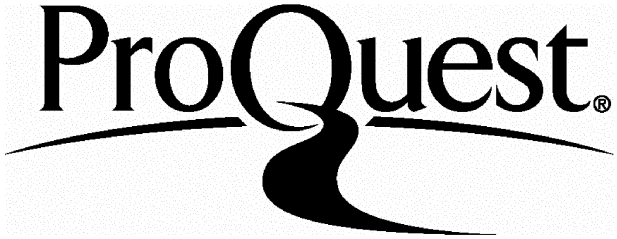
In Vitro DNA Cross-Linking and Sequence Specificity of Bifunctional Alkylating Agents

Submitted by Mark D. Berardini for the PhD degree at University College London



ABSTRACT

The development and implementation of a simple, sensitive and reliable assay to measure the formation of interstrand cross-links induced in isolated DNA by bifunctional alkylating agents is described. A number of compounds were evaluated for their ability to induce interstrand cross-links in DNA including the nitrogen mustard class of alkylating agents, several structurally related analogues of the bioreductive aziridinylbenzoquinone AZQ (diaziquone), and several C8-linked pyrrolobenzodiazepine dimers. The assay involves the reaction of end-labeled linear plasmid DNA with cross-linking agents and the subsequent separation and detection of cross-linked DNA from uncross-linked DNA using agarose gel electrophoreses. The technique compares the extent and rate of cross-link formation of different classes of bifunctional agents and can be extended to measure the conversion of DNA monoadducts to cross-links.

In the second part of this study, the nucleotide sequence preferences for the formation of interstrand cross-links induced in DNA by nitrogen mustard, 2,5-diaziridinyl-1,4-benzoquinone (DZQ) and 3,6-dimethyl DZQ (MeDZQ) were examined using synthetic duplex oligonucleotides and denaturing polyacrylamide gel electrophoresis (PAGE). Nitrogen mustard was found to preferentially cross-link DNA in a 5'-GNC sequence using a panel of oligonucleotides containing a single potential cross-linking site. Reaction of DZQ and MeDZQ with DNA duplexes containing several potential cross-linking sites resulted in the formation of cross-linked DNAs with different electrophoretic mobilities. Analysis of the principal cross-linked products by piperidine fragmentation revealed that the preferential site of

cross-linking was altered from a 5'-GNC to a 5'-GC sequence upon reduction of DZQ to the hydroquinone form by the enzyme DT-Diaphorase. In contrast, the reduced form of MeDZQ was found to preferentially cross-link at 5'GNC sites within the same sequence. Additional minor cross-linked products were characterised and revealed that unreduced DZQ and reduced MeDZQ are both capable of cross-linking across four base pairs in a 5'-GNNC sequence.

INDEX

LIST OF FIGURES AND TABLES

Chapter 2

Tables **Page**

ABBREVIATIONS

ATP	Adenosine triphosphate
AZQ	2,5-diaziridinyl-3,6-bis(carboxyamino)-1,4-benzoquinone
BAP	Bacterial alkaline phosphatase
BRL	Bethesda Research Laboratories
BSA	Bovine serum albumin
BZQ	2,5-diaziridinyl-3,6-bis[(2-hydroxyethyl)amino]-1,4-benzoquinone
DMSO	Dimethylsulphoxide
DTT	Dithiothreitol
DTD	DT-Diaphorase
DZQ	2,5-diaziridinyl-1,4-benzoquinone
EDTA	Ethylenediamine tetra-acetic acid
HN2	Mechlorethamine (nitrogen mustard)
MeDZQ	3,6-dimethyl-2,5-diaziridinyl-1,4-benzoquinone
M	Molar
mM	Millimolar
NADH	Nicatinamide adenine dinucleotide (reduced form)
NBL	Northumbria Biologicals Ltd.
NMR	Nuclear magnetic resonance
ng	Nanogram
PBD	Pyrrolobenzodiazepine
TAE	Tris-acetate-EDTA, pH 8.1
TEA	Triethanolamine
µg	Microgram
µl	Microlitre
µM	Micromolar

PUBLICATIONS CONTAINING DATA PRESENTED IN THIS THESIS

Berardini, M. D., Souhami, R. L., Lee, C-S, Gibson, N. W., Butler, J., and Hartley, J. A. Two structurally related diaziridinylbenzoquinones preferentially cross-link DNA at different sites upon reduction with DT-diaphorase. Biochemistry. In Press (1993):

Bose, D. S., Thompson, A. S., Ching, J., Hartley, J. A., Berardini, M. D., Jenkins, T. C., Neidle, S., Hurley, L. H., and Thurston, D. E. Rational design of a highly efficient irreversible DNA interstrand cross-linking agent based on the pyrrolobenzodiazepine ring system. J. Am. Chem. Soc. 114 (12 1992a): 4939-4941.

Bose, S. D., Thompson, A. S., Smellie, M., Berardini, M. B., Hartley, J. A., Jenkins, T. C., Neidle, S., and Thurston, D. E. Effect of linker length on DNA binding affinity, cross-linking efficiency and cytotoxicity of C8-linked pyrrolobenzodiazepine dimers. J. Chem. Soc., Chem. Commun. (1992b): 1518-1520.

Hartley, J. A., Berardini, M. , Ponti, M., Gibson, N. W., Thompson, A. S., Thurston, D. E., Hoey, B. M., and Butler, J. DNA cross-linking and sequence selectivity of aziridinylbenzoquinones: a unique reaction at 5'-GC-3' sequences with 2,5-diaziridinyl-1,4-benzoquinone upon reduction. Biochemistry 30 (50 1991a): 11719-24.

Hartley, J.A., Berardini, M.D., and Souhami, R.L. An agarose gel method for the determination of DNA interstrand crosslinking applicable to the measurement of the rate of total and "second-arm" crosslink reactions. Anal. Biochem. 193 (1991b): 131-134.

Hartley, J. A., Souhami, R. L., and Berardini, M. D. Electrophoretic and chromatographic separation methods used to reveal interstrand cross-linking of nucleic acids. J. Chromatog. Biomed. Appl. 9 (1993): In Press.

Lee, C. S., Hartley, J. A., Berardini, M. D., Butler, J., Siegel, D., Ross, D., and Gibson, N. W. Alteration in DNA cross-linking and sequence selectivity of a series of aziridinylbenzoquinones after enzymatic reduction by DT-diaphorase. Biochemistry 31 (N11 1992): 3019-3025.

ACKNOWLEDGEMENTS

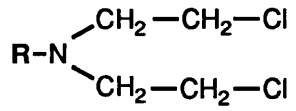
I would firstly like to thank Dr. John Hartley for his encouragement, support, and advice throughout the course of these studies and in particular for his patience during the preparation of this thesis. Special thanks to Professor Bob Souhami for allowing me the opportunity to undertake these studies in the Department of Oncology in addition to his kind advice and helpful discussions. Thanks also go to the people in the lab, Ray Hicks for his computer expertise, and in particular Kathryn Moore for her patient support and the use of her computer. I am also grateful to Dr. Neil Gibson for his help and advice without which a number of these studies would not have been possible.

CHAPTER 1: INTRODUCTION

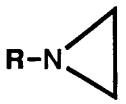
The bifunctional alkylating agents constitute a class of antitumour compounds that continue to be of central importance in cancer chemotherapy. The search for effective chemical agents in the treatment of neoplastic disease has persevered since the landmark discovery following World War II that nitrogen mustard was effective in the treatment of patients with lymphomas (Gilman & Phillips 1946). The development of curative treatments for many of the cancers that afflict children and young adults, including acute lymphocytic leukaemia, Hodgkins disease, and testicular germ cell tumours, has been the result of advances in drug therapy. Such progress has generated considerable interest in the development of new drugs and much effort has been directed toward understanding the biochemical basis for drug interactions as well as to improving the selectivity of drug action against malignant cells. Other areas of research have focused on the modification of existing clinically active agents with a view to enhancing their antitumour effects while minimising unwanted toxic side effects.

The search for synthetic agents as well as naturally occurring compounds that are effective in the treatment of cancer has resulted in a multitude of different agents that differ greatly with respect to structure and reaction mechanism. Such compounds include synthetic chemicals, hormones, antimetabolites, antibiotics, and a host of other useful agents that defy easy classification. However, alkylating agents remain one of the most effective classes of antitumour compounds nearly 50 years after their discovery.

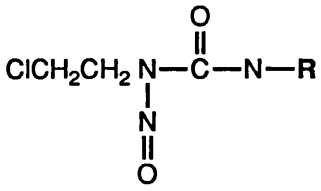
A number of alkylating agents including the nitrogen mustards, nitrosoureas, aziridines, and dimethane sulphonates (figure 1.1) have proven effective in treating a variety of cancers. A common property



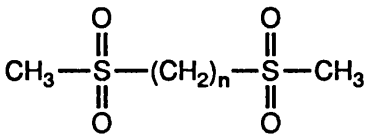
Nitrogen Mustards



Aziridines



Nitrosoureas



Dimethane sulphonates

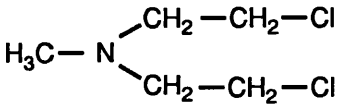
Figure 1.1 Structure of some common alkylating agents

associated with the alkylating agents is their ability to substitute an alkyl group, the structure of which may be complex, for a proton in a nucleophilic molecule. Although not all clinically useful alkylating agents are bifunctional, the presence of two alkylating moieties appears to be crucial with respect to the cytotoxic action of this group of compounds. As the chemical mechanism for many of the alkylating agents became better understood, it was thought that the cytotoxic effects produced by these agents were due to the formation of covalent cross-links between biological macromolecules. It was later proposed that DNA was a critical target for cross-linking by the bifunctional alkylating agents (Goldacre et al., 1949; Stacey et al., 1958). The role of DNA in replication and the finding that alkylation reactions can occur at DNA bases suggested DNA interstrand

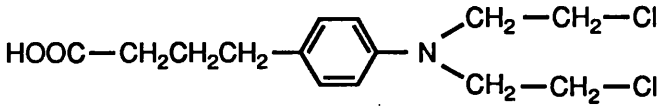
cross-linking as a crucial event (Brooks & Lawley, 1960; Brooks & Lawley, 1961). Even though some of the most effective alkylating agents are bifunctional and that DNA appears to be an important target for these agents, the mechanism(s) by which alkylating agents exert their cytotoxic effects is still not fully understood.

1.1. Nitrogen mustards

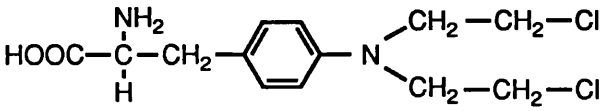
Mechlorethamine was one of the first non-hormonal agents to exhibit significant antineoplastic activity in the clinic and has played an important historic role in the evolution of chemotherapeutic agents (Gilman & Phillips, 1946). Figure 1.2 shows the structures of some of the clinically used nitrogen mustard compounds. The precursor of mechlorethamine, sulphur mustard, was used as a chemical warfare agent during the first World War. Sulphur mustard was known to be an extremely potent vesicant causing severe leukopenia, bone marrow aplasia and ulceration of the gastrointestinal tract in military personnel exposed to high concentrations of the gas (Krumbhaar & Krumbhaar, 1919). Although its use as a chemical warfare agent was never reported, nitrogen mustard was also originally developed during the mid 1930's for use on the battlefield. The known toxic effects of the mustards inspired Gilman and co-workers to test the effects of mechlorethamine in tumour bearing mice. Encouraging early results led to the first clinical trials of nitrogen mustard in 1942 in a patient with lymphosarcoma (Gilman & Phillips, 1946). With the publishing of this paper, it can be argued that the era of modern chemotherapy began.



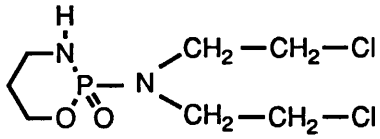
Mechlorethamine



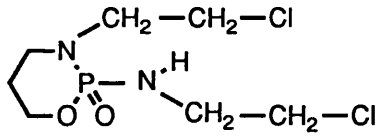
Chlorambucil



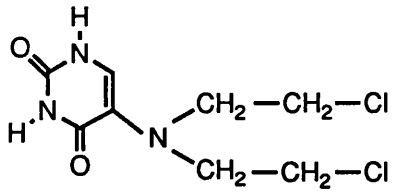
Melphalan



Cyclophosphamide



Ifosfamide



Uracil Mustard

Figure 1.2 Structures of some common nitrogen mustard compounds

Since the discovery of the clinical effectiveness of nitrogen mustard, thousands of analogues continue to be synthesised in an effort to improve the efficacy of the parent compound. As it became clear that the 2,2'-dichlorodiethylamine structure (nor-HN2) was an important feature of the nitrogen mustards, synthetic efforts concentrated mainly on replacement of the methyl group on nitrogen mustard with almost every feasible

constituent. Attempts have been made to synthesise modified mustards in the hope that they would preferentially localise in a specific tissue type. For example, since phenylalanine is a precursor of melanin, it was thought that phenylalanine mustard (Melphalan) might be directed to crucial metabolic sites in melanomas. Using similar reasoning, mustards containing pyrimidines (uracil mustard), phenylbutyric acid (chlorambucil) and other cellular constituents were attached to nor-HN2 in an effort to obtain a site directed effect. Reportedly high levels of phosphoramidases and phosphatases in some tumours prompted the search for suitable phosphorylated nitrogen mustard substrates (Gomori, 1948). The development of cyclophosphamide emerged as a result of such efforts and continues to be one of the most widely used antitumour agents despite the fact that the original premise for its synthesis has largely been discounted. In contrast to mechlorethamine, cyclophosphamide and ifosphamide require metabolic activation by mixed function oxygenases to elicit their antitumour effect (Connors et al., 1974).

In general, the nitrogen mustard alkylating agents are highly reactive compounds that are able to form covalent bonds with various nucleophiles (Price, 1975). Figure 1.3 shows the proposed mechanism of action for a majority of nitrogen mustard compounds. Formation of the aziridinium ion intermediate at neutral or alkaline pH occurs upon loss of the chlorine atom from one of the chloroethyl side chains. The resulting three membered aziridine ring system is highly reactive and capable of forming a covalent bond with biological nucleophiles. Although nitrogen mustards are capable of reacting with a variety of biological macromolecules including the sulfhydryl and amino groups of proteins and other cellular constituents (Connors, 1975), the most critical site for attack is thought to

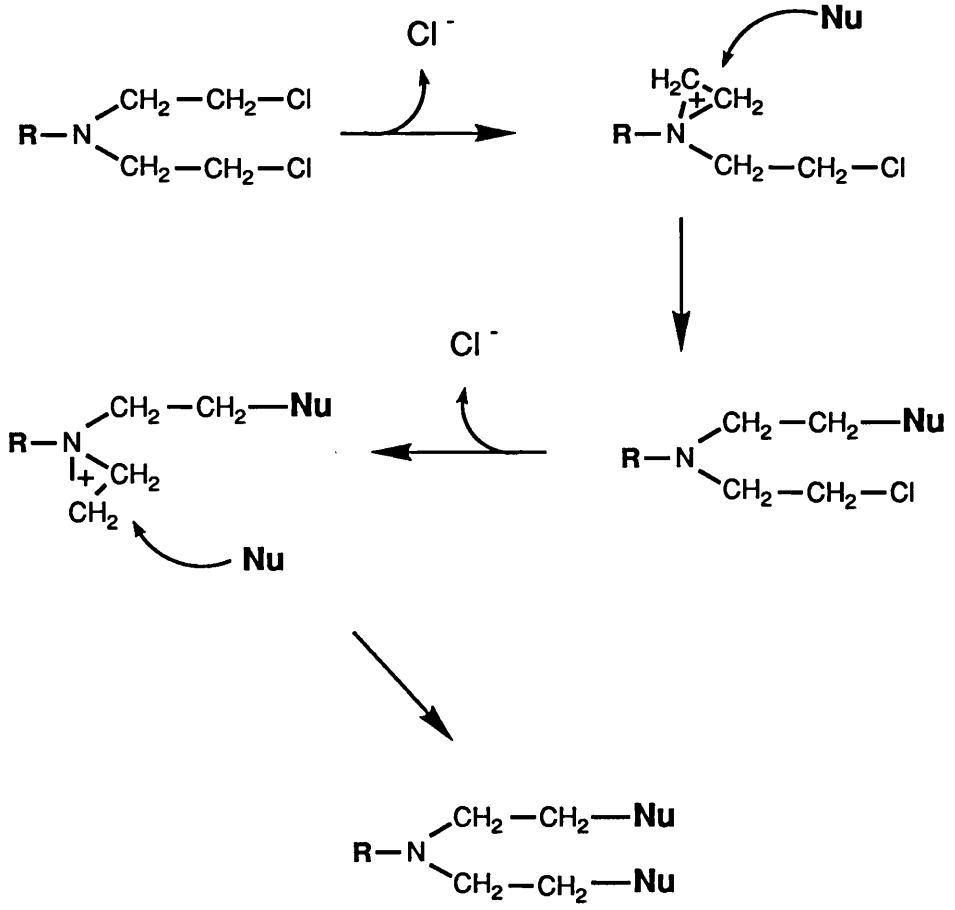


Figure 1.3 Proposed reaction mechanism for nitrogen mustard compounds

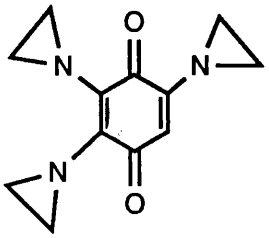
be at the guanine-N7 position of DNA. Upon initial attachment of the mustard to the guanine-N7 position, the second chloroethyl group has the potential to react with other nucleophilic moieties including protein or DNA. Reaction of the second group with the opposite strand of DNA leads to the formation of a DNA interstrand cross-link while hydrolysis of the second arm of the chloroethyl group prevents cross-link formation. Thus the sequence of alkylation of DNA can have a number of results and may lead to different biological effects. Indeed, the number of nitrogen

mustard monoadducts far exceeds the number of cross-links in DNA (Hemminki & Ludlum, 1981).

