the DNA (Morgan and Pulleyblank, 1967). Upon heat denaturation of the duplex DNA in the presence of ethidium bromide at high alkaline pH (>11), the fluorescence enhancement falls to zero due to the high pH preventing the renaturation of regions of self-complimenarity (Morgan and Pulleyblank, 1972). However, if an interstrand crosslink is induced by the action of a crosslinking agent, a return of fluorescence after heat denaturation and cooling is observed, even under high alkaline conditions, since the crosslink serves as a nucleation site for the spontaneous renaturation of the DNA (Lown et al, 1976). This return of fluorescence is then proportional to the amount of duplex DNA containing at least one interstrand crosslink. Therefore, the return of fluorescence is proportional to the amount of crosslinked DNA. An outline of the procedure is shown in Figure I.1.

G. General Crosslink Assay Procedure:

All reactions were performed in vitro at the physiological
Figure I.1. Protocol for the ethidium bromide fluorescence assay.

conditions of pH=7, 37°C and 0.15 M NaCl. A 0.25 to 1 mg sample of the crosslinking agent contained in a microcapsule was pre-dissolved in 20 mM sodium phosphate at pH=7, containing 0.15 M NaCl prior to the initiation of the crosslink reaction. Reactions were then initiated at t=t₀ by mixing equal volumes of the crosslinking agent and CT-DNA at twice the reactions concentrations to a total volume of 300 to 500 µl. At specific reactions times, 30 µl aliquots of the reactions mixture were quenched in 1 ml of standard ethidium bromide assay solution contained in a 1.9 ml polypropylene microcentrifuge tube.

A control sample of the same CT-DNA used in the crosslinking reaction was prepared by diluting 30 µl of the uncrosslinked CT-DNA solution at twice the reaction concentration with 30 µl of 20 mM sodium phosphate at pH=7 containing 0.15 M NaCl. A 30 µl aliquot of this mixture was similarly added to 1 ml of standard ethidium bromide assay solution contained in a 1.9 ml polypropylene microcentrifuge tube.
The fluorescence of the reactions samples and a control sample was measured on a Perkin-Elmer Fluorescence Spectrophotometer MPF 3L. To ensure that ethidium bromide binding sites were not being excluded by extensive crosslinking and that the crosslinking reactions did not lead to any DNA degradation, the measured fluorescence enhancement of the reactions samples \( F_{100}(X) \) was carefully checked to guarantee that it was equal to the measured fluorescence enhancement of the control sample \( F_{100}(C) \), as shown in Figure I.1. The reactions and control sample vials were then placed in drilled holes in plexiglass plates and simultaneously heat denatured for 2 minutes at 98°C in a Fisher High Temp Bath Model 160 filled with sand. The samples were then quenched on ice for 5 minutes and allowed to reach ambient temperature by placing in a water bath at room temperature for 15 minutes. The fluorescence of the samples was remeasured to measure the extent of fluorescence return of the reaction samples \( F_\Delta(X) \), and to measure the background fluorescence of ethidium bromide in the presence of denatured single-stranded DNA \( F_\Delta(X) \), as indicated by the control sample. The percent of crosslinked DNA was then calculated from the fluorescence data.

H. Fluorescence Measurements:

The fluorescence of all samples was measured on a Perkin-Elmer Fluorescence Spectrophotometer MPF 3L set in ratio mode at ratio setting 2. The sensitivity of the instrument was usually set at 10, with the excitation and emission slits both generally set at 16. All measurements were made using a 0.2 cm cell, with the samples at ambient temperature. Fluorescence measurements were made by setting
the excitation wavelength at 520 nm and scanning the emission spectra from 540 to 640 nm with a 3 nm per second scan rate. The fluorescence measurements were also obtained by measuring the fluorescence of the samples at the maximum emission wavelength of 590 nm.

An external ethidium bromide reference containing 6.8 μM ethidium bromide in 20 mM sodium phosphate at pH=7 was used as a control to note any instrumental and/or experimental fluctuations.

I. Analysis of Fluorescence Data:

The measured fluorescence due to intercalated ethidium bromide is directly proportional to the amount of duplex DNA, and at the high pH of the standard ethidium bromide assay solution (11.4) only crosslinked DNA can return to duplex form after heat denaturation. Therefore, the ratio of ethidium bromide fluorescence after heat denaturation for each of the reactions samples \( F_L(Y) \), to the ethidium bromide fluorescence before heat denaturation for each sample \( F_{100}(X) \) is proportional to the amount of crosslinked DNA. The fluorescence of the denatured, uncrosslinked control sample \( F_A(C) \) is subtracted from the previous values to correct for the background fluorescence due to ethidium bromide in the presence of denatured, single-stranded DNA. For Calf-Thymus (Type 1, polymeric) DNA of unknown molecular weight, the ratio is reported as the percent of crosslinked DNA, and can be calculated using Equation I.1. However, since only one crosslink per DNA duplex is sufficient to induce rapid renaturation upon cooling after heat denaturation (Lown et al, 1976), the actual number of crosslinks per DNA molecule is undetermined. Furthermore, since the DNA segments have an unequal
Equation I.1:

\[
\frac{F_\Delta(X) - F_\Delta(C)}{F_{100}(\lambda) - F_\Delta(C)}
\]

% Crosslinked DNA = 

distribution of molecular weights, determination of the actual number of DNA molecules is impossible.

J. Fluorescence of Bound Ethidium Bromide as a function of Duplex DNA Concentration:

In order to accurately measure the amount of duplex DNA by bound ethidium bromide fluorescence, the ethidium bromide concentration must be sufficient to saturate all the possible DNA intercalation sites (1 site/2 base pairs). To ensure that this condition was met for the reaction samples, various sized aliquots of CT-DNA, at the reaction concentration of 0.15 mM nucleotide-phosphate \( (A_{260} = 1) \), were added to 1 ml of standard ethidium bromide assay solution, and the observed fluorescence was measured as a function of DNA duplex concentration. The tested aliquot sizes ranged from 5 to 40 μl, resulting in solutions that were 3.73 to 28.85 μl in nucleotide-phosphate, respectively. The 5 to 40 μl aliquots were obtained using a Gilson Pipetman P100 micropipette. A plot of the measured fluorescence of bound ethidium bromide as a function of duplex DNA concentration indicated that 1 ml of standard ethidium bromide assay solution is sufficient to saturated all the duplex DNA in aliquots up to 35 μl of the 0.15 mM nucleotide-phosphate \( (A_{260} = 1) \) CT-DNA, corresponding to 25.36 μM in nucleotide-phosphate. It was therefore
decided to quench 30 μl aliquots of the reaction mixtures into 1 ml of standard ethidium bromide assay solution, resulting in a solution that is 21.84 μM in nucleotide-phosphate.

K. Determination of Heat Denaturation Time:

Heat denaturation of all samples was accomplished at 98°C in a Fisher Temp Bath Model 160 filled with sand. In order to maximize denaturation, but minimize DNA degradation due to excessive heating, the amount of time for heat denaturation was determined by adding 30 μl aliquots of CT-DNA, at the reaction concentration of 0.15 mM nucleotide-phosphate (A_{260}=1), to 1 ml aliquots of standard ethidium bromide assay solution and heat denaturing the samples for various amounts of time. The fluorescence of the samples was then measured by setting the excitation wavelength at 520 nm and scanning the emission spectra from 540 to 640 nm with a 3 nm per second scan rate. Results indicated that denaturation times below 2 minutes led to incomplete denaturation, and that denaturation times above 2 minutes led to DNA degradation. Based on these results, a 2 minute denaturation time optimized the required conditions and was used for all of the experiments.

L. Modification of the General Assay Procedure to Determine Stability of Crosslinking Isomers in Aqueous Solution:

A 0.25 to 1 mg sample of crosslinking agent, weighted on a Mettler Analytical Microbalance and contained in a microcapsule, was added to a calculated volume of 20 mM sodium phosphate at pH=7 containing 0.15 M NaCl which would result in an equal volume dilution
with the CT-DNA solution to give the desired drug to nucleotide-phosphate concentration ratio. For all reactions, the CT-DNA concentration was held constant by initiating reactions at 0.30 mM nucleotide-phosphate ($A_{260} = 2$). The desired drug to nucleotide-phosphate concentration ratios were accomplished by adding a pre-weighed sample of the crosslinking agent to a sufficient amount of buffer solution which would result in the desired drug concentration. The crosslinking agent was then allowed to remain in the buffer for a series of time intervals ranging from 30 seconds to 30 minutes. At specific time intervals, 30 µl of the drug solution was added to 30 µl of the 0.30 mM nucleotide-phosphate ($A_{260} = 2$) CT-DNA and the reactions initiated to $t = t_0$. Based on previous reaction profiles (Cabral, 1984; Doty, 1984), the 4,9 N-methyl isomer reactions were allowed to react for 2 minutes, the 4,8 N-methyl isomer for 6 minutes, the 4,8 N-piperidinoethyl isomer for 10 minutes and the 4,9 N-piperdinoethyl isomer for 4 minutes. At these reactions times, 30 µl of the reaction mixtures were quenched in 1 ml of standard ethidium bromide assay solution. All samples were then mixed on a Vortex mixer and centrifuged for 1 minute on a Fisher microcentrifuge. The fluorescence of the samples was measured as previously described, the samples were heat denatured and the fluorescence remeasured. The percent of crosslinked CT-DNA was then plotted as a function of time that the drug spent in aqueous solution prior to initiation of reaction to determine the stability of active crosslinking species in aqueous solution.

RESULTS AND DISCUSSION
Any loss of active crosslinking species for each of the drugs was observed by plotting the measured percent of crosslinked CT-DNA at identical reaction times as a function of the amount of time the crosslinking agents were pre-dissolved in aqueous solution prior to initiation of the crosslinking reaction. Since the reaction times were held constant for each of the crosslinking agents, any decrease in the measured extent of crosslink formation could be attributed to the loss of active crosslinking species due to aqueous solution instability.

As shown in Figure I.2, within experimental error, the measured extent of crosslinking by mechlorethamine after a 10 minute reaction appears unaffected by pre-dissolving times of up to 20 minutes. Because mechlorethamine yields an aziridinium ion intermediate upon exposure to aqueous solution, these results indicate that there is no appreciable reduction in the aziridinium ion concentration due to extended exposure to aqueous solution for up to at least the tested 20 minutes. Therefore, the effective concentration of the active crosslinking aziridinium ion intermediate at the time of initiation of the crosslinking reactions, was independent of the pre-dissolving time and was essentially the same as the calculated initial concentration of mechlorethamine. These results are in agreement with previously published work (Kohn, 1966) indicating the aqueous solution stability of the aziridinium ion intermediate of mechlorethamine.

As shown in Figures I.3 and I.4, in contrast to mechlorethamine, the 4,8 and 4,9 N-methyl series of azaadamantanes do exhibit decreasing extents of crosslink formation at identical reaction times.

The figures represent the dependence of the efficiency of crosslinking of Calf-Thymus DNA (Type 1, polymeric) on the amount of time that the drugs were pre-dissolved in aqueous solution prior to initiation of the crosslinking reactions. Reactions were performed at 37°C in 20 mM sodium phosphate buffer at pH=7, 0.15 M NaCl containing 0.15 mM nucleotide-phosphate. Pre-weighed samples of the crosslinking agents were pre-dissolved in a sufficient amount of buffer to give the desired initial drug to nucleotide-phosphate (D/P) concentration ratio. The D/P ratios used for all of the crosslinking agents except the 4,8 N-methyl azaadamantane were set at D/P=6. The D/P ratio for the 4,8 N-methyl azaadamantane was raised to D/P=8 due to the previously observed lower extents of crosslink formation (Cabral, 1984; Doty, 1984). The drugs were pre-dissolved in aqueous buffer for varying amounts of time prior to initiating the crosslinking reactions. The reactions were then initiated by mixing equal volumes of the drug and CT-DNA solutions at twice the reaction concentrations. For each of the crosslinking agents, the time of reaction was held constant to observe any measured decrease in crosslink formation due to a time dependent loss of active crosslinking species in aqueous solution prior to exposure to the DNA.
Figure 1.2. Solution stability of mechlorethamine.
Figure I.3. Solution stability of the 4,8-dichloro-N-methyl azadazotane.
Figure I.4. Solution stability of the 4,9-dichloro-N-methyl azaadamantane.
as a function of increasing amounts of pre-dissolving time. These results indicate a time dependent decrease in the concentration of active crosslinking species at the time of initiating the crosslinking reactions due to aqueous solution instability of the N-methyl isomers.

In order to compare the rate of loss of active species between the two isomers, a quantitative measure was made from the resulting plots of the measured percent of crosslinked CT-DNA as a function of the aqueous solution lifetime (pre-dissolving time). For each isomer, the resulting plots were extrapolated to the maximum percent of crosslinked CT-DNA occurring at $t=t_0$, and the pre-dissolving time for which half of this maximum value occurred was determined from the plot. This pre-dissolving time indicates the time at which half of the initial concentration of active crosslinking species was present at the time of initiating the crosslinking reaction, and was therefore designated as the half-life in aqueous solution for the corresponding isomer.

Similarly, since the pre-dissolving times used for previous experiments (Cabral, 1984; Doty, 1934) were arbitrarily set at 3 minutes, it was necessary to determine the percent of the initial concentration of active crosslinking species remaining after 3 minutes in aqueous solution. From the plots, the extent of crosslink formation occurring after 3 minute pre-dissolving times was expressed as a percent of the extrapolated maximum extent of crosslinking at $t=t_0$. This percent of the maximum extent of crosslinking is then equal to the percent of the initial concentration of active crosslinking species.
The results of the above calculations for the 4,8 and 4,9 N-methyl azaadamantanes are summarized in Tables I.1 and I.2. As shown, the average half-life in aqueous solution for the 4,8 N-methyl isomer was determined to be 5.40 minutes, with a relative error of 1.85 percent. The average half-life of the 4,9 N-methyl isomer was determined to be 1.76 minutes, with a relative error of 2.27 percent. A comparison of the half-lives indicate that the rate of loss of active species in aqueous solution is slower for the 4,8 isomer than for the 4,9 isomer. As would be expected from these results, the percent of the initial concentration of active crosslinking species remaining after the previously employed 3 minute pre-dissolving times was greater for the 4,8 isomer than for the 4,9 isomer, 70.75 and 30.3 percent respectively.

As shown in Figures I.5 and I.6, the 4,8 and 4,9 N-piperdinoethyl series of azaadamantanes also exhibit decreasing extents of crosslink formation as a function of increasing amounts of pre-dissolving times. Therefore, the half-lives in aqueous solution and the percent of the initial concentration of active crosslinking species remaining after 3 minute pre-dissolving times were calculated as previously described.

The results of the calculations for the 4,8 and 4,9 N-piperdinoethyl azaadamantanes are summarized in Tables I.3 and I.4. As shown, the average half-life in aqueous solution for the 4,8 N-piperdinoethyl isomer was determined to be 13.35 minutes, with a relative error of 0.37 percent. The average half-life for the 4,9 N-piperdinoethyl isomer was determined to be 5.55 minutes, with a relative error of 6.31 percent. A comparison of the half-lives
Tables I.1-I.4. Summarized results of the aqueous solution stabilities of the N-substituted azaadamantanes.