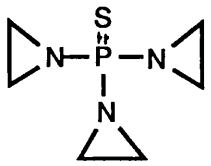
1.2. Aziridines

Interest in the synthesis and evaluation of aziridine compounds grew from the consistent observation that the most effective nitrogen mustards possessed two chloroethyl groups which were able to cyclise to form the highly reactive aziridinium ion intermediate. Figure 1.4 shows some of the bifunctional aziridines that have been effective against some human tumours. Triethylenemelamine (TEM), originally described as a cross-linking agent for wool, showed activity in animal tumours (Burchenal et al., 1950; Lewis & Crossley, 1950) and later in human cancers. A particular advantage with TEM was that it could be orally administered with little or no nausea. Subsequently, TEPA (Hendry et al., 1951) and its thio derivative (Baterman, 1955; Ultmann et al., 1957) preferred for its activity against carcinoma of the breast and ovary , were used extensively in the clinic. However, these aziridine compounds have found limited use today due to lack of therapeutic advantage over the more frequently used nitrogen mustard derivatives.

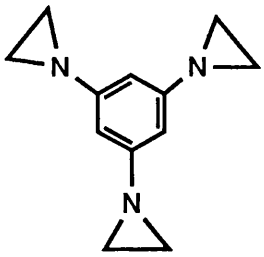
One group of compounds, the mitomycins, are unique in that they are the only known naturally occurring compounds to contain an aziridine ring. Mitomycin C (figure 1.4), first isolated in 1958 (Wakaki et al., 1958), was of particular interest and has been found to be active against a wide variety of solid tumours. A key feature of mitomycin C is the presence of the quinone moiety as part of its structure. Studies on the mechanism of action of mitomycin C have implicated DNA as the relevant cellular target. However, mitomycin C in its oxidised form is inactive and requires enzymatic



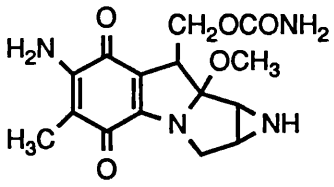
Trenimon



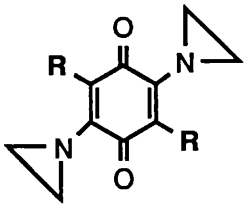
Thio-TEPA



Triethylenemelamine



Mitomycin C



Aziridinylbenzoquinones

Figure 1.4 Structures of alkylating agents containing the aziridine ring

reduction by cellular enzymes such as cytochrome P-450 reductase (Bachur et al., 1979), xanthine oxidase (Pan et al., 1984), or DT-Diaphorase (Fracasso et al., 1983; Siegel et al., 1990b) to produce more active cytotoxic species.

Mitomycin C is capable of undergoing both one and two electron reduction to generate more active metabolites. One electron reduction can lead to the formation of several active metabolites such as the radical semiquinone (Bachur et al., 1978; Kalyanaraman et al., 1980) which may interact with molecular oxygen to produce superoxide radicals or other highly reactive oxygen species (Lown & Chen, 1981; Tomasz, 1976). Such highly reactive metabolites have the potential to interact with DNA to produce single strand breaks. In contrast, two electron reduction of mitomycin C to the dihydroquinone form can lead to a species capable of alkylating and cross-linking DNA (Siegel et al., 1990b).

The requirement for mitomycin C to be reduced by cellular enzymes to elicit its antitumour effect has led to the search for other bioreductive alkylating agents in the hope that tumours with high levels of reductive enzymes may be sensitive to their cytotoxic effects. In addition, the quinone containing drugs may be more selective to hypoxic cells since reduced levels of oxygen in poorly vascularised tumours favour the reduced and hence more reactive form of these compounds. As a result the aziridinyl benzoquinones (figure 1.4) have recently received attention as possible antitumour agents. Like mitomycin C, these compounds also have the potential to undergo bioreductive activation via the one or two electron reduction pathway to generate more reactive metabolites (Begleiter & Leith, 1990; Gutierrez, 1989; Powis, 1989). Formation of DNA cross-links induced by enzymatically activated aziridinylbenzoquinones is thought to play an important role in the cytotoxic activity of these agents (Dzielendziak et al., 1990; Gibson et al., 1992; Pacheco et al., 1991; Siegel et al., 1990a). Enzymatic or chemical reduction of the non aromatic quinones to the aromatic hydroquinone alters the electron distribution and facilitates aziridine ring opening. Protonation of the aziridine ring also

catalyses ring opening and thus an increase in DNA cross-linking has been observed with both the quinone and hydroquinone at acidic pH (Hartley et al., 1991a; Lee et al., 1992).

1.3. Nitrosoureas

The development of the nitrosoureas emerged from a nation-wide research program set up by the US government in the mid-1950's to evaluate and test chemical compounds in animal tumour models for possible use against human cancers (Endicott, 1965). Subsequently, N-methyl-N'-nitro-N-nitrosoguanine (MNNG), formerly used in the preparation of diazomethane, a powerful methylating agent, was found to exhibit a degree of activity against L1210 leukaemia in mice (Montgomery, 1981). Promising results led to the development of N-methyl-N-nitrosourea (MNU) which later proved to be effective in mice with intracerebrally implanted leukaemia. Although the mechanism of action was not fully understood, evidence that MNU was capable of crossing the blood-brain barrier led to a synthetic program at the Southern Research institute to develop related nitroso compounds in an effort to improve the clinical effectiveness of this group of agents (Montgomery, 1981).

Further study indicated the chloroethyl derivatives such as chloroethylnitrosourea and bischloroethylnitrosourea (BCNU) possessed greater antitumour activity than MNU and other similar alkyl derivatives. As a result and because of its lipophilic properties, BCNU was the first compound of its type to undergo clinical trials and has since shown activity against brain tumours and other cancers (DeVita et al., 1965; Nissen et al., 1979). Because of the beneficial activity of BCNU, other chloroethyl derivatives such as cyclohexylchloroethylnitrosourea (CCNU) and methyl

CCNU have emerged as effective antitumour agents (figure 1.5). Because these agents shared cross-resistance with compounds such as the nitrogen mustards it was thought that they were acting as alkylating agents. Several

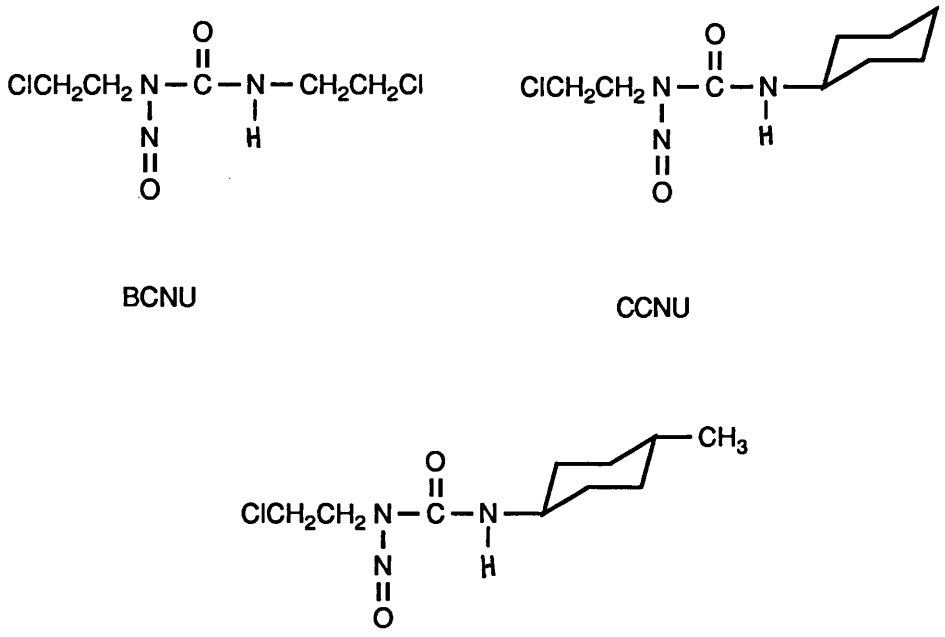


Figure 1.5 Structure of some chloroethylnitrosoureas

studies have confirmed this hypothesis and the chemical mechanism of alkylation has been established (figure 1.6). The spontaneous decomposition of the drug at physiological pH produces the 2-chloroethyldiazene hydroxide and the isocyanate moieties. The breakdown of the 2-chloroethyldiazene hydroxide species then generates the chloroethyl carbonium ion which is capable of alkylating nucleophilic sites within the cell. Largely due to the work of Ludlum and Kohn it was established that the chloroethyl carbonium ion had the potential to

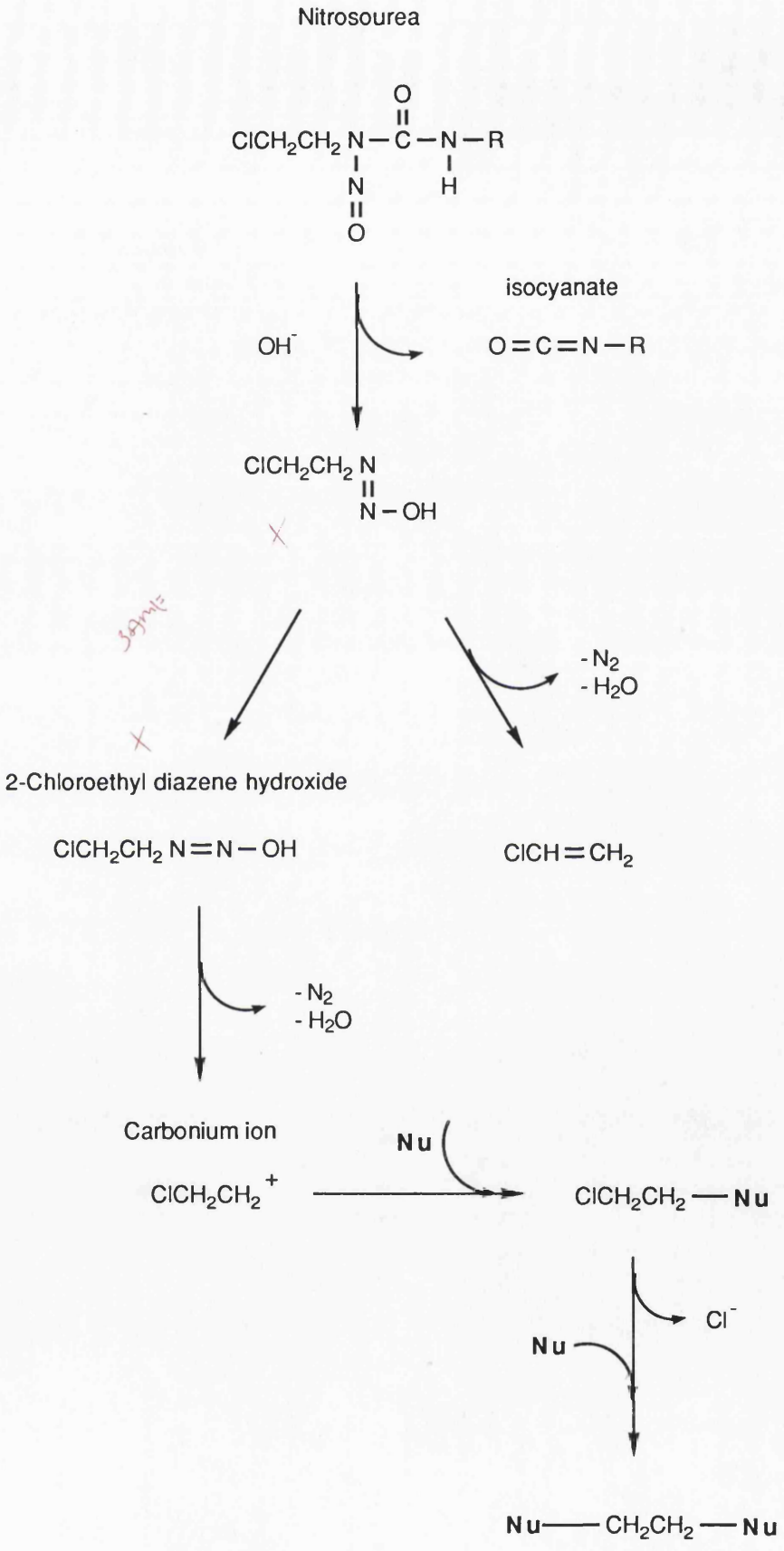


Figure 1.6 Proposed reaction mechanism of the chloroethylnitrosoureas

alkylate bases within DNA and, through loss of the chlorine atom, form DNA-DNA or DNA-protein cross-links (Kohn, 1977; Ludlum et al., 1977).

1.4. Chemistry

The reaction mechanisms of the bifunctional alkylating agents can ordinarily be classified as either S_N1 (nucleophilic substitution, first order) or S_N2 (nucleophilic substitution, second order). S_N1 reactions involve the initial formation of a reactive intermediate followed by the rapid reaction with a nucleophilic substrate to form an alkylated product (figure 1.7). The

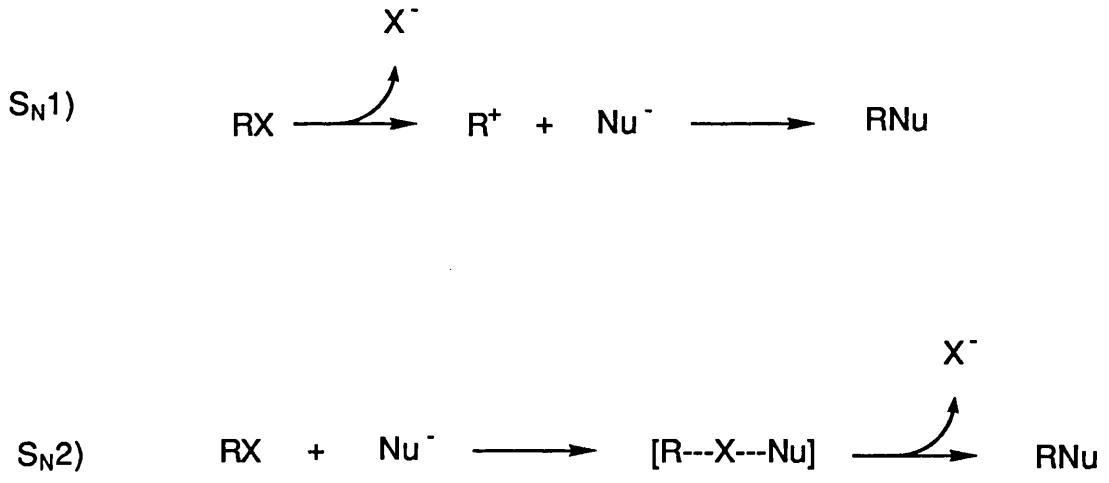


Figure 1.7 Kinetic reaction mechanisms S_N1 and S_N2.

rate of this reaction depends only upon the initial concentration of the alkylating agent and is largely independent of the concentration of the substrate. Formation of the reactive intermediate is therefore the rate limiting step and the reaction follows first order kinetics. The S_N2 reactions are characterised by the formation of an intermediate complex in

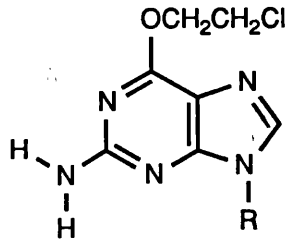
which the rate of reaction depends on the initial concentrations of both the alkylating agent and nucleophilic substrate and thus follows second order kinetics. The reaction of chemotherapeutic alkylating agents can follow either S_N1 reaction kinetics such as the nitrogen mustards and nitrosoureas or exhibit S_N2 characteristics such as busulfan.