Data from Figures I.3-I.6 representing solution stability dependence of the efficiency of crosslinking by the N-substituted azaadamantanes.
### 4,8 N-METHYL AZAADAMANTANE

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<th>T1/2 (min.)</th>
<th>% Remaining After 3 Min. in Aqueous Solution</th>
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</tbody>
</table>

Table I.1. Solution stability summary for the 4,8-dichloro-N-methyl azaadamantane.
### 4,9 N-METHYL AZAADAMANTANE

<table>
<thead>
<tr>
<th>Trial</th>
<th>T1/2 (min.)</th>
<th>% Remaining After 3 Min. in Aqueous Solution</th>
<th>Min. in Aqueous Solution After Which 70.75% Remains</th>
</tr>
</thead>
<tbody>
<tr>
<td>Trial 1</td>
<td>1.80</td>
<td>32.7</td>
<td>0.70</td>
</tr>
<tr>
<td>Trial 2</td>
<td>1.72</td>
<td>27.9</td>
<td>0.80</td>
</tr>
<tr>
<td>Average</td>
<td>1.76</td>
<td>30.3</td>
<td>0.75</td>
</tr>
<tr>
<td>Ave. Dev.</td>
<td>0.04</td>
<td>2.40</td>
<td>0.05</td>
</tr>
<tr>
<td>Stnd. Dev.</td>
<td>0.06</td>
<td>3.39</td>
<td>0.07</td>
</tr>
<tr>
<td>Rel. Error</td>
<td>2.27%</td>
<td>7.92%</td>
<td>6.67%</td>
</tr>
</tbody>
</table>

* 70.75% corresponds to % remaining of 4,8 N-methyl azaadamantane after 3 min. in aqueous solution.

Table I.2. Solution stability summary for the 4,9-dichloro-N-methyl azaadamantane.
Figure 1.5. Solution stability of the 4,8-dichloro-N-piperdinoethyl azaadamantane.
Figure I.6. Solution stability of the 4,9-dichloro-N-piperdinoethyl azaadamantane.
4,8 N-PIPERIDINETHYL AZAADAMANTANE

<table>
<thead>
<tr>
<th></th>
<th>$T_1/2$ (min.)</th>
<th>% Remaining After 3 Min. in Aqueous Solution</th>
</tr>
</thead>
<tbody>
<tr>
<td>Trial 1</td>
<td>13.4</td>
<td>83.3</td>
</tr>
<tr>
<td>Trial 2</td>
<td>13.3</td>
<td>86.2</td>
</tr>
<tr>
<td>Average</td>
<td>13.35</td>
<td>84.75</td>
</tr>
<tr>
<td>Ave. Dev.</td>
<td>0.05</td>
<td>1.45</td>
</tr>
<tr>
<td>Std. Dev.</td>
<td>0.07</td>
<td>2.05</td>
</tr>
<tr>
<td>Rel. Error</td>
<td>0.37%</td>
<td>1.71%</td>
</tr>
</tbody>
</table>

Table I.3. Solution stability summary for the 4,8-dichloro-N-piperidinoethyl azaadamantane.
### 4,9 N-Piperdinoethyl Azaadamantane

<table>
<thead>
<tr>
<th></th>
<th>Tl/2 (min.)</th>
<th>% Remaining After 3 Min. in Aqueous Solution</th>
<th>*Min. in Aqueous Solution After Which 84.75% Remains</th>
</tr>
</thead>
<tbody>
<tr>
<td>Trial 1</td>
<td>5.20</td>
<td>71.6</td>
<td>1.60</td>
</tr>
<tr>
<td>Trial 2</td>
<td>5.90</td>
<td>74.0</td>
<td>1.70</td>
</tr>
<tr>
<td>Average</td>
<td>5.55</td>
<td>72.8</td>
<td>1.65</td>
</tr>
<tr>
<td>Ave. Dev.</td>
<td>0.35</td>
<td>1.20</td>
<td>0.05</td>
</tr>
<tr>
<td>Std. Dev.</td>
<td>0.49</td>
<td>1.70</td>
<td>0.07</td>
</tr>
<tr>
<td>Rel. Error</td>
<td>6.31%</td>
<td>1.65%</td>
<td>3.03%</td>
</tr>
</tbody>
</table>

* 84.75% corresponds to % remaining of 4,8 N-piperdinoethyl azaadamantane after 3 min. in aqueous solution.

Table I.4. Solution stability summary for the 4,9-dichloro-N-piperdinoethyl azaadamantane.
indicate that, like the N-methyl series, the rate of loss of active
crosslinking species in aqueous solution is slower for the 4,8 isomer
than for the 4,9 isomer. And similarly, the percent of the initial
concentration of active crosslinking species remaining after 3 minute
pre-dissolving times is greater for the 4,8 isomer than for the 4,9
isomer, 84.75 and 72.8 percent, respectively.

From the preceding results, it can be seen that for both the
N-methyl series and the N-piperdinoethyl series of azaadamantanes,
the 4,8 isomers exhibit longer half-lives than do the 4,9 isomers.
Since the only difference between the 4,8 and 4,9 isomers within the
same series is the stereochemistry of the chloride substituents,
these factors must somehow influence the stability of the adamantyl
aziridinium ion intermediate in aqueous solution. At present, the
exact nature of these stereochemical influences is unknown.

In comparisons of the same isomers between the two series, as
shown in Table I.5, it can be seen that the N-piperdinoethyl isomers
exhibit longer half-lives than do the corresponding N-methyl isomers.
These observed differences in the aqueous solution stability for the
same isomers can only be attributed to the differences in the nitro-
gen substituents. Replacement of the methyl group by the
piperdinoethyl moiety appears to help stabilize the adamantyl
aziridinium ion intermediate. Possible factors for this increased
stabilization include electrostatic and/or steric interactions due to
the increased size and nature of the piperdinoethyl substituent. To
date, the exact cause for the increased stabilization is unclear.

SUMMARY AND CONCLUSIONS
<table>
<thead>
<tr>
<th></th>
<th>4,8-METHYL</th>
<th>4,9-METHYL</th>
<th>4,8-PIPERIDINETHYL</th>
<th>4,9-PIPERIDINETHYL</th>
</tr>
</thead>
<tbody>
<tr>
<td>HALF-LIFE (MIN.)</td>
<td>5.40</td>
<td>1.76</td>
<td>13.35</td>
<td>5.55</td>
</tr>
<tr>
<td>RATIO 4,8/4,9</td>
<td>3.07</td>
<td></td>
<td></td>
<td>2.41</td>
</tr>
<tr>
<td>RATIO 4,8-METHYL/4,8-PIPERIDINETHYL</td>
<td></td>
<td>0.404</td>
<td></td>
<td></td>
</tr>
<tr>
<td>RATIO 4,9-METHYL/4,9-PIPERIDINETHYL</td>
<td></td>
<td></td>
<td></td>
<td>0.317</td>
</tr>
</tbody>
</table>

Table I.5. Solution stability summary for the N-substituted azaadamantanes.
It can now be concluded that for the previous experiments (Cabral, 1984; Doty, 1984) the reduction in the measured extent of crosslinking observed for when the crosslinking agents were added to the DNA in aqueous form was due to susceptibility of the adamantyl aziridinium ion intermediates to hydrolysis in aqueous solution as a function of time, as shown in Scheme I. The reduction in the measured extent of crosslink formation would therefore depend on the effective concentration of active crosslinking species at the time of initiation of the crosslinking reaction, which is a function of the rate of loss of active species and the amount of time that the drug was allowed to spend in aqueous solution prior to addition to the DNA.

Scheme I.1. Hydrolysis of the 4,8-dichloro-N-methyl azaadamantane.

Similar to mechlorethamine, the azaadamantanes spontaneously form an aziridinium ion intermediate through an anchimeric effect of the nitrogen. This aziridinium ion is then subject to hydrolysis allowing for the formation of the second aziridinium ion which is
also subject to hydrolysis. A single resulting hydroxyl group would render the compound ineffective as a crosslinking agent since it has been previously shown, for nitrosoureas, that hydroxyl moieties are inadequate leaving groups for allowing alkylation of the DNA nucleic strands (Lawn et al, 1980).

Brookes and Lawley (1961) have previously reported the aqueous solution half-life of mechlorethamine to be approximately 2.2 hours. This increase in aqueous solution stability is probably due to the increased stability of the mechlorethamine aziridinium ion intermediate. The freedom of rotation associated with mechlorethamine allows for the formation of a stable aziridinium ion, whereas the conformational rigidity of the azaadamantane framework increases the distance between the electron donating nitrogen and the electron deficient carbon atom. This increased distance would be expected to decrease the stability of the adamantyl aziridinium ion intermediate allowing for hydrolysis under milder conditions than necessary for the aziridinium ion intermediate of mechloethamine. Therefore, it can be rationalized that the reaction conditions of 37°C, pH=7 and a maximum of 20 minutes in aqueous solution prior to initiation of the crosslinking reactions were insufficient to significantly hydrolyze the aziridinium ion of mechlorethamine, but were adequate to allow hydrolysis of the less stable adamantyl aziridinium ion intermediate. It can further be rationalized that, because of this susceptibility to rapid hydrolysis, the azaadamantane isomers would be of little or no pharmacological significance.

These results also raise some interesting problems. If the aqueous solution stability of the adamantyl aziridinium ion interme-
mediate is increased, one may expect that the crosslinking efficiency would decrease due to possible low reactivity with the nucleic acid bases. Conversely, if the aqueous solution stability of the adamantyl aziridinium ion is decreased, one may expect a faster rate of hydrolysis yielding inactive crosslinking species resulting, again, in a decreased crosslinking efficiency. However, with the azaadamantane isomers, the strict stereochemistries and the fixed distances between alkylating centers must also be taken into consideration. These problems will be the focus of Chapter II.
CHAPTER II

AZAADAMANTANE CROSSLINKING REACTION ISOTHERMS

INTRODUCTION

In order to more accurately compare the crosslinking efficiencies and the initial rates of crosslink formation of the 4,8 and 4,9 azaadamantane isomers both within and between the two N-substituted series, it was necessary to initiate the crosslinking reactions at equivalent concentrations of active crosslinking species. Therefore, the pre-dissolving times for the 4,8 isomers of both series were arbitrarily set at 3 minutes, and the pre-dissolving times for the 4,9 isomers which resulted in the same percentage of remaining active crosslinking species as the 4,8 isomer of the same series was determined from the plots of the measured percent of crosslinked CT-DNA as a function of pre-dissolving time. As shown in Tables I.2 and I.4 in Chapter I for the 4,9 N-methyl and 4,9 N-piperdinoethyl isomers, these pre-dissolving times were determined to be 0.75 and 1.65 minutes, respectively.

In this manner, the drug to nucleotide-phosphate concentration (D/P) ratios are no longer based on the initial weight of the crosslinking agents, but are more accurately based on the concentrations of the active crosslinking species at the time of initiation of the crosslinking reactions. Therefore, any observed differences in the initial rates of crosslink formation by each of the isomers in the
same N-substituted series at identical D/P ratios may be attributed to the different stereochemistries of the two isomers. Similarly, any observed differences in the crosslinking behavior exhibited by the same isomer between the two N-substituted series at the same D/P ratios may be attributed to the effects of the different nitrogen substituents. If, however, the efficiency of crosslinking formation is unaffected by the differing stereochemistries or the varying nitrogen substituents, but only effected by the different rates of hydrolysis both before and during the crosslinking reactions, the greater crosslinking efficiency may be expected for the more hydrolysis resistant isomers. That is, the efficiency of crosslink formation may be expected to be in the order of 4,8 N-piperdinoethyl > 4,9 N-piperdinoethyl > 4,8 N-methyl > 4,9 N-methyl.

EXPERIMENTAL

A. Materials and Methods:

The crosslinking agents, CT-DNA solution, standard ethidium bromide assay solution and the general crosslinking assay procedure were all as outlined in Chapter I.

All reactions were performed in vitro at the physiological conditions of pH=7, 37°C and 0.15 M NaCl. The reaction concentration of the CT-DNA was held constant at 0.15 mM in nucleotide-phosphate, and the extent of crosslinking was measured as a function of time for each isomer at various drug to nucleotide-phosphate concentration ratios. Reactions with mechlorethamine, a well established crosslinking agent, were performed with each azaadamantane crosslinking
reaction to serve as a positive control.

B. Modification of the General Assay Procedure to Determine the Time Dependent Crosslinking Reaction Profiles of the Azaadamantane Isomers:

A 0.25 to 1 mg. sample of the crosslinking agents contained in a microcapsule was added to 0.25 to 20 ml. of 20 mM sodium phosphate at pH=7 containing 0.15 M NaCl which would result in an equal volume dilution with the CT-DNA at 0.30 mM in nucleotide-phosphate to give the desired drug to nucleotide-phosphate concentration ratio at the time of initiating the crosslinking reactions.

In order to account for the different aqueous solution stabilities of the various isomers, it was necessary to establish the actual drug to nucleotide-phosphate (D/P) concentration ratios based on the concentrations of active crosslinking species at the time of initiating the crosslinking reactions, [D(r)]. Therefore, the pre-dissolving times for the 4,8 isomers of both the N-methyl and N-piperdinoethyl series were arbitrarily set at 3 minutes, and the percent of remaining active crosslinking species was determined from the aqueous solution stability plots. The pre-dissolving times for the 4,9 isomers of each series which resulted in the same percent of remaining active crosslinking species as the 4,8 isomer of the same series were also determined from the aqueous solution stability plots. The pre-dissolving times used and the corresponding percent of remaining active crosslinking species for each isomer are summarized in Table II.1. Therefore, by holding the DNA concentration constant at 0.30 mM in nucleotide-phosphate and knowing the percent of remaining
### Table II.1. Pre-dissolving times used and the corresponding percent of remaining active species for each of the azaadamantane isomers.

<table>
<thead>
<tr>
<th></th>
<th>4,8-METHYL</th>
<th>4,9-METHYL</th>
<th>4,8-PIPERIDINETHYL</th>
<th>4,9-PIPERIDINETHYL</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>HALF-LIFE (MIN.)</strong></td>
<td>5.40</td>
<td>1.76</td>
<td>13.35</td>
<td>5.55</td>
</tr>
<tr>
<td><strong>MIN. IN AQUEOUS</strong></td>
<td>3.00</td>
<td>0.75</td>
<td>3.00</td>
<td>1.65</td>
</tr>
<tr>
<td><strong>SOLUTION USED BEFORE</strong></td>
<td>3.00</td>
<td>0.75</td>
<td>3.00</td>
<td>1.65</td>
</tr>
<tr>
<td><strong>INITIATING REACTION</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>% REMAINING AFTER</strong></td>
<td>70.75</td>
<td>70.75</td>
<td>84.75</td>
<td>84.75</td>
</tr>
<tr>
<td><strong>TIME USED</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
active species at the time of initiating the crosslinking reactions and the initial weight of the crosslinking agent, the initial concentration of crosslinking agent, \([D(i)]\), can be calculated using the following equations:

\[
[D(r)] = \frac{D}{P} \cdot [\text{CT-DNA}]
\]

\[
[D(r)] = [D(i)] \times (\text{fraction remaining})
\]

\[
[D(i)] = \frac{[D(r)]}{(\text{fraction remaining})}
\]

An example can be shown for the 4,8 N-methyl isomer at a drug to nucleotide-phosphate concentration ratio of 7.08 using the set pre-dissolving time of 3 minutes which results in 70.75 percent of remaining active crosslinking species as follows:

7.08 mM active drug

\[
[D(r)] = \frac{7.08 \text{ mM}}{1 \text{ mM}} \times 0.30 \text{ mM nucleotide-phosphate} = 2.124 \text{ mM active drug}
\]

\[
[D(i)] = \frac{2.124 \text{ mM active drug}}{0.7075} = 3.002 \text{ mM crosslinking agent}
\]

The volume of buffer in which the crosslinking agent is then pre-dissolved can then be adjusted to give the desired concentration.

Since mechlorethamine is stable in aqueous solution, the pre-dissolving time was arbitrarily set at 3 minutes and the concentration of active crosslinking species was determined from the
initial weight.