1.5. Sites of Alkylation

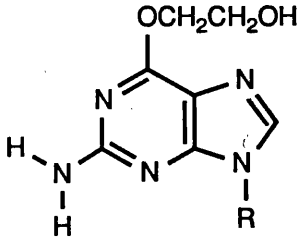
Although bifunctional alkylating agents are known to react with a variety of nucleophilic sites within cellular biological macromolecules (Skipper et al., 1951), it has been difficult to ascertain which sites within the cell have been critical with respect to the cytotoxic actions of these agents. Cytotoxicity might be associated with such factors as damage to the plasma membrane, inactivation of crucial enzymes, depletion of amino acid or nucleotide precursors, or a combination of these deleterious effects. Although these considerations cannot be totally discounted, the fact that alkylating agents are frequently active at extremely low concentrations appears to implicate the inactivation of macromolecules such as DNA. The demonstration that bifunctional agents are capable of inhibiting DNA synthesis, seems to support such ideas (Drysdale et al., 1958; Wheeler, 1962). In general, the preponderance of evidence appears to support the contention that inactivation of the DNA template by alkylating agents is principally responsible for inhibition of synthesis rather than damage to polymerase enzymes (Roberts et al., 1971; Ruddon & Johnson, 1968; Wheeler & Alexander, 1969).

In light of these observations, attention turned to the sites within DNA which are modified by alkylating agents. The DNA molecule presents a number of nucleophilic sites which have the potential to react with

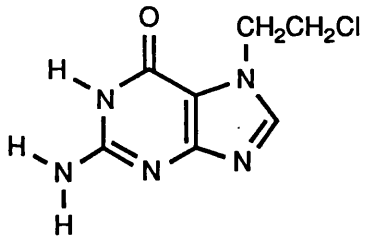
alkylating agents. Several studies have shown that the oxygen atoms of the sugar phosphate group of DNA have the potential to undergo alkylation reactions (Bannon & Verly, 1972; Lawley, 1973; Singer & Fraenkel-Courat, 1975). Even though the biological significance of phosphate alkylation is not well understood, it is not thought to make a substantial contribution to cytotoxicity due to the slow rate of phosphotriester hydrolysis (Verly, 1974). The heterocyclic purine and pyrimidine bases of DNA present additional sites for alkylation and a number of studies have shown that most nitrogen and oxygen atoms are susceptible to varying degrees of alkylation by both monofunctional and bifunctional compounds (Singer, 1976). Alkylation of base sites, in particular the O6 position of guanine, is thought to play a role in carcinogenesis (Loveless, 1969; Weinstein et al., 1976). Thus, in addition to their cytotoxic activity, the alkylating agents are potential carcinogens and mutagens. Studies of the relative electrostatic potential of purine bases reveal that the most electronegative sites in DNA are located at the N7 and O6 position of guanine and the adenine N3 and N1 positions (Pullman & Pullman, 1981). It is clear however, that the guanine N7 position is the most electronegative position in DNA and therefore the site which is most readily alkylated. Indeed, reaction of a monofunctional nitrogen mustard with individual nucleosides in solution produced a distribution of adducts between the four bases with the guanine-N7 adduct as the major product, followed by the N1 of adenine, N3 of cytosine, and N3 of thymidine (Price et al., 1968). In contrast, only the guanine-N7 adduct was produced in any appreciable quantities upon brief incubation of the agent with intact DNA. In similar studies, hydrolysis of DNA treated with nitrogen mustard and busulfan also yielded primarily guanine-N7 adducts upon HPLC analysis (Brooks & Lawley, 1961; Mehta et al., 1980; Tong & Ludlum, 1980).



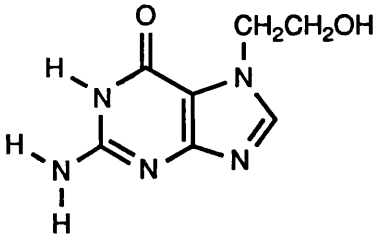
O^6 - chloroethylguanine



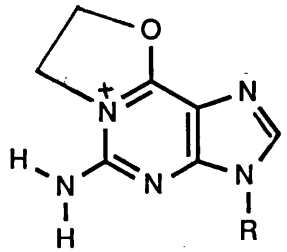
O^6 - hydroxyethylguanine



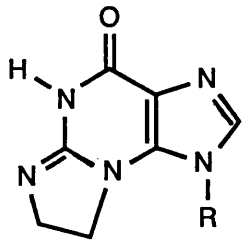
7-chloroethylguanine



7-hydroxyethylguanine



1,O^6-ethanoguanine



N^2,3-ethanoguanine

Figure 1.8 Modified guanine products isolated from DNA treated with chloroethylnitrosoureas

In contrast to the mustard compounds, a complex series of adducts has been isolated upon hydrolysis of DNA treated with the chloroethylnitrosoureas (Ludlum, 1990). For example, figure 1.8 shows

some of the guanine adducts generated from chloroethylnitrosourea treatment of DNA. In some instances, these adducts may be due to direct attack of the chloroethyl carbonium ion, or rearrangement of alkyl groups initially attached at other base positions. Formation of additional heterocyclic rings by the alkyl group between two atoms on the same base may account for some of the adducts. Other adducts are believed to originate from DNA cross-linked by the alkyl group. Interestingly, unlike the nitrogen mustards, the distribution of specific DNA base adducts by the chloroethylnitrosoureas has been shown to be compound specific (Tong et al., 1982). Although the 7-chloroethylguanine and the 7-hyroxyethylguanine DNA adducts have been shown to comprise a majority of the substituted base products, the guanine O6 alkyl lesion is believed to be the most important in the formation of DNA cross-links.

1.6. DNA Cross-Linking

The initial event in the formation of a DNA interstrand cross-link involves the non covalent binding of the drug with double stranded DNA. This interaction may be influenced by a number of factors including the ability of the drug to intercalate into the bases of DNA, hydrogen bond formation and the distribution of nucleophilic centres along the DNA molecule as well as the structure of the DNA reactive species. The covalent modification of a specific site on one strand of the DNA by the alkylating agent to form a monoadduct is the first step in cross-link formation. Depending on the nature of the reactive species, the second reactive part of the molecule may be hydrolysed and prevent cross-link formation. The second reaction may also take place on the same strand to produce a DNA intrastrand cross-link. Reaction with the opposite DNA strand results in the production of the DNA interstrand cross-link.

Based on the finding that the N-7 position of guanine was the primary site of attachment for many of the nitrogen mustards, it was suggested that these compounds exert their cytotoxic effect by forming DNA interstrand cross-links (Brooks & Lawley, 1961; Brooks & Lawley, 1963). Loveless and Ross showed that the bifunctional alkylating agents were much more effective antitumour agents than their corresponding monofunctional derivatives (Loveless & Ross, 1950). In addition, the antitumour activity of these agents was not significantly enhanced upon increasing the number of alkylating units beyond two. Although a number of cellular constituents can serve as substrates for chemical attack by alkylating agents (Connors, 1975), detailed studies of the effect of nitrogen mustards on the various biochemical functions of bacterial cell-free systems have shown the most critical cellular processes were those involved with the ability of DNA to act as a template for DNA and RNA synthesis (Johnson & Ruddon, 1967; Ruddon & Johnson, 1968). Early studies of the effects of nitrogen mustard on cultured mouse fibroblasts showed inhibition of DNA synthesis was much more pronounced than that of RNA or protein synthesis (Booth et al., 1964; Brewer et al., 1961).

Additional evidence for the inhibition of DNA replication by cross-linking agents has been reported for other agents. In one study, low concentrations of free mitomycin C did not inhibit DNA polymerase systems in-vitro while the addition of a small amount of cross-linked DNA decreased its activity (Tanaka & Yoshida, 1981). Inhibition of polymerase activity was reversed by increasing the concentration of template primer while increased concentrations of dideoxyribonucleotide triphosphates (dNTP) had no effect. It has been demonstrated that treatment of cells in culture with psoralen plus UV light arrested cells in the S and G2 phases of the cell

cycle (Varga et al., 1982). In contrast, the monofunctional alkylating agent angelicin had no such effect. Thus the inhibition of DNA replication and synthesis is a likely mechanism by which cross-linking agents are thought to exert their cytotoxic effect.

Other early studies implicating DNA interstrand cross-linking as an important cytotoxic lesion were based on the observation by Loveless and Stock that T2 phage was inactivated a short time after treatment with either nitrogen mustard or di(2:3-epoxypropyl)ether (Loveless & Stock, 1959a; Loveless & Stock, 1959b). Delayed inactivation of the phage was thought to be due to the formation of DNA interstrand cross-links from monoadducts. Continued inactivation of the phage was shown to be prevented upon subsequent treatment with thiosulphate which inactivates any unreacted monoadducts and precludes cross-link formation. Although it was proposed that inactivation of T2 phage might be attributed to formation of cross-links between the DNA and capsid protein (Loveless & Stock, 1959c), it was later demonstrated that bacteriophage T7 were much more sensitive to inactivation by the bifunctional agent (di-(2-chloroethyl)sulphide) than its corresponding monofunctional derivative (Lawley et al., 1969). In the latter study it was found that the monofunctional agent required approximately 40 fold more alkylations to inactivate the average T2 bacteriophage than its bifunctional counterpart.

Although physical techniques such as sedimentation velocity techniques and thermal denaturation methods have provided direct evidence for the presence of DNA interstrand cross-links induced in isolated and cellular DNA (Brooks & Lawley, 1963; Geiduschek, 1961), it was not until the development of the alkaline elution technique that DNA damage could be evaluated at therapeutic levels of alkylation (Kohn et al., 1976). Using this

technique it was possible to begin to assess the role of DNA cross-linking in the cytotoxic action of a variety of bifunctional alkylating agents at pharmacologically relevant concentrations (Ewig & Kohn, 1977; Ross et al., 1978). Important evidence for the role of DNA interstrand cross-linking has emerged from alkaline elution studies involving cell lines with differential levels of DNA repair enzymes. Day and co-workers were able to demonstrate that some human tumour cell lines (designated Mer-) were deficient in the ability to remove alkylations from the 06 position of guanine while other cell lines (termed Mer+) were proficient in this repair process (Day et al., 1980). Since the initial step in the formation of DNA cross-links induced by chloroethylnitrosoureas was proposed to be an addition of the chloroethyl group to the O6 position of guanine with the subsequent conversion to a cross-link over a period of hours (Kohn, 1977), it was hypothesised that the repair enzyme, guanine 06 alkyltransferase (GOAT), would also remove chloroethyl alkylations during this delay period to prevent cross-link formation (Erickson et al., 1980a). It was later demonstrated that the Mer+ phenotype was indeed resistant to the cytotoxic effects of the chloroethylnitrosoureas and exhibited far fewer DNA cross-links than did the Mer- cell strain despite the fact that both cell strains showed equivalent levels of drug uptake and protein cross-links (Erickson et al., 1980b). In addition, DNA cross-linking in Mer- cell lines has been associated with cytotoxicity in other studies (Scudiero et al., 1984). Pretreatment of Mer+ cell lines with MNNG, conditions which deplete intracellular GOAT levels, has resulted in an increase in the level of DNA interstrand cross-links following exposure to chloroethylnitrosoureas (Zlotogorski & Erickson, 1983; Zlotogorski & Erickson, 1984). Furthermore, transfection of DNA from a chloroethylnitrosourea resistant tumour cell line (Mer+) to a sensitive tumour cell line (Mer-) results in a tumour cell

line resistant to chloroethylnitrosourea with increased GOAT levels and decreased levels of DNA cross-links (Yarosh et al., 1986).

The production and removal of DNA cross-links induced by nitrogen mustard and melphalan has been suggested to be a measure of sensitivity in cell lines (Hansson et al., 1987; Zwelling et al., 1981). It has recently been shown that a correlation between the kinetics of DNA cross-link formation and loss of colony forming ability existed for a number of nitrogen mustard compounds (O'Connor & Kohn, 1990). In studies with other agents, the in-vitro cytotoxicity of a series of dimethane sulphonates has been attributed to their ability to form DNA cross-links but not protein cross-links (Bedford & Fox, 1983).

Although it became clear that bifunctional attack was an important aspect associated with cytotoxicity, the role of intrastrand cross-linking remains unresolved. This problem was partially addressed in a study relating platinum induced damage in λ DNA phage to loss of transfecting activity in E. coli (Filipski et al., 1980). It was found that both cis- and trans-platinum were capable of generating cross-links in the purified DNA. Drug reactions were carried out in such a way as to ensure any remaining reactive monoalkylations were inactivated (Filipski et al., 1979). For the case of trans-platinum, only uncross-linked DNA molecules retained transfective properties. Although DNA cross-linked by cis-platinum was also capable of inhibiting transfection, a significant proportion of uncross-linked DNA treated with this agent inactivated λ DNA phage. These findings suggested that DNA cross-linked by both cis- and trans-platinum were capable of inactivating viral DNA but only cisplatin treated DNA contained non cross-linked adducts capable of inhibiting transfection . Such lesions are likely to be intrastrand cross-links.

1.7. In-Vitro DNA Cross-Linking Assays

Structure function relationships have indicated that bifunctionality and therefore DNA interstrand cross-linking play a critical role in the cytotoxic action of bifunctional alkylating agents. Unfortunately, alkylation reactions take place at a number of sites within the cell of which only a small percentage are of the crucial cross-linking type. The challenge is therefore to detect and identify DNA lesions, such as interstrand cross-links, induced by bifunctional alkylating agents in order to assess their role in cytotoxicity.

Several methods have been described for the measurement of DNA interstrand cross-linking both in isolated DNA and in cells. Detection of interstrand cross-links in cells treated with bifunctional agents has been accomplished using sedimentation velocity and denaturation-renaturation studies. More recently, the technique of alkaline elution has been instrumental in the analysis of cross-link damage induced in cellular DNA. A number of techniques have been applied to the analysis of cross-links formed in isolated DNA. The action of various enzymes including endonucleases, exonucleases and some polymerases is inhibited by DNA containing an interstrand cross-link. Under appropriate conditions, the presence of a cross-link between the complementary strands of a DNA fragment will cause that fragment to migrate with an increased apparent molecular weight through polyacrylamide or agarose gels. Cross-linked DNA can also be analysed by various chromatographic methods and detected by an increase in the retention time compared to uncross-linked DNA.

Many cross-linking assays are based on the principle that treatment of double stranded DNA with heat or alkali under appropriate conditions will denature the DNA. Raising the temperature of double stranded DNA fragments in solution to a critical point disrupts the hydrogen bonding between bases and will denature the DNA into its component single strands. Rapid cooling of the denatured DNA will prevent the strands from re-associating. Alternatively, double stranded DNA is denatured under alkaline conditions at sufficiently high pH values (pH>12). The time required for the complete separation of DNA strands depends on a number of factors including the length of the fragment, temperature, and ionic strength.

The presence of an interstrand cross-link in a double stranded DNA fragment will prevent the complete separation of the strands under either heat or alkaline denaturing conditions. The interstrand cross-link in DNA serves as a point for the re-association of the denatured strands upon cooling or neutralisation while uncross-linked molecules remain separated (Jolley & Ormerod, 1973). The extent of cross-linking in a population of DNA fragments can thus be evaluated by subjecting the sample to the denaturing/renaturing process and separating the fragments using a variety of methods.

1.7.1. Sedimentation

The analysis of cross-linking and repair in cellular DNA has been demonstrated by both alkaline sucrose sedimentation and isopicnic caesium chloride centrifugation methods(Ball & Roberts, 1971/72; Cole, 1973; Cole et al., 1976; Fujiwara & Tatsumi, 1977; Iyer & Szybalski, 1964; Jolley & Ormerod, 1973; Pera et al., 1981). These techniques usually involve

labeling cells in culture prior to treatment with the cross-linking agent. With the alkaline sucrose method, cells are harvested, lysed and subjected to alkaline denaturing conditions. The lysate is then layered onto an alkaline sucrose gradient (ca. pH 12.5), and centrifuged under appropriate conditions. Estimation of the molecular weight can be obtained by incorporating DNAs of known size as internal standards. After centrifugation, the sample is eluted from the bottom of the centrifuge tube and collected as a number of fractions. The radioactivity in each fraction is counted to obtain a sedimentation profile. A plot of radioactivity versus fraction number will show DNA from drug treated cells sedimenting more rapidly through the gradient (i.e.. early fraction numbers) than the DNA from untreated cells. Thus a qualitative measure of the extent of cross-linking can be obtained from the resulting sedimentation profiles. A recent study has applied alkaline sucrose sedimentation to the analysis of cross-link formation and repair in actively transcribed gene sequences by hybridising specific probes to eluate fractions blotted to nitrocellulose filters (Islas et al., 1991). The process of analysing DNA repair with this method is somewhat complicated by the need to separate out drug treated parental DNA from newly synthesised DNA using bromodeoxyuridine incorporation.

Moreover, the rate at which DNA migrates through alkaline sucrose gradients depends on a number of factors including centrifugation time and speed, DNA fragment size, and the number of cross-links per fragment. Quantitative analysis of DNA cross-linking using this method is complicated by the fact that DNA fragments containing many cross-links will sediment more rapidly through the gradient and can lead to an overestimation of cross-linking. To avoid such difficulties the DNA must be sheared by vortex to obtain DNA containing roughly one cross-link per fragment. Other

methods utilise x-rays to induce random breaks in the treated DNA. An underestimation of the extent of DNA cross-linking can occur if the cross-link is converted to strand breaks during the alkaline denaturing step. Precise quantitation of cross-linked DNA also becomes cumbersome from the analysis gradient profiles.

1.7.2. Alkaline Elution

The technique of alkaline elution was originally developed by Kohn and co-workers for analysing cellular DNA damage in mammalian cells (Kohn, 1979; Kohn, 1981; Kohn et al., 1976; Kohn et al., 1981). Essentially, cells are layered onto a membrane filter and lysed with a detergent solution. Cell lysates are allowed to flow through the filter leaving the DNA largely intact on the filter. The DNA is then slowly pumped through the filter with a high pH solution (ca. pH 12) which separates the DNA into single strands. The majority of denatured untreated DNA remains on the filter under these conditions due to the extreme length of the strands. However, if the DNA is fragmented into smaller pieces by exposure to x-rays, the DNA will elute through the filter at an increased rate and is proportional to the x-ray dose. Strand breaks induced by x-rays are random in nature and the DNA will elute from the filter according to first order kinetics. The level of sensitivity is high enough to measure one break per 10^9 daltons.

In order to assess the level of DNA interstrand cross-links induced in the cell it is necessary to induce a defined level of random strand breaks with x-ray after treatment with the drug of interest. This allows the DNA from untreated cells to elute at a defined rate. Production of cross-links in the DNA fragments of treated cells will diminish the rate at which the irradiated DNA will elute from the filter. This effect is due to the increase

in apparent fragment size imparted by the cross-link between the strands of DNA. The extent of cross-linking can be determined by measuring the difference in the treated and untreated elution profiles.

The technique of alkaline elution is arguably one of the best methods for evaluating damage, particularly cross-linking, induced in mammalian cells by DNA reactive agents at pharmacologically meaningful dosages. It has the added advantage of measuring other types of DNA damage including DNA protein cross-links, DNA double and single strand breaks as well as alkali labile lesions. Although the technique is extremely sensitive, the measurement of DNA cross-links is nevertheless indirect and depends on the decreased rate of elution of radiolabelled DNAs. Any limitations might include expensive and specialised equipment and the time necessary to set up and execute the experiments.

1.7.3. Ethidium Bromide Fluorescence Assay

Ethidium bromide is a fluorescent dye containing a planar structure which is capable of intercalating into the stacked bases of DNA (Maniatis et al., 1982). Dye which is bound to DNA displays an increased fluorescent yield compared to dye in free solution. Radiant energy absorbed by DNA at 254 nm is transmitted to the bound dye and subsequently re-emitted at 590 nm in the red-orange region of the visible spectrum. Ethidium bromide can be used to detect both single- and double stranded DNA. However, the fluorescent yield of ethidium bromide bound to double stranded DNA is much greater than that for single stranded DNA. This differential fluorescence can be exploited to measure cross-links induced in DNA.

The use of ethidium bromide to detect cross-links induced in DNA by mitomycin C was demonstrated by Lown et. al. (1976). Essentially, a small aliquot of drug treated DNA is added to a solution containing ethidium bromide. After an equilibration period, during which the ethidium bromide intercalates into the DNA, a fluorescence measurement is taken. The sample is then subjected to thermal denaturation followed by rapid cooling. Under these conditions, DNA fragments containing a cross-link will renature to become double stranded while uncross-linked fragments remain single stranded. Following thermal treatment and renaturation, ethidium bromide will only incorporate into DNA fragments containing an interstrand cross-link. The extent of cross-linking can be estimated by calculating the ratio of fluorescence values before and after thermal treatment.

Using this approach, it was demonstrated that DNA cross-links induced by mitomycin C increased with time and were pH dependent. However, at high concentrations of mitomycin C, a decrease in the level of fluorescence was reported (Lown et al., 1976). The decrease in fluorescence was attributed to a decrease in the number of intercalation sites available to ethidium bromide at high levels of alkylation.

1.7.4. Chromatography

A number of investigators have reported the preparation and analysis of cross-linked DNAs using hydroxyl apatite, sephadex and HPLC chromatographic methods (Bernges & Holler, 1991; Borowy-Borowski et al., 1990a; Borowy-Borowski et al., 1990b; Norman et al., 1990; Tomasz et al., 1974; Tong & Ludlum, 1980). A method for the separation of mitomycin C cross-linked DNA has been described using hydroxyl apatite

chromatography (Fujiwara, 1983). One approach involves radiolabeling of cells in culture with either ^{3}H or ^{14}C thymidine, followed by chasing the label into high molecular weight DNA in label-free medium. The DNA is then purified from cells and subjected to denaturing-renaturing conditions. Typically 10 μg of DNA is loaded onto a column packed with 1 ml of hydroxylapatite equilibrated with phosphate buffer (0.1-0.5M), pH 6.8 (Fujiwara, 1983). The DNA is then pumped through the column at room temperature with a linear phosphate buffer gradient (0.05-0.4M). Denatured single stranded DNA elutes first followed by cross-linked DNA. Fractions can then be counted directly by scintillation counting or as TCA insoluble radioactivity.

Preparation of oligonucleotides cross-linked by mitomycin C and characterisation of the resulting base modifications has recently been reported using sephadex chromatography (Borowy-Borowski et al., 1990a) and HPLC methods (Borowy-Borowski et al., 1990b). Preparation of mitomycin C cross-linked oligomers with yields as high as 50% have been reported. Other studies have used chromatographic methods in the preparation of short DNA duplexes for characterisation by NMR (Norman et. al., 1990). Since isolation of the cross-linked oligomer product using sephadex chromatography often requires a second step of purification it is generally more suitable to the preparative stage of analysis and not applicable to the detailed time course studies involving the quantitative determination of the extent of cross-linking.

1.7.5. S1 Nuclease

S1 nuclease is an enzyme which degrades single stranded DNA while leaving double stranded DNA intact. Untreated DNA which has been

subjected to a denaturing-renaturing process will be single stranded and thus becomes susceptible to degradation by the enzyme. However, the presence of a covalent cross-link between the complementary strands of DNA permits the re-association of the two strands to the double stranded form. Hence, cross-linked DNA will be resistant to the action of the enzyme. The preference of S1 nuclease for digestion of single stranded DNA has previously been used to measure the extent of DNA cross-linking in mammalian cells (Boyer et al., 1988). The DNA of cells were radiolabeled in culture prior to treatment with a cross-linking agent. The cells were then exposed to x-rays to create DNA fragments of a defined size and then extensively digested with S1 nuclease. Precipitation of the undigested DNA followed by scintillation counting of the insoluble fraction provides a measure of the extent of cross-linking.

1.7.6. Gel Based Assays

The extent of cross-linking in a population of DNA fragments can be evaluated by subjecting the sample to the denaturing/renaturing process and separating the fragments using agarose gel electrophoresis. Under these conditions, native undenatured and cross-linked denatured DNA will migrate with diminished mobility in an agarose gel compared to uncross-linked denatured DNA. Electrophoresis of a drug treated sample of homogenous DNA fragments will produce two bands of different electrophoretic mobility within the gel. Quantitation of DNA cross-linking can therefore be determined by measuring the amount of DNA migrating as double stranded.

The evaluation of cross-linking in defined human genomic DNA sequences has been demonstrated utilising the differential migration of cross-linked

and uncross-linked DNA fragments in agarose gels (Vos & Hanawalt, 1987). DNA from cells treated with a cross-linking agent is isolated and digested with an appropriate restriction enzyme. Fragments are then applied to an alkaline agarose gel in either the native or denatured form. After electrophoresis the DNA is transferred to a nitrocellulose filter and probed for the sequence of interest. The signal from native (double stranded) DNA fragments from untreated cells appears as a single band on the autoradiograph. Similarly, the signal from denatured (single stranded) DNA from untreated cells also appears as a single band but migrates with an increased mobility through the gel compared to the native DNA fragment. If a proportion of the DNA fragments containing the gene of interest is cross-linked, then two bands appear on the autoradiograph. Those fragments containing DNA-DNA cross-links will migrate as double stranded through the gel while the uncross-linked fragments remain denatured and migrate as single stranded.

Using this approach it was possible to detect cross-links and cross-linkable monoadducts induced in the actively transcribed dihydrofolate reductase gene (DHFR) in cultured human cells. It was demonstrated that 80% of cross-links induced by 4'-hydroxymethyl-4,5',8-trimethylpsoralen (HMT) and UV light in the DHFR gene were removed after 24 hours (Vos & Hanawalt, 1987). Extension of the method to measure the conversion of monoadducts to cross-links required the separation of newly synthesised daughter DNA from parental DNA in order to assess damage to the DHFR gene prior to replication.

In a similar study, heterogeneity of damage induced in specific genomic regions was reported for nitrogen mustard and dimethyl sulphate (Wassermann et al., 1990). DNA from treated cells is extracted, restricted

and the parental DNA purified. Strand breaks are created at sites of N-alkylpurine damage (presumably cross-links and monoadducts) by heating the DNA at neutral pH and subsequently treating with alkali. DNA fragments are then separated in alkaline agarose gels and transferred to nylon support membranes. Radioactive probes are then hybridised to specific regions of various genes to measure the extent of damage and repair. Nitrogen mustard damage in actively transcribed genes was found to be preferentially repaired compared to transcriptionally silent genes in CHO cells. Interestingly, no such differential repair between active and inactive genes was observed after damage induced by dimethyl sulphate. Similar patterns of heterogeneous repair of DNA cross-linking damage have been demonstrated for nitrogen mustard in specific gene sequences (Futscher & Erickson, 1990).

Other studies have focused on the ability of DNA interstrand cross-links to alter the mobility of non-cellular restriction fragments of plasmid DNAs in agarose gels. One such study has reported that the electrophoretic mobility of Hae III ϕX174 DNA fragments is reduced upon reaction with HMT and UV light both in neutral and alkaline agarose gels (Kohn, 1979). However, only slight shifts in mobility were detected for fragments in neutral gels even at very high drug concentrations. More pronounced shifts occurred under alkaline electrophoresis conditions although, somewhat surprisingly, the shift in mobility for low concentrations of HMT was much greater than for high concentrations. Several years later this technique was used to compare the efficiency of cross-linking for a series of structurally similar CC-1065 dimers (Mitchell et al., 1989). Although the effect of DNA cross-linking can be detected by band shifting, there is no physical separation of cross-linked and uncross-linked DNA in the gel and

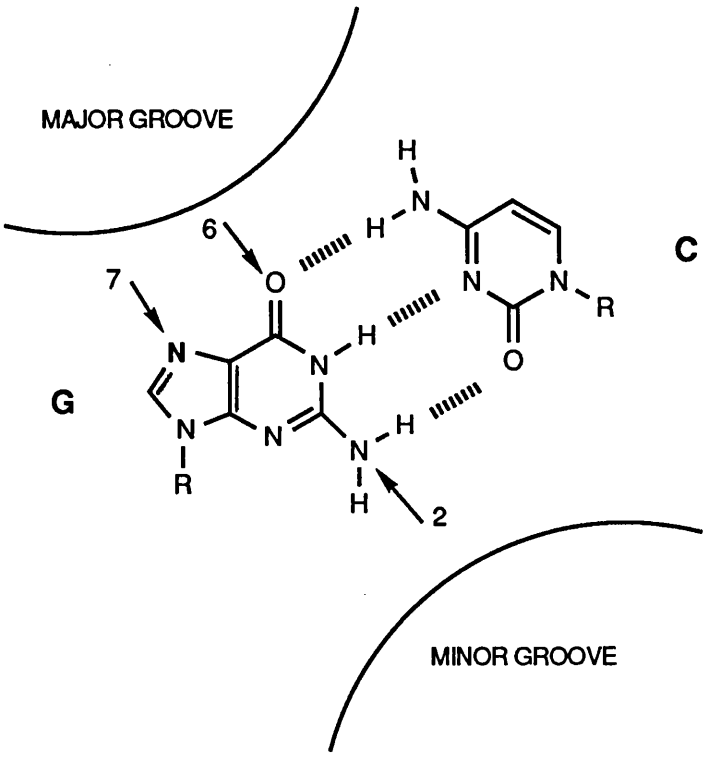
detection by ethidium bromide staining requires a relatively large amount of DNA per sample.

1.7.7. Summary

Several techniques are currently available for the measurement of DNA interstrand cross-linking both in cells and in isolated DNA. However, many of these techniques are time consuming and often require large amounts of DNA or drug. In addition, a number of these methods suffer from the lack of sensitivity and are not generally applicable to detailed time course studies.

1.8. Sequence Specificities of Alkylating Agents

Until recently alkylating agents have been discounted as being poorly sequence selective mainly due to the majority of studies focusing primarily on DNA base adduct formation requiring high concentrations of drug to permit detection. Only within the past fifteen years or so have techniques become available for the direct analysis of the sequence specificity of bifunctional alkylating agents. The choice of method for detecting DNA adducts depends to a large degree on the type of adduct produced by the agent of interest. For example, the nitrogen mustards react primarily at the N7 position of guanine in the major groove while mitomycin C has been shown to covalently bind with the exocyclic amino group of guanine in the minor groove (figure 1.9). The sequence selectivity of alkylating agents which react with either the guanine-N7 or adenine-N3 positions on DNA can be determined by converting these adducts to strand breaks using adaptations of the chemical sequencing reactions described by Maxam and Gilbert (Maxam & Gilbert, 1977; Maxam & Gilbert, 1980) . Alternatively, the



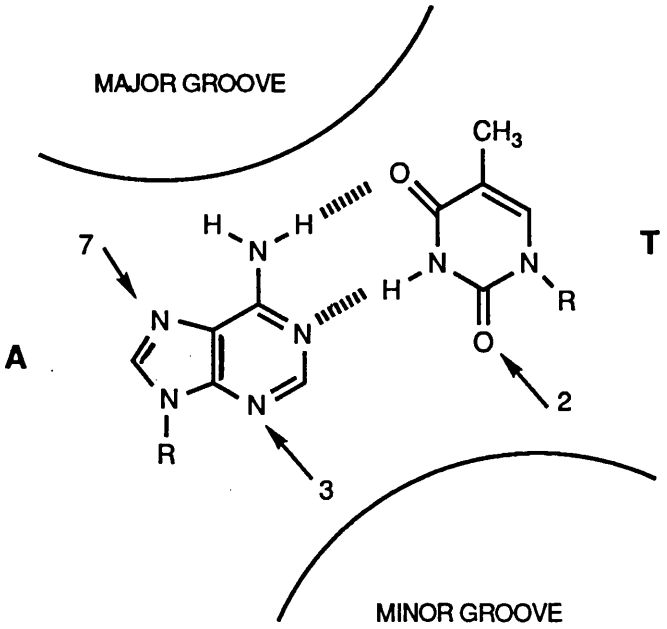


Figure 1.9 Chemical structures of GC and AT base pairs (from Nielson, 1991)

use of DNA polymerase reactions or nuclease enzymes have been used to detect lesions which cannot easily be converted to DNA strand breaks. In addition, DNA footprinting methods have been used for those agents which do not form covalent complexes with DNA.