After the crosslinking agents were pre-dissolved for the specific amounts of time, an equal volume dilution was made with the CT-DNA and the reactions were initiated at \( t=t_0 \). At various reaction times, 30 \( \mu l \) aliquots of the reaction mixtures were quenched in 1 ml of standard ethidium bromide assay solution contained in a 1.9 ml polypropylene microcentrifuge tube. The samples were then treated as previously described, and the percent of crosslinked DNA was plotted as a function of time.

RESULTS AND DISCUSSION

The measured percent of crosslinked CT-DNA was plotted as a function of the reaction time for each of the azaadamantane isomers at various drug to nucleotide-phosphate concentration ratios. The resulting time dependent crosslinking profiles for each of the drugs were then compared to note the effect of varying the initial concentration of active crosslinking species and the effects of the various stereochemistries and the nitrogen substituents on the efficiency of crosslink formation.

The crosslinking reaction profiles for each of the separate isomers at three different drug to nucleotide-phosphate concentration ratios are shown in Figures II.1-II.4. For each isomer, an increase in the initial concentration of active crosslinking species at the time of initiating the crosslinking reactions results in an increase in the maximum extent and initial rate of crosslink formation. However, the reaction time at which the maximum extent of crosslink
Figures II.1-II.4. Time dependent crosslinking reaction profiles for each of the individual N-substituted azaadamantanes.

The figures represent the time dependent crosslinking of Calf-Thymus DNA (Type 1, polymeric) by each of the individual N-substituted azaadamantanes at three different drug to nucleotide-phosphate concentration ratios. Reactions were performed at 37°C in 20 mM sodium phosphate buffer at pH=7, 0.15 M NaCl containing 0.15 mM in nucleotide-phosphate CT-DNA. Pre-weighed samples of the crosslinking agents were pre-dissolved in a sufficient amount of buffer to give the desired drug to nucleotide-phosphate concentration ratio after the determined pre-dissolving times and taking into account the aqueous solution stability of the azaadamantane isomers. The reactions were then initiated by mixing equal volumes of the drug and CT-DNA solutions at twice the reaction concentrations. The percent of crosslinked DNA was then measured as a function of reaction time.
Figure II.1. Time dependent crosslinking of CT-DNA by the 4,8-dichloro-N-methyl azaadamantane at three drug to nucleotide-phosphate concentration ratios.
Figure II.2. Time dependent crosslinking of CT-DNA by the 4,9-dichloro-N-methyl azaadamantane at three drug to nucleotide-phosphate concentration ratios.
Figure II.3. Time dependent crosslinking of CT-DNA by the 4,8-dichloro-N-piperdinoethyl azaadamantane at three drug to nucleotide-phosphate concentration ratios.
REACTION PROFILES FOR 4,9-PIPERDINOETHYL

Figure II.4. Time dependent crosslinking of CT-DNA by the \( \Delta,9 \)-dichloro-N-piperdinoethyl azaadamantane at three drug to nucleotide-phosphate concentration ratios.
formation is achieved for each isomer is essentially unaffected by
the change in the drug to nucleotide-phosphate concentration ratio,
with the 4,8 N-methyl isomer reaching a maximum extent of crosslink
formation after approximately 8 minutes, the 4,9 N-methyl isomer
after approximately 4 minutes, the 4,8 N-piperdinoethyl isomer after
approximately 27 minutes and the 4,9 N-piperdinoethyl isomer after
approximately 8 minutes.

The reaction profiles for each isomer also show an apparent loss
of DNA crosslinks after reaching the maximum extent of crosslink
formation. This phenomena will be the topic of Chapter III, in which
it will be discussed in greater detail.

From the reaction profiles for each isomer, cross-sectional
plots were made of the percent of crosslinked CT-DNA at specific
reaction times as a function of the drug to nucleotide-phosphate
concentration ratio. As shown in Figures II.5 and II.6 for the 4,9
N-methyl and the 4,9 N-piperdinoethyl isomers, respectively, the
percent of crosslinking appears to be stoichiometric with the drug to
nucleotide-phosphate concentration ratio up to a point after which
the percent of crosslink formation begins to level off due to
saturation of the available DNA binding sites. The drug to
nucleotide-phosphate concentration ratios at which saturation begins
is approximately 2.5 for the 4,9 N-methyl isomer and approximately 3
for the 4,9 N-piperdinoethyl isomer.

Figures II.7 and II.8 indicate the cross-sectional plots of the
percent of crosslinked CT-DNA at specific reaction times as a
function of the drug to nucleotide-phosphate concentration ratio for
the 4,8 N-methyl and the 4,8 N-piperdinoethyl isomers, respectively.
Figures II.5-II.8. Drug to nucleotide-phosphate concentration ratio dependence on the efficiency of crosslinking for each of the N-substituted azaadamantane isomers.

Data from Figures II.1-II.4 representing the time dependent crosslinking of CT-DNA by each of the N-substituted azaadamantanes.
Figure II.5. Percent of crosslinked CT-DNA at specific reaction times as a function of the drug to nucleotide-phosphate concentration ratio for the 4,9-dichloro-N-methyl azaadamantane.
Figure II.6. Percent of crosslinked CT-DNA at specific reaction times as a function of the drug to nucleotide-phosphate concentration ratio for the 4,9-dicloro-N-piperdinoethyl azaadamantane.
Figure II.7. Percent of crosslinked CT-DNA at specific reaction times as a function of the drug to nucleotide-phosphate concentration ratio for the 4,8-dichloro-N-methyl azaadamantane.
Figure II.8. Percent of crosslinked CT-DNA at specific reaction times as a function of the drug to nucleotide-phosphate concentration ratio for the 4,8-dichloro-N-piperdinoethyl azaadamantane.
As opposed to the 4,9 isomers, the percent of crosslink formation for both of the 4,8 isomers appears to be stoichiometric up to a point after which an increase is observed. For the 4,8 N-methyl isomer, the percent of crosslink formation appears to be stoichiometric with the drug to nucleotide-phosphate concentration ratio up to a ratio of about 2.5, after which a large increase is observed until reaching a ratio of approximately 3. A leveling off is then observed, probably due to the saturation of available DNA binding sites. For the 4,8 N-piperdinoethyl isomer, the percent of crosslinking appears stoichiometric with the drug to nucleotide-phosphate concentration ratio up to a ratio of approximately 1.5, after which a leveling off is observed, again, probably due to a saturation of the available DNA binding sites. An increase in the percent of crosslinking is then observed after a ratio of approximately 4.5. These results would suggest a possible cooperative behavior and/or the involvement of more than one binding site for both of the 4,8 isomers. And, since similar results were not observed for either of the 4,9 isomers, this behavior can be attributed to the differences in stereochemistry and/or the differences in the distance between alkylating centers between the 4,8 and 4,9 isomers.

To note the possible effect of the different stereochemistry of the isomers on the efficiency of crosslinking, comparisons were made of the reaction profiles of the 4,8 and 4,9 isomers of the same series at identical starting drug to nucleotide-phosphate concentration ratios. Figures II.9-II.11 show the reaction profiles for the N-methyl isomers and mechlorethamine at three different drug to nucleotide-phosphate concentration ratios. As shown, the 4,9
Figures II.9-II.11. Comparisons of the efficiency of crosslinking by the N-methyl azaadamantanes.