1.8.1. Monoalkylation Specificity

The initial reaction of bifunctional alkylating agents with DNA begins with the covalent modification of one strand of the DNA duplex to form a DNA monoadduct. Depending on the type of adduct, the site at which these agents covalently modify DNA bases can be detected using a number of strategies.

1.8.1.1. Chemical Cleavage

Several compounds have been evaluated for their ability to alkylate guanines in a sequence selective manner using a modification of the guanine - specific chemical cleavage reaction described by Maxam and Gilbert (figure 1.10). The guanine specific sequencing reaction involves treating singly end - labeled DNA with dimethylsulphate (DMS), cleaving the modified residues with piperidine at elevated temperature and separating the cleavage products using denaturing polyacrylamide gel electrophoresis (PAGE). This technique takes advantage of the fact that DMS methylates DNA primarily at guanine-N7 positions and to a lesser extent at adenine-N3 positions. Substitution of a methyl group for the hydrogen atom at the guanine-N7 position creates a positive charge shared by the N7, C8, and N9 atoms on the purine ring. Under alkaline conditions, the bond between C8 and N9 is broken on reaction of a hydroxyl ion with the C8 atom. Thermal treatment with piperidine then displaces the ring opened 7-methyl-

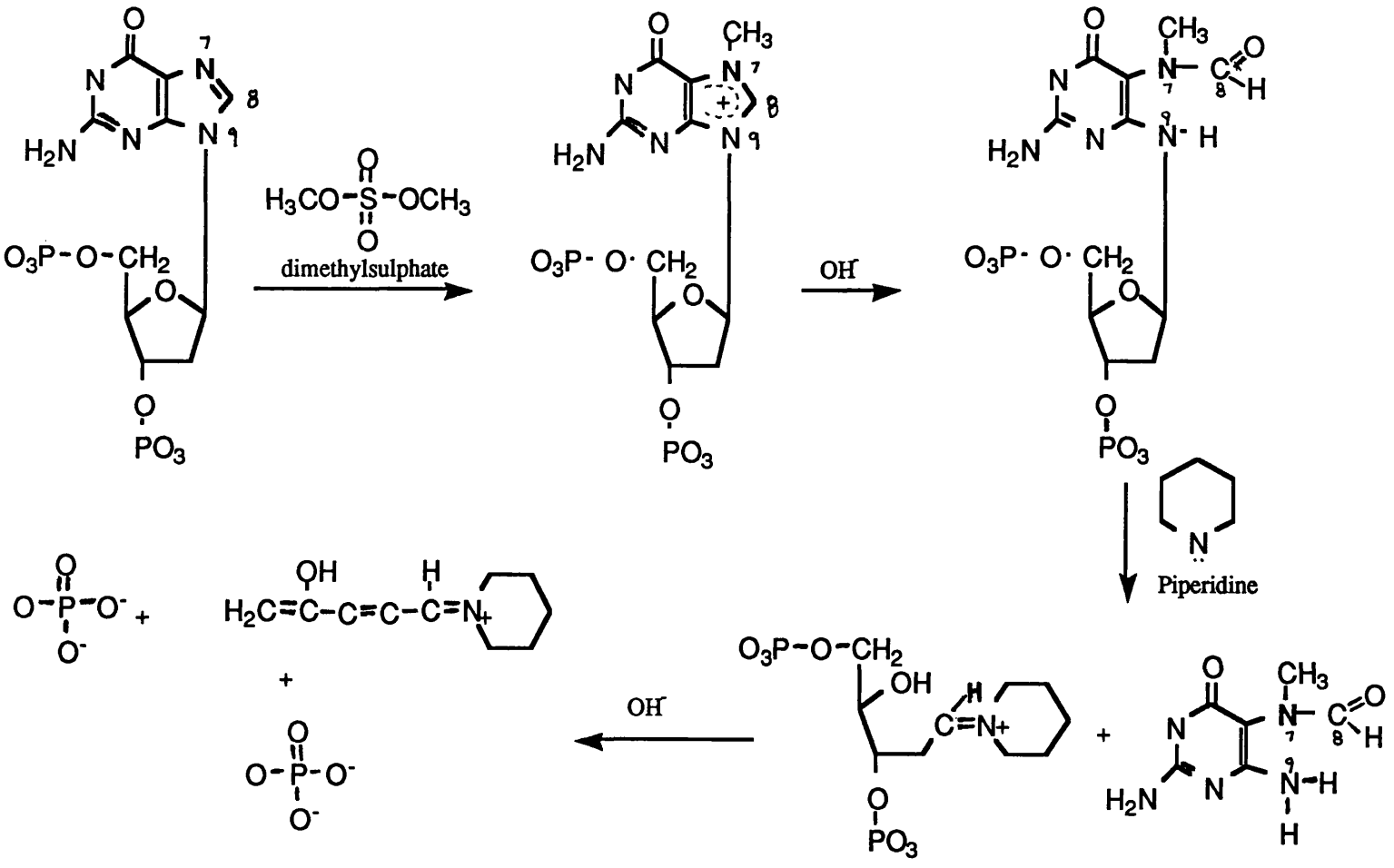


Figure 1.10 Schematic representation of the guanine specific chemical cleavage reaction.

guanine and catalyses the β-elimination of both phosphates from the DNA. Methylated adenines are stable under these reaction conditions and does not lead to DNA strand cleavage. The position of guanine residues in a given DNA sequence can be determined by adjusting the conditions of the methylation reaction so that approximately one guanine residue per DNA fragment is alkylated. Following piperidine treatment, a distribution of fragments of different length are generated from cleavage of the fragment wherever there was a guanine.

The sequence preferences for those compounds which react predominantly at the N7 position of guanine in the major groove can be evaluated by substituting an alkylating agent for DMS in the guanine specific sequencing reaction provided the DNA sequence is known and the concentration of the drug is adjusted to maintain roughly one alkylation per fragment (figure 1.11). This modification of the chemical cleavage sequencing reaction was first adapted to the study of aflatoxin B1 adduct distribution in DNA (D'Andrea & Hazeltine, 1978). Among the first class of antitumour agents to be evaluated using the modified method were the chloroethylating agents including several chloroethylnitrosoureas (Hartley et al., 1986; Wurdeman & Gold, 1988). These compounds showed unexpected specificity for guanines located in the middle of a stretch of three or more guanines in a DNA sequence. The intensity of reaction was shown to increase with the number of consecutive flanking guanines although the intensity of the reaction at individual guanines within the run was found to vary. Several nitrogen mustard alkylating agents have also shown variations with respect to preferential alkylation of guanines in a DNA sequence.

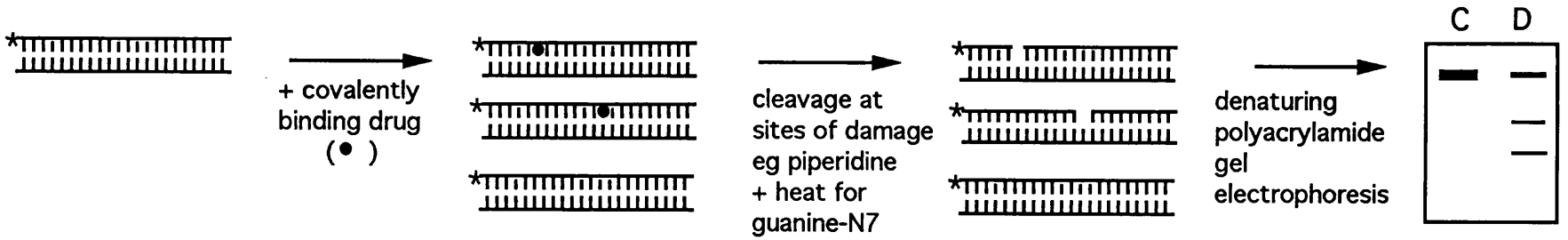


Figure 1.11 Chemical cleavage method for determining sequence specificity of covalently modified DNA. C is control, D is drug treated DNA.

Unlike the chloroethylnitrosoureas, the sequence specificity of certain nitrogen mustards was influenced by the substituent attached to the reactive part of the molecule. Alkylation of central guanines in stretches of three or more guanines was also characteristic of many of the nitrogen mustards with the notable exception of uracil and quinacrine mustard.

Uracil mustard was found to preferentially react at 5'-PyGC sites which is poorly alkylated by other mustards including mechlorethamine. It has been suggested that preferential reaction of uracil mustard at these sites may be due to hydrogen bonding between the cytosine 3' amino group and the O4 atom of uracil mustard (Kohn et al., 1987). The ability of quinacrine mustard to intercalate between the bases of DNA is thought to be responsible for the extremely low concentration required for reaction. Previous studies have shown that alkylation of guanine residues by quinacrine mustard is enhanced if the two bases situated to the 3' side of the reactive site are either GPu or TPu (Kohn et al., 1987).

Many other classes of chemotherapeutic alkylating agents have been evaluated for their ability to alkylate guanine residues in a sequence specific manner including the triazenes (Hartley et al., 1988b), dimethanesulphonates (Ponti et al., 1991b) and diaziridinylbenzoquinones (Hartley et al., 1991a; Lee et al., 1992) . Included in these studies was the analysis of various structural analogues of the clinically useful bioreductive agent AZQ (diaziquone). Among the key factors influencing the reactivity of several of these symmetrically substituted analogues were the substitutions at the 3 and 6 positions of the ring, the ease of reduction of the quinone function, and pH. Reactivity of several of these analogues was increased at low pH and upon reduction of the quinone to the hydroquinone by either ascorbic acid or by the obligate two electron

reductase enzyme DT-Diaphorase (Hartley et al., 1991a; Lee et al., 1992). The pattern of guanine-N7 alkylation generated by several of these compounds in their quinone form was similar to that of the nitrogen mustard compounds. However, upon reduction of one of the AZQ analogues, DZQ, the reactivity was significantly increased and the sequence specificity was substantially enhanced to alkylation of guanines in 5'-GC and in particular 5'-TGC sequences. Molecular modelling studies have suggested that intercalation of the hydroquinone form of DZQ at 5'-GC sites is facilitated by hydrogen bonding of the hydroxyl groups with the O2 and C4 groups of cytosine. This allows the reactive aziridine carbons to be in a favourable position above the plane of the aromatic ring for covalent bond formation to the guanine-N7 position (Hartley et al., 1991a). Such bonding would not be possible with the quinone form of DZQ and would be inhibited by bulky groups located at the 3 and 6 position of the hydroquinone. In addition to the preferential alkylation of guanines at 5'-GC sites by the hydroquinone form of DZQ, new sites of alkylation were detected at adenine residues centrally located in 5'-AAA or 5'-TAA sequences. A 3,6-dimethyl analogue of DZQ, MeDZQ, also exhibited similar new sites of adenine alkylation upon reduction but did not exhibit preferential alkylation of guanines in 5'-GC sequences (Lee et. al., 1992). These findings demonstrate that interactions of these compounds with DNA in a sequence selective manner is influenced to a large extent by subtle alterations in the structure of the moiety attached to the reactive component of the molecule.

The sequence preferences for alkylating agents which react principally at the N3 position of adenine residues can be determined by using a variation of the guanine-adenine specific chemical sequencing technique described by Maxam and Gilbert (Maxam & Gilbert, 1977; Maxam & Gilbert, 1980). In the original sequencing method, detection of adenines residues was

achieved by treating singly end-labelled DNA with dimethylsulphate in much the same way as for the guanine specific sequencing reaction (Maxam & Gilbert, 1977). Heating the resulting methylated purine DNA at neutral pH leads to depurination of the modified guanine and adenine residues. Subsequent strand scission upon treatment with dilute NaOH gives a pattern of strong bands at guanines and weak bands at adenines upon electrophoresis. In the modified method, the 3-alkyladenine residue is depurinated by acid treatment (0.1 M HCl) and the production of strand breaks is catalysed by 0.1 M NaOH. The sequence preference for adenine-N3 alkylation in the early promoter region of SV40 has been investigated for the exceedingly potent antitumour antibiotic CC-1065 using the above variation of the chemical sequencing technique (Hurley et al., 1984; Reynolds et al., 1985). CC-1065 displayed a high level of specificity and was shown to react with adenines located in 5'-PuNTTA sequences and to the 3' terminal adenine of 5'-AAAAA sequences.

Compounds which cause direct breakage of DNA represent the most straightforward approach to the analysis of sequence specificity using the chemical cleavage method. Agents producing these types of lesions include bleomycin and neocarzinostatin (D'Andrea & Hazeltine 1978) but little sequence specificity was reported. One of the early compounds to demonstrate preferential cleaving of DNA with a sequence specificity was a penta-N-methylpyrrolecarboxamide-EDTA-Fe(II) (Schultz & Dervan, 1983). The finding that coupling of the synthetic footprinting tool methidium propyl EDTA to DNA binding ligands led to the synthesis of a number of sequence specific cleaving molecules (Dervan, 1986). Extension of the modified Maxam-Gilbert procedures has also been used to study the mechanism of repair of UV-induced pyrimidine dimers. It was previously thought that repair proceeded from a nick 5' to the pyrimidine dimer but it was

subsequently shown that the site of breakage actually occurred between the dimerized pyrimidines (Haseltine et al., 1980).

The strength of the chemical cleavage method can be attributed to the ease at which the technique can be carried out and that chemical modifications of guanine-N7 sites on the DNA are quantitatively converted to strand breaks (Mattes, et. al., 1986). The potential to evaluate DNA adduct distribution both in isolated DNA and in various parts of the genome may provide insight into specific DNA repair mechanisms. Unfortunately, simple alkylating agents produce other adducts in addition to guanine-N7 alkylations which cannot be analysed with this method (Lawley et al., 1975; Mattes, 1990).

1.8.1.2. Polymerase Inhibition

DNA adducts can act as a block to the action of certain polymerase enzymes (figure 1.12). Modification of the Sanger-dideoxy method has been utilised to measure adduct distribution for a variety of chemically reactive compounds (Sanger et al., 1977). It should be pointed out that dideoxy nucleotide triphosphates are not used in the evaluation of strand termination from drug treated DNAs. Studies have shown that E. Coli DNA polymerase is normally inhibited one nucleotide before sites of UV induced pyrimidine dimers (Moore et al., 1981). Other polymerase enzymes that have displayed the inability to bypass DNA adducts include AMV reverse transcriptase, alpha polymerase and Taq DNA polymerases (Moore & Strauss, 1979; Ponti et al., 1991a; Strauss et al., 1979). A weakness associated with techniques based on Sanger protocols is that drug treated duplex DNAs must be rendered single stranded and annealed to a primer prior to initiation of synthesis with the polymerase enzyme.

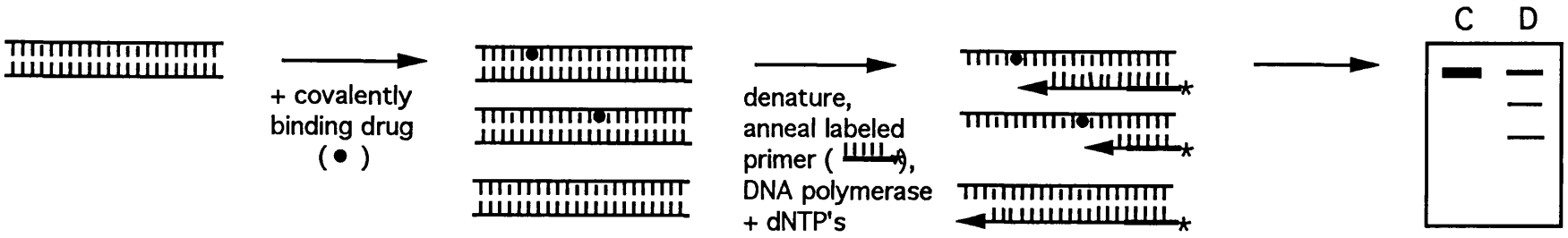


Figure 1.12 Polymerase assay used to determine the sequence specificity of covalently binding DNA ligands

The presence of spurious bands in control reactions is often observed and can be due to factors such as contaminating nucleases, tertiary DNA structure, and the presence of short fragments, all of which can cause premature termination of synthesis from the template DNA.

1.8.1.3. Enzymatic Digestion

The sequence selectivity of DNA reactive compounds has also been assessed by their ability to block the action of certain nucleases. In a typical experiment, end-labeled DNA fragments of known sequence are treated with the agent of interest and digested with an exonuclease. The presence of bulky DNA adducts can act as a barrier to further sequential DNA digestion. As with the chemical cleavage assays, the products of the reaction are usually separated on denaturing polyacrylamide sequencing gels and the presence of bands in the treated lanes give an indication of the sequence specificity of binding (figure 1.13).

The sites of DNA covalent modification have been analysed for a group of natural and synthetic pyrrolo-[1,4]benzodiazepines (PBD's) using their ability to inhibit the action of exonuclease III (Hurley et al., 1988). Anthramycin and tomaymycin are the best known examples of the PBD compounds and it has been proposed that these agents react with the N2 position of guanine in the minor groove of DNA. In a similar study, the binding sites of a number of cis-platin analogues have recently been studied for their ability to inhibit the 3' to 5' exonuclease activity of T4 polymerase using a synthetic DNA duplex (Farrell et al., 1990). Binding of a compound to a restriction endonuclease recognition site can interfere with the ability of the enzyme to cut DNA at that site.

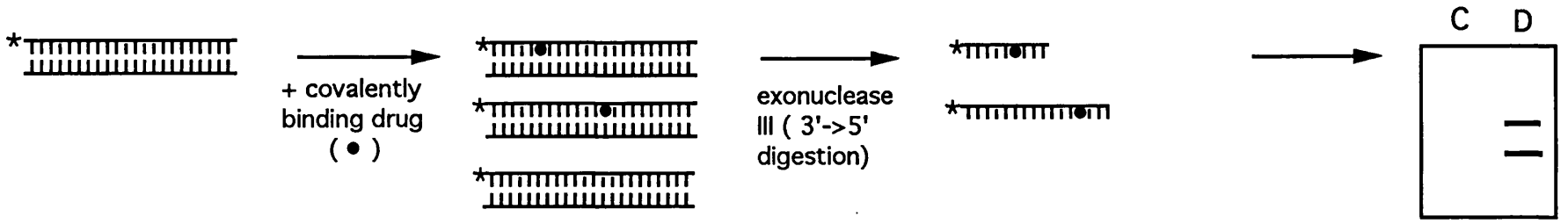


Figure 1.13 Exonuclease method for determining the sequence specificity of covalent drug binding.

Using this approach, restriction enzymes have been used to study the sequence binding sites of N-ethyl-nitrosourea (ENU) and N-methyl-N-nitrosourea (MNU) (Ahmed & Hadi, 1988). The conversion of plasmid DNA from open circular to linear form was shown to be inhibited by the presence of a guanine-O6 adduct located in the restriction site of PstI (Green et al., 1984). More recent studies have provided information on the base sequence selectivity of anthracycline compounds by taking advantage of their ability to protect the cleavage of plasmid DNA by restriction endonucleases (Barcelo & Miro, 1991).

An interesting technique to evaluate the effect of DNA sequence on guanine adduct formation was demonstrated using several synthetic oligomers with identical base composition but differing in the position of the guanine residue in the sequence (Briscoe & Cotter, 1984; Briscoe & Cotter, 1985). Essentially, duplex oligonucleotides of defined sequence were alkylated and subsequently hydrolysed with acid to liberate O6 alkyl- and N7-guanine residues. The relative amounts of both adducts were quantitated by high pressure liquid chromatography and the ratio of adducts were compared for each oligomer. In this way, the influence of DNA sequence on formation of O6 and-N7 guanine adducts can be evaluated. An analogous method for analysing sequence specificity of DNA adduct formation in synthetic oligonucleotides was described by (Richardson et al., 1989). Oligonucleotides of identical sequence but differing in the position of a radiolabelled guanine nucleoside were independently alkylated and enzymatically digested. The hydrolysed products are then separated by HPLC and fractions collected for liquid scintillation counting. Although the method suffers from a lack in sensitivity, it offers the potential to study the effect of sequence on adduct

distribution and repair for lesions at different bases in the same duplex oligomer.

1.8.1.4. Footprinting

DNA footprinting was first used to determine the sequences in DNA which were bound by proteins (Drew & Travers, 1984; Galas & Schmitz, 1978). Essentially, protein bound to a singly end-labeled DNA fragment of known sequence is subjected to limited cleavage with DNAse. DNA bound protein prevents the nuclease from cutting the DNA at the binding site. Separation of the cleavage products on a DNA sequencing gel shows bands at each base position for regions not protected by protein (figure 1.14). In contrast, the DNA sequence bound by protein is revealed by an absence of bands in the sequencing gel. The principle of DNA footprinting can also be extended to determine the sites of drug-DNA contacts. Although the sequence specificity of some non covalently binding compounds have been evaluated using the DNAse footprinting method (Lane et al., 1983), it is not the method of choice due to the slight preference for DNAse I to cleave DNA at certain sequences (Hertzberg & Dervan, 1984). The finding by Dervan and co-workers that methidiumpropyl EDTA (MPE) cleaves DNA with almost no sequence specificity prompted the use of this reagent to define the sequence specificity of actinomycin D and distamycin (Dervan, 1986; Hertzberg & Dervan, 1984; Van Dyke et al., 1982). MPE also has the advantage of increased resolution over DNAse I for determining the binding of smaller molecules such as many of the antitumour drugs.

In 1986, Tullius and Dombroski described a simple hydroxyl radical footprinting method to precisely define DNA-protein contacts. This

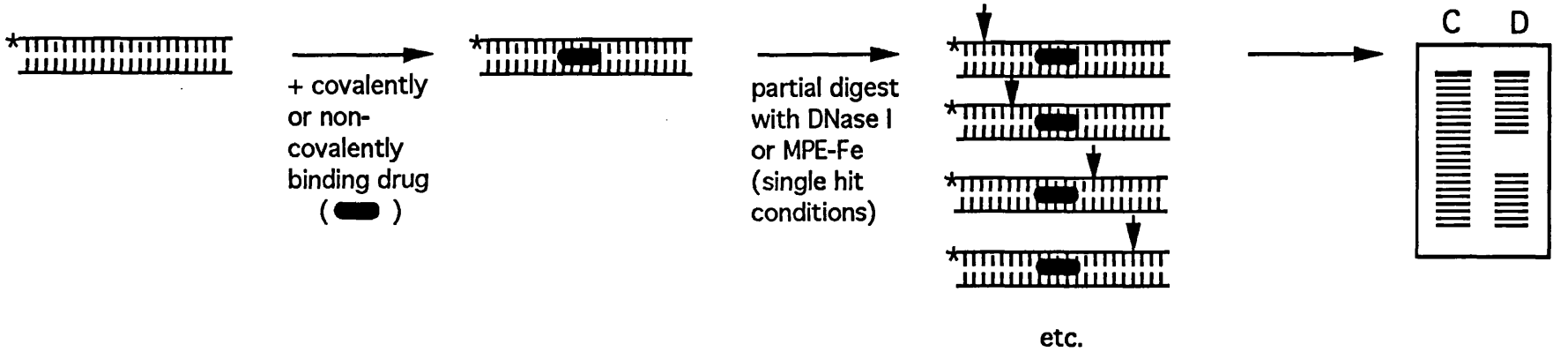


Figure 1.14 Footprinting technique for the determination of the sequence specificity of non-covalently binding DNA ligands

approach had an important advantage over both the DNAse I and MPE footprinting methods in that the production of hydroxyl radicals could be produced simply from readily available chemicals (Tullius & Dombroski, 1985; Tullius & Dombroski, 1987).

1.8.2. Cross-Linking Specificity

Although there are a variety of techniques available to measure the sequence specificity of monoalkylation induced by bifunctional agents, much less information has been obtained concerning the sequence preferences for the formation of DNA interstrand cross-links. The principle challenge in determining the cross-linking specificities of bifunctional agents is that the cross-link is only a minor component of a complex mixture, due to the inefficiency of many bifunctional agents.

Several enzymatic methods have been applied to the determination of the sequence specificity of DNA interstrand cross-links. For example, sites of DNA interstrand cross-links have been evaluated using the double strand specific 3'-5' exonuclease activity of Bal31 (Zhen et al., 1986). Treatment of cross-linked DNAs with endonucleases and polymerases have also been used to study patterns of cross-link formation. In the absence of deoxynucleotide triphosphates, the 3' to 5' exonuclease activity associated with T4 DNA polymerase was shown to be blocked by psoralen monoadducts and cross-links in DNA of defined sequence (Sage & Moustacchi 1987; Boyer et. al., 1988). Piette and Hearst have demonstrated that the *E. coli* DNA polymerase I haloenzyme is capable of bypassing psoralen induced DNA monoadducts but not DNA interstrand cross-links using a nick translation assay (Piette & Hearst 1983). The polymerase and exonuclease techniques are particularly informative in that they allow the evaluation of many

potential cross-link sites simultaneously. In general, most enzyme assays can provide some insight into the sequence specificity of cross-linking but are often limited by the resolution of the resulting fragments and an uncertainty as to whether these enzymes can be inhibited with different types of DNA damage inflicted by other bifunctional agents. These methods show inhibition of enzymes at sites of DNA covalent modification but may not be specific for sites of DNA cross-links.

Another approach which has been used to investigate the sequence preferences for DNA cross-link formation involves the reaction of bifunctional agents with synthetic oligonucleotides of defined sequence. Separation of cross-linked fragments from uncross-linked fragments can be achieved using denaturing PAGE in thin sequencing gels. The presence of a high concentration of urea in denaturing polyacrylamide gels and the increased running temperature during electrophoresis ensures untreated DNA fragments migrate as single strands through these gels. The formation of a cross-link in a duplex DNA fragment will prevent the complete denaturation of the two complementary strands during separation and thus cross-linked DNAs will migrate with a diminished mobility through the gel. Radiolabeling of the DNA prior to electrophoretic separation affords a high degree of sensitivity for the detection of cross-linked products which often constitute only a very small percentage of the total reaction products. In a sample of DNA in which some of the duplexes are cross-linked, two bands will appear upon separation with the cross-linked fragments migrating with a diminished electrophoretic mobility. Cross-linking in synthetic DNA fragments and separation of the reaction products using denaturing PAGE has been reported for a number of agents including nitrogen mustard and mitomycin C (Cera et al., 1989; Hopkins et

al., 1991; Millard et al., 1990; Millard et al., 1991; Millard et al., 1990; Teng et al., 1989; Weidner et al., 1990).

Although denaturing PAGE readily separates cross-linked DNA from uncross-linked DNA, no information is obtained regarding the exact site of cross-linking at the atomic or nucleotide level. Instead, the sequence preference for cross-linking is inferred since the sequences of the duplex oligonucleotides are known. In some cases, the cross-linked product has been excised from the gel and subjected to chemical cleavage reactions in an effort to determine the exact site of linkage. A method for locating the preferred site of DNA interstrand cross-linking for nitrogen mustard has recently been described using a piperidine fragmentation assay (Millard et al., 1990). It was found that nitrogen mustard preferentially cross-linked DNA duplexes through deoxyguanosine residues in a 5'-GNC sequence and not at 5'-CG sequences as previously thought (Brooks & Lawley, 1961; Brooks & Lawley, 1963). A similar approach for the determination of DNA cross-linking sequence specificity at single nucleotide resolution has been described using hydroxyl radical footprinting techniques (Weidner et al., 1989). The method involves separation of singly end labeled cross-linked DNA oligonucleotides by denaturing PAGE in an initial preparative step. The cross-linked DNA is then subjected to random fragmentation using hydroxyl radical. Only cleavage between the radiolabel and the cross-linked residue will generate fragments shorter than the full length single strand. Fragmentation at any other position in the cross-linked duplex will result in much larger fragment due to the presence of the cross-link. Hence a discontinuity is produced in the banding pattern produced from a cross-linked fragment which is diagnostic for the site of cross-linking. With this technique, the sequence preferences for the formation of DNA cross-links in synthetic duplexes has been reported for a number of agents

including nitrogen mustard, cis-platin, mitomycin C and a series of structural analogues of mitomycin C (Hopkins et al., 1991; Millard et al., 1991; Weidner et al., 1990).

The strength of this method lies in the fact that hydroxyl radicals are thought to cleave DNA by extracting a hydrogen atom from the ribose sugar of the DNA backbone and thus cleave DNA with almost no sequence specificity (Tullius & Dombroski, 1987). Unfortunately, the efficiency of the fragmentation reaction is quite low and requires a great deal of radioactively labeled cross-linked product to determine the site of cross-linking. In addition, the detection of the site of cross-linking is at the nucleotide level and no information is gained about the nature of the cross-link at the atomic level.

1.9. Objectives for Experimental Work

The continuing importance of bifunctional alkylating agents in cancer chemotherapy together with their ability to cross-link DNA underlines the necessity for suitable methods to study this important aspect of drug DNA interaction. A variety of techniques has been utilised for the detection and measurement of DNA interstrand cross-linking. However, with the continuous development and synthesis of new cross-linking agents it becomes increasingly impractical, time consuming and expensive to assess the extent of cross-linking using existing techniques. In addition, many of the techniques used to detect DNA cross-linking in isolated DNA lack sensitivity and are generally not well suited to quantitative analysis. As a result, one of the objectives of this study was to develop a simple and sensitive assay to evaluate the extent and rate of DNA cross-link formation applicable to all cross-linking agents.

The second objective of this study was to gain some insight into the sequence preferences for the formation of DNA interstrand cross-links induced by structurally distinct bifunctional agents. Although the sequence preference for monoalkylation has been well documented for many of the alkylating agents, the sequence actually cross-linked by these compounds is much less clear. The studies outlined below attempt to unambiguously define the sequence preferences for the formation of DNA cross-links induced in synthetic DNA fragments using a piperidine fragmentation assay and denaturing polyacrylamide gel electrophoresis.

CHAPTER 2: DEVELOPMENT AND EVALUATION OF AN AGAROSE GEL BASED METHOD FOR THE DETECTION OF DNA INTERSTRAND CROSS-LINKS

The development and implementation of an agarose gel based assay to measure interstrand cross-links in isolated DNA is described. The technique is based on the principle that, following complete denaturation, DNA fragments containing a covalent cross-link can be separated from uncross-linked fragments upon electrophoresis (figure 2.1). An untreated double stranded DNA fragment will migrate as a single band upon neutral agarose gel electrophoresis. Upon denaturation, the DNA becomes single stranded and migrates with an increased mobility through the gel relative to that of double stranded DNA . The presence of a cross-link between the two complementary strands of a DNA fragment will prevent complete separation of the strands upon denaturation and will serve as a point for the re-association of the DNA upon renaturation (Eigen & Porschke 1970). Thus, DNA fragments containing a cross-link will migrate as double stranded through an agarose gel. For example, if 50% of the DNA fragments in a given sample are cross-linked, two bands of equal intensity but different electrophoretic mobility will appear in the gel. The slower migrating band represents the fraction of DNA fragments which are cross-linked while the band migrating with increased mobility comprises the remaining uncross-linked single-stranded DNA. Quantitation of the amount of DNA migrating as double stranded in a drug treated sample provides an accurate measurement of the extent of DNA cross-linking.

The following studies describe the development and evaluation of an agarose gel based assay for the measurement of DNA interstrand cross-links induced by several different classes of bifunctional alkylating

agents. Among the agents studied were the clinically useful nitrogen mustard compounds and platinum complexes, as well as recently developed bifunctional agents of the aziridinylbenzoquinone and pyrrolobenzodiazepine class.