Data from Figures II.1 and II.2 representing the time dependent crosslinking of CT-DNA by the 4,8 and 4,9 N-methyl azaadamantanes, respectively.
Figure II.9. Time dependent crosslinking of CT-DNA by mechlorethamine and the 4,8 and 4,9 N-methyl azaadamantanes at a drug to nucleotide-phosphate concentration ratio of 1.82.
Figure II.10. Time dependent crosslinking of CT-DNA by mechlorethamine and the 4,8 and 4,9 N-methylazaadamantanes at a drug to nucleotide-phosphate concentration ratio of 4.25.
Figure II.11. Time dependent crosslinking of CT-DNA by mechlorethamine and the 4,8 and 4,9 N-methyl azaadamantanes at a drug to nucleotide-phosphate concentration ratio of 7.08.
N-methyl isomer crosslinks to a greater extent than does the 4,8 N-methyl isomer, and both crosslink to a lesser extent than does mechlorethamine. Similarly, the initial rate of crosslink formation is greater for the 4,9 N-methyl isomer than for the 4,8 N-methyl isomer. However, the distinct loss of DNA crosslinks occurs sooner for the 4,9 N-methyl isomer than for the 4,8 N-methyl isomer, with this loss occurring for both isomers much sooner than for mechlorethamine. Figures II.12-II.14 show the reaction profiles for mechlorethamine and the 4,8 and 4,9 N-piperdinoethyl isomers at three identical starting drug to nucleotide-phosphate concentration ratios. As shown, the 4,9 N-piperdinoethyl isomer crosslinks to a greater extent than does the 4,8 N-piperdinoethyl isomer, and both isomers crosslink to a lesser extent than does mechlorethamine. The 4,9 N-piperdinoethyl isomer also crosslinks at a higher initial rate than does the 4,8 N-piperdinoethyl isomer, and begins to show a loss of crosslinks sooner than does the 4,8 N-piperdinoethyl isomer. Both of the N-piperdinoethyl isomers exhibit a loss of crosslinks sooner than does mechlorethamine.

In order to note the possible effect of varying the nitrogen substituent on the efficiency of crosslinking, comparisons were made of the reaction profiles of the same isomers between the two series at identical starting drug to nucleotide-phosphate concentration ratios. As shown in Figures II.15 and II.16 for the N-methyl and N-piperdinoethyl 4,8 isomers, the 4,8 N-piperdinoethyl isomer crosslinks to a greater extent than does the 4,8 N-methyl isomer, and the 4,8 N-methyl isomer shows a loss of crosslinks sooner than does the 4,8 N-piperdinoethyl isomer. Similarly, as shown in Figures

Data from Figures II.3 and II.4 representing the time dependent crosslinking of CT-DNA by the 4,8 and 4,9 N-piperdinoethyl azaadamantanes, respectively.
Figure II.12. Time dependent crosslinking of CT-DNA by mechlorethamine and the 4,8 and 4,9 N-piperdinoethyl azaadamantanes at a drug to nucleotide-phosphate concentration ratio of 1.82.
Figure II.13. Time dependent crosslinking of CT-DNA by mechlorethamine and the 4,8 and 4,9 N-piperdinoethyl azaadamantanes at a drug to nucleotide-phosphate concentration ratio of 4.25.
Figure II.14. Time dependent crosslinking of CT-DNA by mechlorethamine and the 4,8 and 4,9 N-piperdinoethyl azaadamantanes at a drug to nucleotide-phosphate concentration ratio of 7.08.
Figures II.15 and II.16. Comparisons of the efficiency of cross-linking by the 4,8 N-methyl and N-piperdinoethyl azaadamantanes.

Data from Figures II.1 and II.3 representing the time dependent crosslinking of CT-DNA by the 4,8 N-methyl and 4,8 N-piperdinoethyl azaadamantanes, respectively.
Figure II.15. Time dependent crosslinking of CT-DNA by the 4,8 N-methyl and 4,8 N-piperdinoethyl azaadamantanes at a drug to nucleotide-phosphate concentration ratio of 1.82.
Figure II.16. Time dependent crosslinking of CT-DNA by the 4,8 N-methyl and 4,8 N-piperdinoethyl azaadamantanes at a drug to nucleotide-phosphate concentration ratio of 7.08.
II.17 and II.18 for the N-methyl and N-piperdinoethyl 4,9 isomers, the 4,9 N-piperdinoethyl isomer crosslinks to a greater extent than does the 4,9 N-methyl isomer, and the 4,9 N-methyl isomer exhibits a loss of crosslinks sooner than does the 4,9 N-piperdinoethyl isomer.

SUMMARY AND CONCLUSIONS

The maximum extent of crosslinking, the reaction time at which this maximum extent occurs and the initial rate of crosslink formation for each of the azaadamantane isomers studied at the various drug to nucleotide-phosphate concentration ratios used are summarized in Tables II.1 and II.2 for the N-methyl and N-piperdinoethyl series of isomers, respectively.

As shown, for both the N-methyl and N-piperdinoethyl series of azaadamantanes, all of the compounds crosslinked to a lesser extent than did mechloretamine. This decreased efficiency of crosslink formation may be due to the decreased aqueous solution stability of the azaadamantanes leading to the formation of inactive crosslinking species before interaction with the available DNA, to possible steric hinderances due to the bulk of the adamantyl framework, or to possible decreased stability of the induced crosslinks themselves as a result of increased strain on the crosslinking bonds due to the fixed distance between alkylating centers for the azaadamantanes as compared to the relative freedom of rotation allowed for mechloretamine to form more stable crosslinks.

Additionally, there are differences in crosslinking behavior for both between and within the two N-substituted series of the
Figures II.17 and II.18. Comparison of the efficiency of cross-linking by the 4,9 N-methyl and 4,9 N-piperdinoethyl azaadamantanes.

Data from Figures II.2 and II.4 representing the time dependent crosslinking of CT-DNA by the 4,9 N-methyl and 4,9 N-piperdinoethyl azaadamantanes, respectively.
Figure II.17. Time dependent crosslinking of CT-DNA by the 4,9 N-methyl and 4,9 N-piperidinoethyl azaadamantanes at a drug to nucleotide-phosphate concentration ratio of 1.82.
Figure II.18. Time dependent crosslinking of CT-DNA by the 4,9 N-methyl and 4,9 N-piperdinoethyl azaadamantanes at a drug to nucleotide-phosphate concentration ratio of 7.08.
Table II.2. Time dependent crosslinking summary for the N-methyl azaadamantane isomers.
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<td>7.7</td>
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<td>65</td>
<td>48.0</td>
</tr>
</tbody>
</table>

Table II.3 Time dependent crosslinking summary for the N-piperdioethyl azaadamantane isomers.
azaadamantine isomers. That is, at identical starting drug to nucleotide-phosphate concentration ratios, the 4,9 isomers crosslink to a greater extent than do their corresponding 4,8 isomers within the same series and the 4,8 and 4,9 N-piperdinoethyl isomers crosslink to greater extents than do the 4,8 and 4,9 N-methyl isomers, respectively.

The increased crosslinking by the 4,9 isomers within the same series is in contrast as to what may be expected from the aqueous solution stability results, since the 4,9 isomers of both series are less stable than the corresponding 4,8 isomers of the same series suggesting that the 4,9 isomers may undergo more rapid hydrolysis than the 4,8 isomers to yield inactive crosslinking species. However, these results may suggest that the decreased aqueous solution stability of the 4,9 isomers could lead to increasingly reactive intermediates followed by increased DNA crosslink formation. More simply, these results may be directly due to the intrinsic stereochemical differences between the two isomers and have very little to do with the differences in the aqueous solution stabilities between the two isomers within the same series.

The increased crosslinking by the 4,8 and 4,9 N-piperdinoethyl isomers as compared to the 4,8 and 4,9 N-methyl isomers, respectively, may be due to some intrinsic differences in the two different nitrogen substituent interactions with the DNA. This would seem most likely, since unlike the case of the less stable 4,9 isomers crosslinking to greater extents than the corresponding more stable 4,8 isomers within the same series, the more stable N-piperdinoethyl isomers crosslink to greater extents than do the
corresponding N-methyl isomers. Similarly, since the 4,8 N-methyl isomer and the 4,9 N-piperdinoethyl isomer have similar aqueous solution half-lives of 5.4 minutes and 5.55 minutes, respectively, the increased crosslinking by the 4,9 N-piperdinoethyl isomer would indicate that the stereochemical and/or nitrogen substituent interactions, as well as the possible aqueous solution stability factors, must be taken into consideration.
CHAPTER III

STABILITY OF AZAADAMANTANE INDUCED CROSSLINKS

INTRODUCTION

Figures II.1, II.2 and II.4 of Chapter II show a significant loss of existing crosslinks at extended reaction times for the 4,8 N-methyl, 4,9 N-methyl and 4,9 N-piperdinoethyl azaadamantane isomers, respectively. As shown in Figures III.1 and III.2, a similar loss of existing crosslinks is also seen for the 4,8 N-piperdinoethyl isomer as well as for mechlorethamine, which exhibits this loss of existing crosslinks at much longer reaction times than those for the azaadamantane isomers.