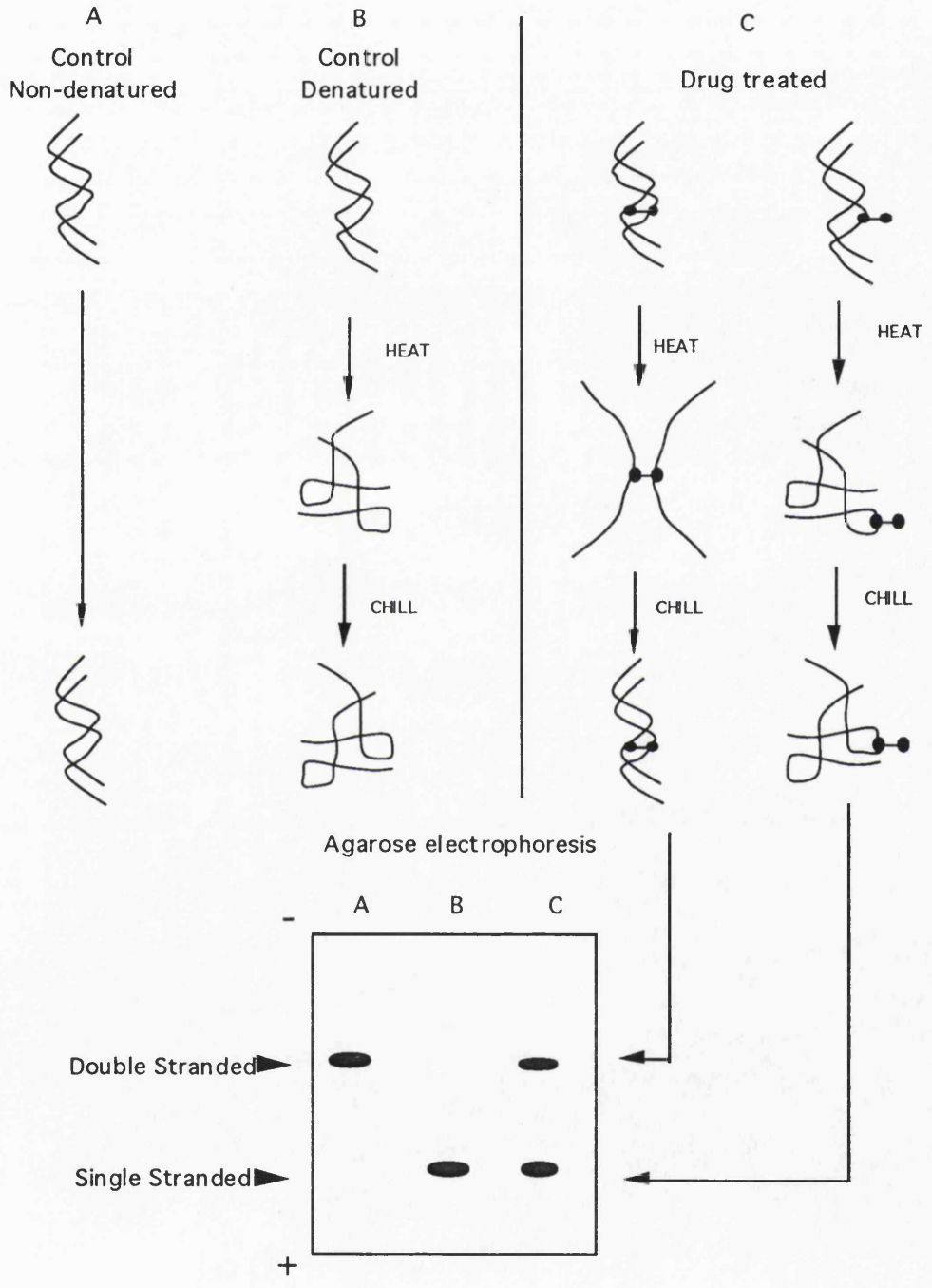


Figure 2.1. Schematic diagram of the agarose gel based cross-linking assay.

2.1 Materials

DNA:

Plasmid pBR322 DNA: (500 mg/ml) was obtained from Northumbria Biologicals (NBL) and stored in 10 mM Tris-HCl, pH 7.8, and 1mM EDTA at -20^{o} C.

Enzymes:

Hind III: 10 units/ml in 10 mM Tris-HCl, pH 7.4; 0.2 M NaCl; 0.5 mM EDTA; 1 mM DTT; 0.5 mg/ml BSA. Purchased from BRL.

T4 polynucleotide kinase (PNK): 5 units/ml in 50 mM Tris-HCl, pH 7.5; 25 mM KCl; 5 mM DTT; 0.1 mM ATP; 50% v/v glycerol; 0.2 mg/ml BSA). Purchased from BRL .

Bacterial alkaline phosphatase (BAP): 100 units/ml in 10 mM Tris-HCL, pH 8.0; 0.12 M NaCl; 50% v/v glycerol. Purchased from BRL

Radioisotope:

[g - ^{32}P] ATP: 5000 Ci/mmol, 10 mCi/ml was obtained from Amersham International.

General buffers:

Hind III (10x): 50 mM Tris-HCl, pH 8.0; 10 mM $MgCl_2$ and 50 mM NaCl.

PNK (5x): 200 mM Tris-HCl, pH 7.6; 50 mM $MgCl_2$; 25 mM DTT; and 5 mM ATP.

BAP (5x): 50 mM Tris-HCl, pH 8.0; 600 mM NaCl.

TEA: 25 mM Triethanolamine, 1 mM EDTA.

TE: 10 mM Tris, pH 7.2; 1 mM EDTA.

Alkylation stop solution: 0.6 M sodium acetate, 20 mM EDTA and 0.1 mg/ml yeast tRNA.

Agarose gel buffers:

TAE (running buffer) : 40 mM Tris, 20 mM acetic acid, 2 mM EDTA, pH 8.1.

Strand separation loading buffer: 30% dimethyl sulphoxide; 1 mM EDTA; 0.04% bromophenol blue; 0.04% xylene cyanol in 1x TAE.

Non-denaturing loading buffer: 0.6% sucrose, 0.04% bromophenol blue in 1x TAE.

Chemicals:

Ultrapure agarose, Tris base, disodium EDTA, phenol, and chloroform were purchased from BRL.

Drugs:

Mechlorethamine, melphalan and cisplatin were obtained from Sigma. Carboplatin was purchased from Bristol Laboratories, UK. Uracil mustard was from the Upjohn Co. Phosphoramide mustard and isophosphoramide mustard were from the Drug Synthesis and Chemistry Branch, Division of Cancer Treatment, National Cancer Institute, USA. Aziridinylbenzoquinones were provided by Dr. J. Butler at the Patterson Institute for Cancer Research, Manchester, UK. Pyrrolobenzodiazepine compounds were obtained from Dr. D. Thurston at the University of Portsmouth, UK.

Mechlorethamine and cisplatin were dissolved in ultrapure dH_2O and used immediately. Diaziridinylbenzoquinones, uracil mustard, melphalan, phosphoramide mustard and isophosphoramide mustard were dissolved in dimethyl sulphoxide. The pyrrolobenzodiazepine compounds were resuspended in HPLC grade methanol. Vehicle concentrations did not exceed 2% in reaction mixtures.

2.2 Methods

2.2.1. Preparation of DNA

2.2.1.1. Linearisation of pBR322

Approximately 20 µg of closed circular pBR322 DNA was digested with 30 units of Hind III in 60 µl of 1x Hind III buffer. The reaction was carried out at 37° C for 30 min. Following enzymatic digestion, an equal volume of buffer saturated (TE pH 7.2) phenol was added to the sample and briefly mixed by use of a vortex mixer. The sample was then briefly centrifuged to separate the aqueous and organic phases. The upper aqueous phase was carefully removed and placed into a clean 1.5 ml microfuge tube. An equal volume of TE buffer was added to the organic phase, vortex mixed and briefly centrifuged. The aqueous phase was removed from above the phenol and combined with the aqueous phase from the first extraction. Any traces of phenol were removed from the aqueous phase by adding an equal volume of chloroform: isoamyl alcohol (24:1), vortex mixed and briefly centrifuged. The upper aqueous phase was removed as before and placed in a clean 1.5 ml centrifuge tube. Extraction with chloroform: isoamyl alcohol (24:1) was repeated and the DNA sample subsequently ethanol precipitated. Unless otherwise specified ethanol precipitation was performed by adjusting the DNA solution to 0.3 M in sodium acetate and 1 mM EDTA prior to addition of 3 volumes of 95% ethanol. The DNA/salt containing sample was then thoroughly mixed using a vortex mixer and immersed into a dry ice/95% ethanol bath for at least 15 min. Precipitated DNA was subsequently centrifuged for 15 min at 4° C. The supernatant was carefully removed by tilting the microfuge tube slightly and drawing off the liquid with a pasteur pipet. Any remaining ethanol was evaporated under vacuum. The DNA pellet was washed with 100 µl of ice cold 70%

ethanol, dried briefly under vacuum and resuspended in 50 µl of distilled deionised water.

2.2.1.2. Dephosphorylation

Dephosphorylation reactions were carried out in 100 µl of 1x BAP buffer containing 10 units of BAP. The sample was incubated for 60 minutes at 65° C and allowed to cool slowly on the bench to room temperature. The dephosphorylated DNA was extracted 2x with one volume of buffer saturated phenol (TE pH 7.2) and 2x with one volume of chloroform: isoamyl alcohol (24:1). The sample was then ethanol precipitated, dried and washed with 100 µl of 70% ethanol. All traces of ethanol were carefully removed with a pasteur pipet. The sample was then briefly dried under vacuum and resuspended in 50 µl of distilled deionised water.

Mini-gel Analysis: A small representative sample of DNA (1 µl) was analysed after each DNA modification step to check that the enzyme modifications did not damage the DNA. The samples from each step were ethanol precipitated and resuspended in either 5 µl of denaturing or non-denaturing buffer. Electrophoreses was through a 0.8% agarose mini-gel at 50V for 3 h on a 10 cm support. Samples were stained in 0.001 mg/ml ethidium bromide in distilled water for 1 h at 20° C. Excess ethidium bromide was removed by soaking the gel in distilled water for 30-40 minutes. DNA was visualised under UV light using a transilluminator.

2.2.1.3. Determination of DNA Concentration

A small aliquot (5 µl) of the Hind III, BAP treated DNA was removed and diluted to 1 ml with dH_2O (1 : 200 dilution). The absorbance of the solution

was measured at 260 nm. An absorbance of 1.0 OD was assumed to correspond to a double stranded DNA concentration of 50 μg/ml (Maniatis et al., 1982).

2.2.1.4. 5' End Labeling

Approximately 1 μg of Hind III, BAP treated pBR322 DNA was labeled in 20 μl of forward reaction buffer containing 20 uCi of [γ -^{32}P] ATP and 5 units of T4 polynucleotide kinase. The sample was incubated at 37^{o} C for 45 minutes prior to ethanol precipitation.

The DNA was precipitated a second time by resuspending the pellet in 50 μl of 0.3 M sodium acetate, 10 mM EDTA and adding 3 volumes of 95% ethanol. After the second precipitation, the pellet was washed with 100 μl of cold 70% ethanol, dried briefly under vacuum and resuspend at 10 ng/μl in distilled deionised water. The labeled DNA stock solution was stored shielded at -20^{o} C until required.

2.2.2. Drug Treatments

Reactions were carried out in TEA buffer containing 10-25 ng of DNA at 37^{o} C in the appropriate volume. Reactions were terminated by addition of an equal volume of alkylation stop solution and ethanol precipitated. After careful removal of the supernatant, the DNA pellet was dried briefly under vacuum.

2.2.3. Preparation of Agarose Gels

Agarose solutions (0.8% w/v) were prepared by bringing 2.4 g of ultrapure agarose to 300 ml with TAE buffer in a 500 ml conical flask. The agarose was dissolved by heating the solution in a microwave oven on high setting for 4 minutes with occasional swirling until all agarose had dissolved. Alternatively, the solution was placed on a hot plate with constant stirring until all agarose was dissolved. For mini-gels 0.24 g of agarose was brought to 30 ml and prepared as described above. The hot solution was allowed to cool to 50° C before pouring it into a 20 x 25 cm gel support containing well forming combs.

2.2.4. Electrophoresis

Samples were resuspended in 10 µl of strand separation buffer by use of a vortex mixer and briefly centrifuged to remove all buffer from the microfuge tube walls. The samples were then heated to 90° C for 2 minutes and immediately immersed in an ice/water bath. Control undenatured samples were resuspended in 10 µl of non-denaturing buffer, briefly centrifuged and loaded directly into the gel.

Electrophoresis was carried out using a 0.8% submerged agarose gel (20 cm x 25 cm x 0.5 cm) in TAE buffer. Samples were initially electrophoresed at 100 V for 5 min and then at 40 V for 14-16 hr.

2.2.5. Autoradiography

Gels were dried for 2 h at 80° C onto one layer of Whatman 3 MM and one layer of DE81 filter papers on a Bio-Rad model 583 gel dryer under vacuum.

Autoradiographic images were obtained after exposure of Hyperfilm MP (Amersham) to the dried gel for 4-6 h at -70°C using a DuPont-Cronex Lightening-plus intensifying screen. Alternatively, film was exposed to the dried gel without a screen to obtain sharper images.

2.2.6. Densitometry

Microdensitometry of the autoradiogram was performed using a Pharmacia LKB enhanced Ultrascan-XL laser densitometer. Integration and quantitation was carried out using Pharmacia GSXL 2.1 software. Calculation of percentage double stranded DNA was determined by the following equation;

% Cross-linked DNA = $\{A_{(ds\ DNA)}/[A_{(ds\ DNA)} + A_{(ss\ DNA)}]\} \times 100$; where $A_{(ds\ DNA)}$ is the area under the curve calculated for DNA migrating as double stranded and $A_{(ss\ DNA)}$ corresponds to the area under the curve for DNA migrating as single stranded.

2.2.7. Spectrophotometry

Spectrophotometric analysis of quinone reduction by DT-Diaphorase was carried out on a Hewlett Packard 8452A diode array spectrophotometer under aerobic conditions. Reactions were performed in 1 ml of TEA buffer (pH 5.8) containing 100 μM NADH and 100 μM quinone. The rate of reduction was measured at the absorbance maximum for the quinone-NADH mixture and was initiated by the addition of 1 μl of DT-Diaphorase (0.173 μg).

2.3 Development of the Assay

2.3.1. Determination of Optimal DNA Denaturing Conditions

Linearised double stranded pBR322 DNA (4362 bp) migrates as a single band through a 0.8% agarose gel under non-denaturing conditions. Under denaturing conditions the DNA becomes single stranded and migrates with increased mobility through the gel relative to that of undenatured DNA. To assess the extent of cross-linking induced by a given agent it is desirable to achieve complete denaturation of the control DNA. In order to develop a consistent and reliable method for denaturing double stranded DNA, both alkaline and thermal denaturation conditions were evaluated.

In order to determine the optimal conditions for thermal denaturation, native DNA was incubated in strand separation buffer for various times and temperatures and immediately immersed in an ice water bath prior to electrophoresis. Figure 2.2A shows that after 2 min at 90^{o} C, more than 95% of the DNA becomes denatured and migrates as single stranded upon electrophoresis. In contrast, native DNA was not denatured after 2 min at temperatures less than 65^{o} C.

The ability of alkali to denature double stranded DNA was investigated using electrophoresis sample buffer containing various concentrations of sodium hydroxide figure 2.2B. DNA samples were resuspended in 10 µl of alkaline buffer, allowed to stand at room temperature for 2 minutes and loaded directly into the gel. Alkaline electrophoresis buffer containing at least 100 mM NaOH was required to achieve complete denaturation of the DNA while only partial denaturation was observed at 50 mM. Partial degradation of the DNA incubated with 500 mM could be detected upon electrophoresis.