There are a number of known mechanisms of action which could possibly lead to this observed loss of existing DNA interstrand crosslinks. A number of known DNA crosslinking agents show a loss of crosslinks as a result of DNA strand breakage. Treatment of DNA with 1-(2-chloroethyl)-1-nitrosoureas and certain aryltriazenes, for example, have led to DNA strand scission due to alkylation of the DNA phosphates, rendering the resulting phosphotriester susceptible to rapid basic hydrolysis leading to DNA strand breakage (Lown, 1982). Similarly, a slower DNA strand scission by other alkylating agents as a result of base alkylation followed by depurination or depyrimidination and subsequent facile base hydrolysis (Lown, 1982). Agents such as dimethyl sulfate, which are known to preferentially alkylate
Figures III.1 and III.2. Time dependent crosslinking reaction profiles for the 4,8 N-piperdinoethyl azaadamantane and mechlo-ethamine at extended reaction times.

The figures represent the time dependent crosslinking of Calf-Thymus DNA (Type 1, polymeric) by the 4,8 N-piperdinoethyl azaadamantane isomer and mechloethamine at drug to nucleotide-phosphate concentration ratios of 4.25 and 1, respectively. The reactions were performed as described in Chapter II.
Figure III.1. Time dependent crosslinking of CT-DNA by the 4,8-dichloro-N-piperdinoethyl azaadamantane at a drug to nucleotide-phosphate concentration ratio of 4.25.
Figure III.2. Time dependent crosslinking of CT-DNA by mechlorethamine at a drug to nucleotide-phosphate concentration ratio of 1.
at the N7 of guanine have been shown to render the alkylated DNA unstable at neutral pH and 37°C yielding 7-alkyguanines due to acid catalyzed hydrolysis followed by DNA strand scission (Lawley, 1966).

Studies with mechlorethamine (HN2) have indicated that the agent preferentially reacts with the N7 of guanines to form the resulting DNA interstrand crosslinks (Kallama and Hemminki, 1984). As with dimethyl sulfate, evidence indicates that the guanine N7 alkylations by HN2 also result in depurination followed by DNA strand breakage at neutral pH, with the rate of depurination increasing with increased temperature (Brookes and Lawley, 1961). However, under alkaline conditions, N7 guanine alkylations have been shown to be stable due to alkaline induced imidazole ring opening of the alkylated guanines rather than depurination (Lown, 1982).

Since the sites of alkylation for the azaadamantane isomers are presently unknown, it is possible that the induced azaadamantane crosslinks are unstable at the high alkaline pH of the ethidium bromide assay solution (11.4). Similarly, since the crosslinked DNA is heat denatured at this high alkaline pH, it is also possible that the azaadamantane, as well as the HN2, induced crosslinks are heat liable in alkali. Therefore, it was deemed necessary to test for the possible heat liability of the induced crosslinks at the high alkaline pH of the standard ethidium bromide assay solution.

The heat liability of the azaadamantane and mechlorethamine induced crosslinks at the high alkaline pH of the standard ethidium bromide assay solution (11.4) will be tested for by using a modification of the general assay procedure. The crosslinked DNA will be exposed to dilute NaOH in excess of 0.05 N, which will denature the
duplex structure of the DNA. The crosslinked DNA will then be renatured by neutralization of the excess hydroxide by acid. This method thereby eliminates the need for exposing the crosslinked DNA to heat for denaturation which could effect heat liable crosslinks. Identical reaction samples will also be heat denatured to allow for a direct comparison of the resulting crosslinking reaction profiles.

EXPERIMENTAL

A. Materials and Methods:

The crosslinking agents, CT-DNA solution, standard ethidium bromide assay solution and the general crosslink assay procedure were all as outlined in Chapter I.

All reactions were performed in vitro at the physiological conditions of pH=7, 37°C and 0.15 M NaCl. The reaction concentration of the CT-DNA was held constant at 0.15 mM in nucleotide-phosphate, and the extent of crosslinking was measured as a function of time for the 4,8 N-methyl isomer and mechlorethamine.

B. Determination of Alkaline Denaturation Conditions:

In order to insure that the alkaline denaturation step was resulting in complete denaturation of the CT-DNA, a 30 µl aliquot of the CT-DNA at the reaction concentration of 0.15 mM in nucleotide-phosphate was added to 1 ml of standard ethidium bromide assay solution, and various amounts of 0.197 N NaOH were added for varying amounts of time. The fluorescence of the samples was then compared to the fluorescence of a sample, prepared as above, which underwent
normal heat denaturation. Results indicated that exposure of the CT-DNA to a 150 μl addition of the 0.197 N NaOH for 1 minute was sufficient for complete denaturation. Further testing indicated that a 135 μl addition of 0.197 HCl was sufficient to return the reaction sample to the pH of the standard ethidium bromide assay solution (11.4). Therefore, reaction samples will be exposed to 150 μl additions of 0.197 N NaOH and allowed to stand for 1 minute, after which a 135 μl addition of 0.197 N HCl will be used to return the samples to the pH of the standard ethidium bromide assay solution, thereby stopping the denaturation process.

C. Determination of the Extent of DNA Degradation Due to Hydrolysis:

Since the modification used will employ alkaline denaturation of the crosslinked DNA, it was necessary to determine the extent of DNA degradation due to alkaline hydrolysis. Therefore, a crosslinking reaction with mechlorethamine at a drug to nucleotide-phosphate concentration ratio of 1 was performed as described in Chapter II. The reaction was allowed to continue until, according to previous results, the maximum extent of crosslinking was achieved, approximately 90 minutes. Two 30 μl aliquots of the reaction mixture were then separately added to 1 ml of standard ethidium bromide assay solution. The extent of crosslinking was then measured by employing the normal heat denaturation method for one sample and the alkaline denaturation method for the other sample. Results indicated that approximately 15 percent of the DNA present in the alkaline denatured sample was lost due to alkaline hydrolysis.

A 0.25 to 1 mg. sample of the crosslinking agents contained in a microcapsule was added to 0.25 to 20 ml of 20 mM sodium phosphate at pH=7 containing 0.15 M NaCl which would result in an equal volume dilution with the CT-DNA at 0.30 mM in nucleotide-phosphate to give the desired drug to nucleotide-phosphate concentration ratio. Reactions were performed for mechlorethamine and the 4,8 N-methyl isomer at drug to nucleotide-phosphate concentration ratios of 2 and 10, respectively. It is important to note that the drug to nucleotide-phosphate concentration ratio used for the 4,8 N-methylazaadamantane was not corrected for the aqueous solution stability, but was based on the initial weight of the crosslinking agent. The crosslinking agents were then pre-dissolved in the calculated volume of buffer for 3 minutes, after which the equal volume dilution with 0.30 mM nucleotide-phosphate CT-DNA was made, and the reactions initiated at t=t_0. At specific reaction times, two 30 μl aliquots of the reaction mixture were separately added to 1 ml of standard ethidium bromide assay solution. One of the duplicate samples for each of the reaction times was assayed by the normal heat denaturation technique as described in Chapters I and II.

The other set of duplicate samples was treated in the same manner except for substitution of the heat denaturation technique with the alkaline denaturation modification. Each of the samples was exposed to a 150 μl addition of 0.197 N NaOH and allowed to stand for 1 minute, after which a 135 μl addition of 0.197 N HCl was made
to return the pH to the pH of the standard ethidium bromide assay solution.

Because of the dilution factors involved with the alkaline denatured samples, it was necessary to provide separate control samples to measure both the maximum expected fluorescence and the fluorescence of the denatured, uncrosslinked DNA. Therefore, 30 μl of 0.15 mM nucleotide-phosphate CT-DNA was added to 1 ml of standard ethidium bromide assay solution and exposed to a 150 μl addition of 0.197 N NaOH for 1 minute, after which 135 μl of 0.197 N HCl was added to serve as a measure of the background fluorescence of ethidium bromide in the presence of single-stranded DNA. Similarly, 150 μl of NaOH and 135 μl of HCl were added to 1 ml of standard ethidium bromide assay solution, after which 30 μl of 0.15 mM nucleotide-phosphate CT-DNA was added to serve as a measure of the maximum expected fluorescence.

The percent of crosslinked DNA was then determined for each of the series of samples, as previously described, and plotted as a function of the reaction time.

RESULTS AND DISCUSSION

For both the 4,8 N-methyl and mechlorethamine reactions, a 15 percent decrease in the maximum extent of crosslinking was observed as determined by the heat denatured samples. This decrease was attributed to the loss of 15 percent of the total DNA as a result of alkaline hydrolysis and was therefore corrected for by adding 15 percent to each of the measured percent of crosslinked DNA values
obtained by the alkaline denaturation technique.

As shown in Figure III.3 and III.4 for mechlorethamine and the 4,8 N-methyl isomer, respectively, after correction for the 15 percent loss of DNA due to alkaline hydrolysis, there appears to be no difference, within experimental error, in the measured extent of crosslinking between the two methods. More importantly, both of the methods employed show a loss of existing crosslinks for both of the crosslinking agents at extended reaction times. These results would suggest that the loss of the existing DNA interstrand crosslinks previously observed by the heat denaturation technique are not the result of heat liability of the induced crosslinks at the high alkaline pH of the standard ethidium bromide assay solution (II.4).

SUMMARY AND CONCLUSIONS

As previously stated, the results indicate that the loss of the induced DNA interstrand crosslinks by mechlorethamine and the 4,8 N-methyl isomer are not the result of heat liability of the crosslinks at the high alkaline pH of the standard ethidium bromide assay solution. However, due to the intrinsic differences between the 4,8 and 4,9 azaadamantane isomers, it would be expected that the two isomers would alkylate at different specific sites on the DNA molecule. This would suggest that the two crosslinks by the two isomers might have different characteristic properties and, even though the 4,8 N-methyl isomer induced crosslinks are not heat liable at the high alkaline pH of the standard ethidium bromide assay solution, the induced crosslinks by the 4,9 isomers may be.
Figures 3 and 4. Time Dependent Crosslinking Reaction Profiles of Mechlorethamine and the 4,8 N-methyl Azaadamantane Isomer Using Heat and Alkaline Denaturation.

The figures represent the time dependent crosslinking of Calf-Thymus DNA (Type 1, polymeric) by mechlorethamine and the 4,8 N-methyl azaadamantane isomer at the drug to nucleotide-phosphate concentration ratios of 2 and 10, respectively. Reactions were performed at 37°C in 20 mM sodium phosphate buffer at pH=7, 0.15 M NaCl containing 0.15 mM nucleotide-phosphate CT-DNA. Pre-weighed samples of the crosslinking agents were predissolved in a sufficient volume of buffer which would give the desired drug to nucleotide-phosphate concentration ratios after an equal volume dilution with the CT-DNA. The drug to nucleotide-phosphate concentration ratio used for the 4,8 N-methyl azaadamantane was uncorrected for the aqueous solution instability, and was based on the initial weight of the crosslinking agent. The reactions were then initiated by mixing equal volumes of the drug and CT-DNA solutions at twice the reaction concentrations. At specific reaction times, two identical 30 ul. samples of the reaction mixture were separately added to 1 ml. of standard ethidium bromide assay solution. The percent of crosslinked DNA by the heat denaturation technique was determined as previously described in Chapters I and II. The alkaline denatured samples were denatured by exposing the crosslinked DNA in the assay solution to 150 ul. of 0.197 N NaOH for 1 minute, after which the denaturation was stopped by the addition of 135 ul. of 0.197 N HCL to bring the pH back to
the pH of the standard ethidium bromide assay solution. The percent of crosslinked DNA was then measured as a function of the reaction time.
Figure 3. Time Dependent crosslinking of CT-DNA by mechlorethamine at a drug to nucleotide-phosphate concentration ratio of 2 using the heat denaturation and alkaline denaturation techniques.
Figure 4. Time dependent crosslinking of CT-DNA by the 4,8-dichloro-N-methylazaadamantane at a drug to nucleotide-phosphate concentration ratio of 10 using the heat denaturation and alkaline denaturation techniques.
Furthermore, since there appears to be no significant differences in the resulting time dependent crosslinking reaction isotherms, and assuming that the induced crosslinks by the 4,9 isomers are not heat liable at the high alkaline pH of the standard ethidium bromide assay solution, it can be assumed that the time dependent crosslinking reaction isotherms obtained by the original heat denaturation technique are correct.

In the case of mechlorethamine, both methods indicate a half-life for the loss of existing crosslinks of approximately 8 hours. These results are in good agreement with previously published results indicating the half-life to be approximately 7 hours (Ross, Ewig and Kohn, 1978). These previously published results were obtained by exposure of mechlorethamine to L1210 cells and the loss of existing crosslinks was attributed to enzymatic repair in vivo. However, the in vitro loss of existing crosslinks in the absence of additional cellular enzymes observed here suggest that the loss of existing crosslinks previously observed was not due to enzymatic repair in vivo, but rather due to the intrinsic instability of the mechlorethamine induced crosslinks themselves.

The increased stability of the mechlorethamine induced crosslinks over the crosslinks induced by the 4,8 N-methyl isomer, as well as the other azaadamantane isomers, may be due to a number of possible reasons. Because of the freedom of rotation of the mechlorethamine bonds as compared to the strict rigidity and the fixed distance between alkylating centers of the azaadamantanes, it is possible that the covalent crosslinking bonds formed by mechlorethamine are less strained than those created by the azaadamantane isomers, thereby
increasing the stability of the formed interstrand crosslinks. It is also possible that the large bulk of the adamantyl framework results in some steric hinderance, again decreasing the stability of the induced covalent crosslinking bonds.

The exact cause of the observed loss of existing DNA interstrand crosslinks for the 4,8 N-methyl isomer, as well as the other azaadamantane isomers, is, as yet, undetermined. A similar adamantyl compound, N-(2-chloroethyl)-N'-(2-adamantyl)-N-nitrosourea has been shown to crosslink DNA and induce depurination leading to DNA strand scission (Lown et al, 1978), and it may seem logical to assume that the azaadamantane isomers studied here similarly lead to depurination. It remains to be determined directly, however, whether the observed loss of the azaadamantane induced DNA crosslinks are indeed the direct result of depurination or depyrimidination followed by strand scission, induced strand scission without depurination or depyrimidination, or simply a loss of the existing crosslinks without damage to the DNA. To distinguish between these possible causes, more research must be done to try to determine the exact site of DNA alkylation for each of the azaadamantane isomers and to determine the effects of this alkylation on the DNA molecule itself.
LIST OF TABLES

CHAPTER I. STABILITY OF AZAADAMANTANE ISOMERS IN AQUEOUS SOLUTION

Table I.1. Solution stability summary for the 4,8-
   dichloro-N-methyl azaadamantane.........................5?
Table I.2. Solution stability summary for the 4,9-
   dichloro-N-methyl azaadamantane.........................5^4
Table I.3. Solution stability summary for the 4,8-
   dichloro-N-piperdinoethyl azaadamantane...............5^7
Table I.4. Solution stability summary for the 4,9-
   dichloro-N-piperdinoethyl azaadamantane...............5^8
Table I.5. Solution stability summary for the
   N-substituted azaadamantanes............................60

CHAPTER II. AZAADAMANTANE CROSSLINKING REACTION ISOTHERMS

Table II.1. Pre-dissolving times used and the corresponding
   percent of remaining active species for each of
   the azaadamantane isomers...............................67
Table II.2. Time dependent crosslinking summary for the
   N-methyl azaadamantane isomers.........................9^6
Table II.3. Time dependent crosslinking summary for the
   N-piperdinoethyl azaadamantane isomers.................9^0
REFERENCES


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