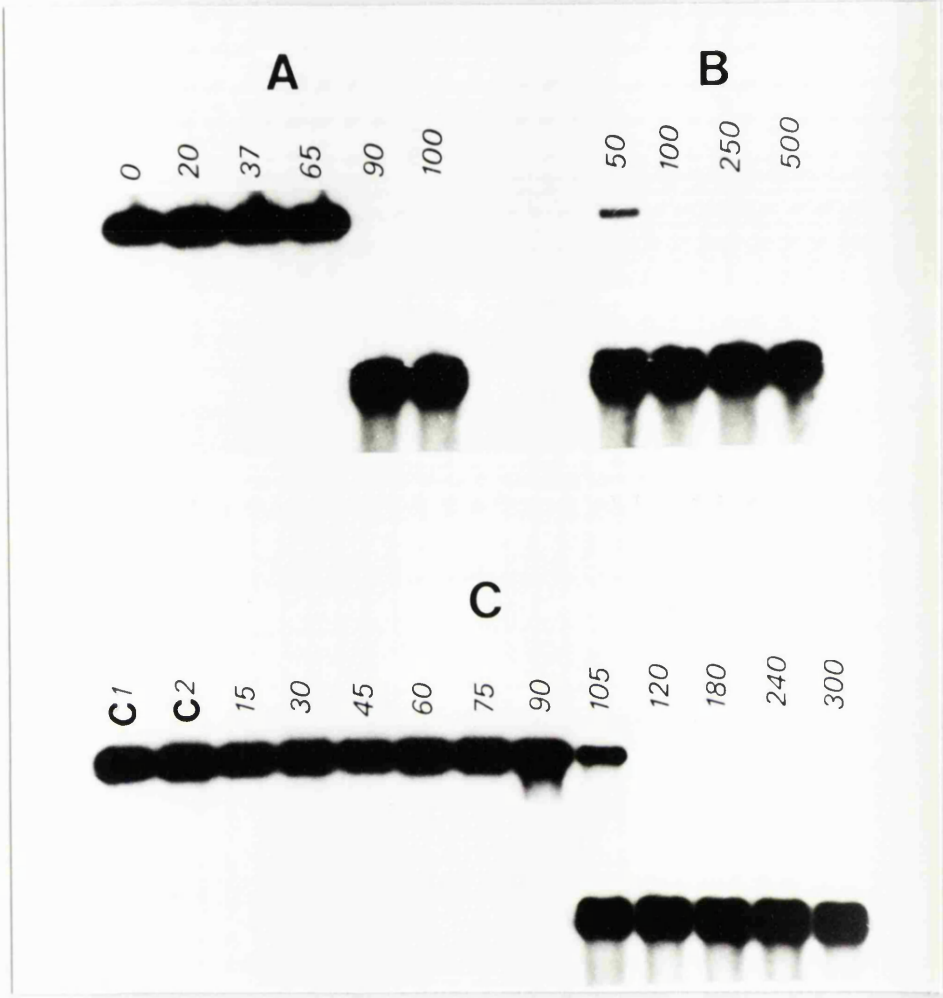


Figure 2.2 A) Thermal denaturation of DNA in strand separation buffer for 2 min at the indicated temperature (^{o}C). B) Alkaline denaturation of DNA incubated for 2 min in alkaline buffer containing 6% sucrose, 0.4% bromophenol blue, 0.4% xylene cyanol at the indicated concentration of NaOH (mM). C) Time course for the thermal denaturation of DNA at 90^{o} C in strand separation buffer. C1 was control DNA in non-denaturing buffer and C2 was control DNA in strand separation buffer. Both C1 and C2 were kept at 0^{o} C prior to electrophoresis.

A time course for the thermal denaturation of native DNA in strand separation buffer was carried out at 90° C to determine the minimum time required to achieve complete denaturation figure 2.2C. Partial denaturation of the DNA could be detected after 90 seconds at 90°C and DNA became completely denatured after 2 min. In addition, no thermal degradation of the denatured DNA was observed at incubation times up to 5 minutes.

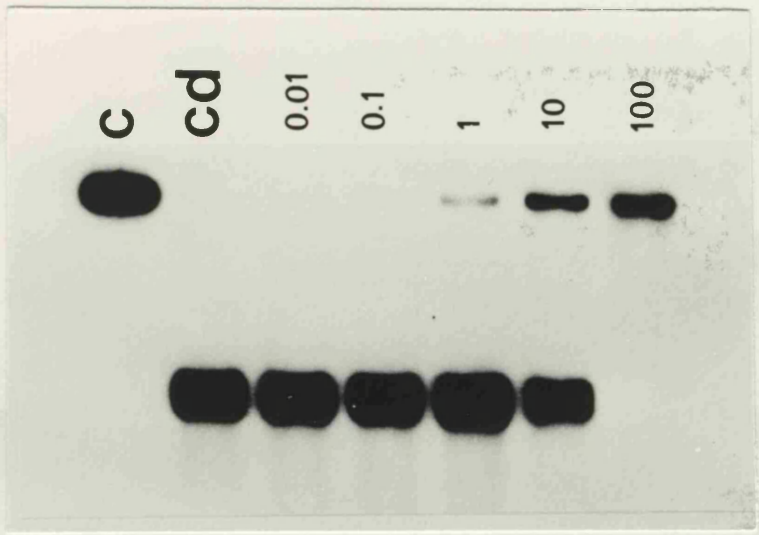
2.3.2. Applications

2.3.2.1. Dose Response

The formation of cross-links induced in DNA by bifunctional agents can be evaluated as a function of drug concentration. Dose response experiments were carried out in TEA buffer containing 10-25 µg of DNA in a final volume of 50 µl for a range of drug concentrations. Figure 2.3a shows the formation of cross-links induced in DNA by different concentrations of melphalan. Quantitation of the amount of cross-linking can be determined by microdensitometry of the autoradiogram. The resulting data can be expressed as the extent of cross-linking as a function of drug concentration and is shown in figure 2.3b for melphalan.

2.3.2.2. Time course studies

DNA cross-linking can be measured as a function of time by incubating the drug with DNA and terminating the reaction at defined time points by ethanol precipitation. Time course studies were carried out at 37° C by incubating the drug with 1-2.5 ng of labeled DNA/µl of TEA buffer. At defined times, a 10 µl aliquot was removed from the reaction mixture and



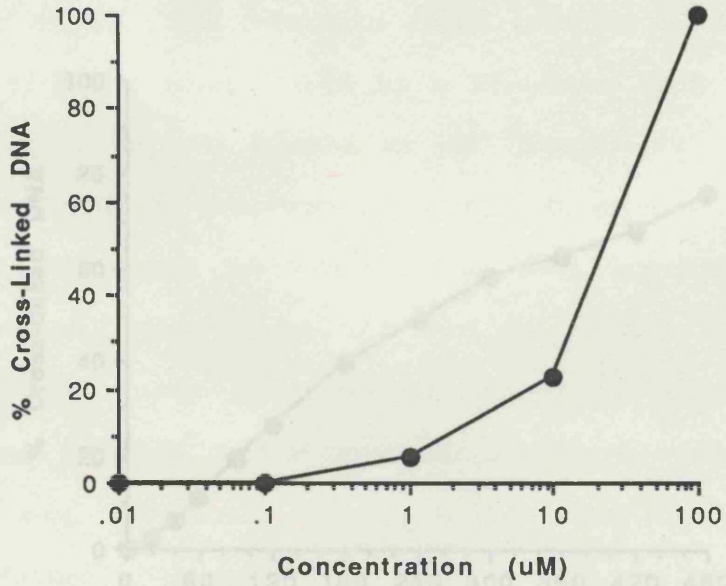
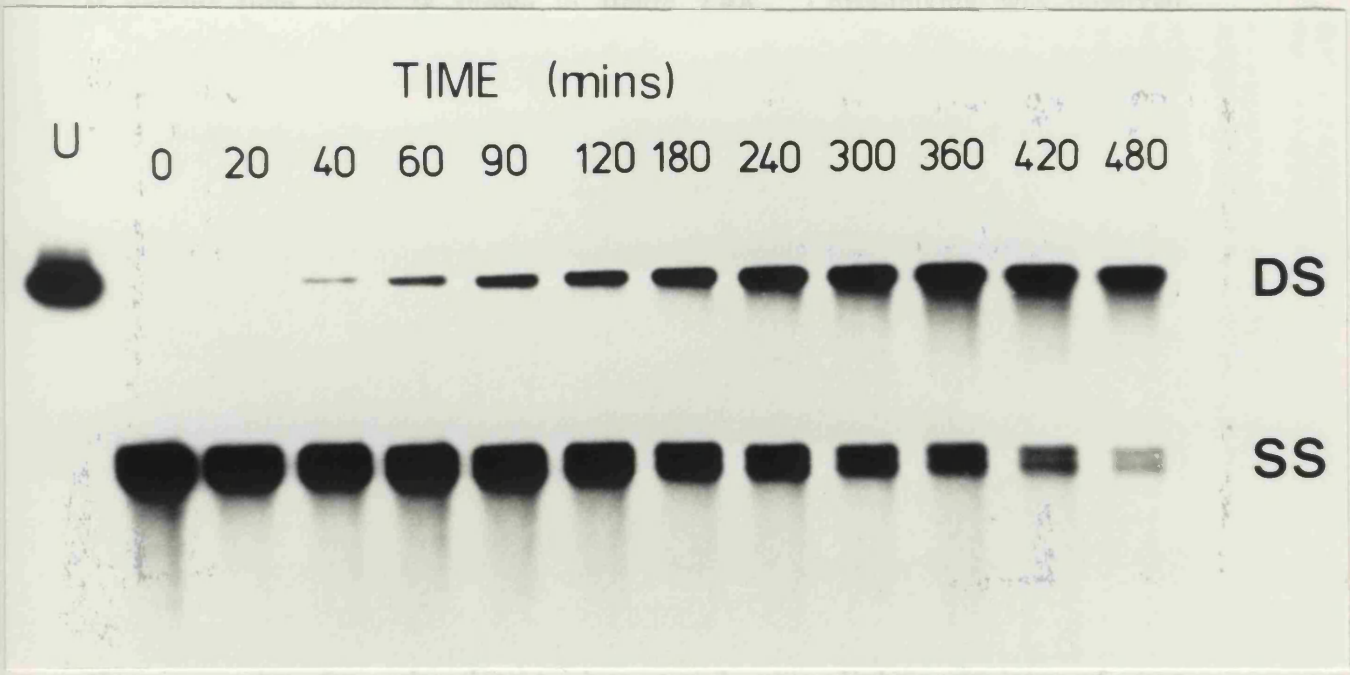


Figure 2.3 A) Autoradiograph showing cross-linking induced in DNA after 2 hr by various concentrations of melphalan (μM). B) Graph representing the results as the percent cross-linked DNA versus concentration.



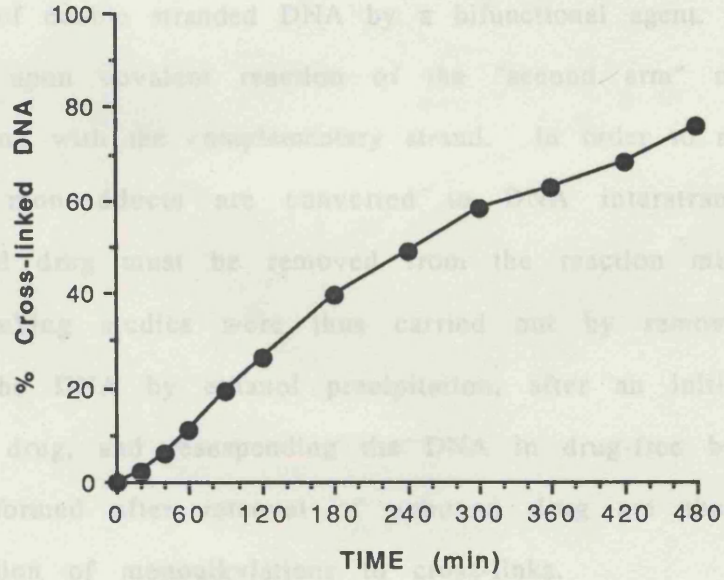


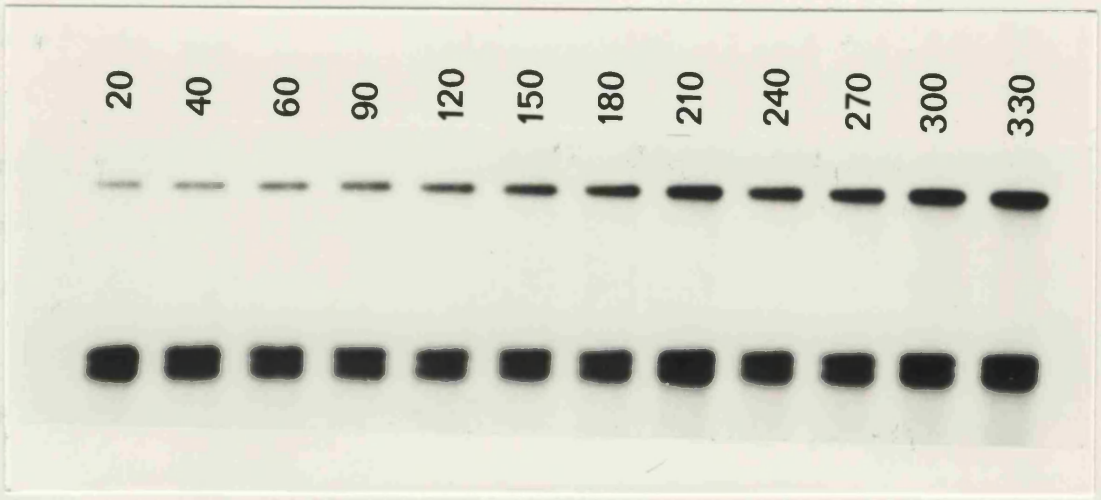
Figure 2.4 A) Autoradiograph showing the time course for DNA cross-linking induced by 10 μM melphalan. U indicates untreated nondenatured DNA; DS, double stranded; SS, single stranded. B) Graph representing the results as the percent cross-linked DNA versus time.

ethanol precipitated. The extent of DNA cross-linking for 10 μM melphalan at various time points is shown in figure 2.4A. Cross-linking was observed to increase with time and did not reach a plateau after 8 hr. Following quantitation of the autoradiograph by densitometry, the data from time course studies can be expressed as the percentage of cross-linked DNA as a function of time. Results for melphalan are shown graphically in figure 2.4B.

2.3.2.3. Second Arm Cross-Link formation

Dose response and time course experiments give information on the overall extent and rate of DNA cross-link formation in the presence of excess drug. However, the formation of DNA interstrand cross-links consists of two separate events. The first event occurs upon the initial alkylation on one strand of double stranded DNA by a bifunctional agent. The second event occurs upon covalent reaction of the "second arm" of the bifunctional compound with the complementary strand. In order to measure the rate at which monoadducts are converted to DNA interstrand cross-links, all unbound drug must be removed from the reaction mixture. Second arm cross-linking studies were thus carried out by removing unbound drug from the DNA by ethanol precipitation, after an initial incubation with excess drug, and resuspending the DNA in drug-free buffer. Any cross-links formed after removal of unbound drug are therefore due to the conversion of monoalkylations to cross-links.

Figure 2.5A shows cross-links induced by melphalan at defined times points after removal of free drug. After an initial incubation of 60 min in the presence of 10 μM drug, approximately 10% of all DNA fragments in the sample are cross-linked. The amount of cross-linked DNA in the sample



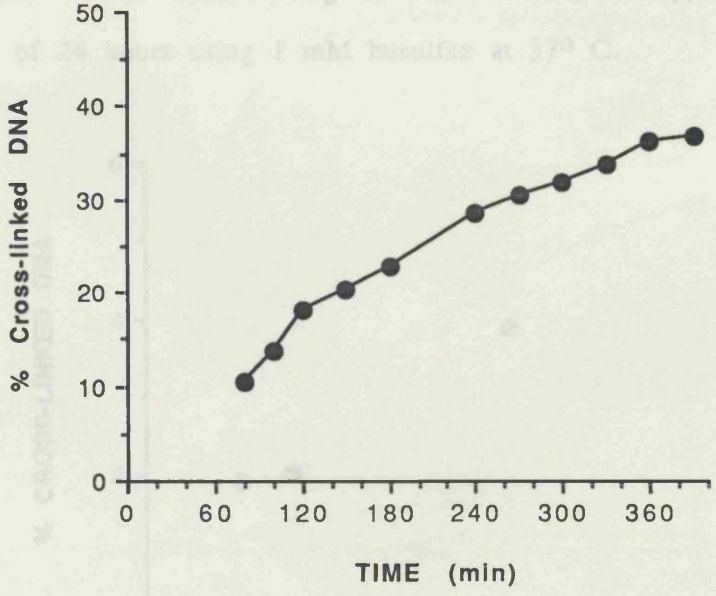


Figure 2.5 A) Autoradiogram showing a time dependent increase in DNA cross-link formation upon removal of unbound melphalan (10 μM) at 60 min. The DNA sample was resuspended in drug-free buffer and stopped at the indicated times (min). B) Results expressed as the percent cross-linked DNA with time. Cross-links formed after removal of unbound drug represent the conversion of monoadducts to cross-links.

increased 30% to nearly 40% nearly 5 hours after removal of unbound drug (figure 2.5B).

2.3.3. Sensitivity of the Assay

Nitrogen mustards such as melphalan are efficient cross-linking agents. However, the assay is sensitive enough to measure cross-links induced in DNA by relatively slow and inefficient cross-linking compounds such as the clinically used antitumour agent busulfan. Busulfan is a bifunctional alkylating agent capable of forming cross-links in DNA but the formation of busulfan cross-links has not previously been demonstrated using physicochemical methods such as caesium chloride gradients. Figure 2.6 shows the time course for the formation of DNA cross-links induced by busulfan. DNA cross-linking increased slowly to approximately 5% over a period of 24 hours using 1 mM busulfan at 37° C.

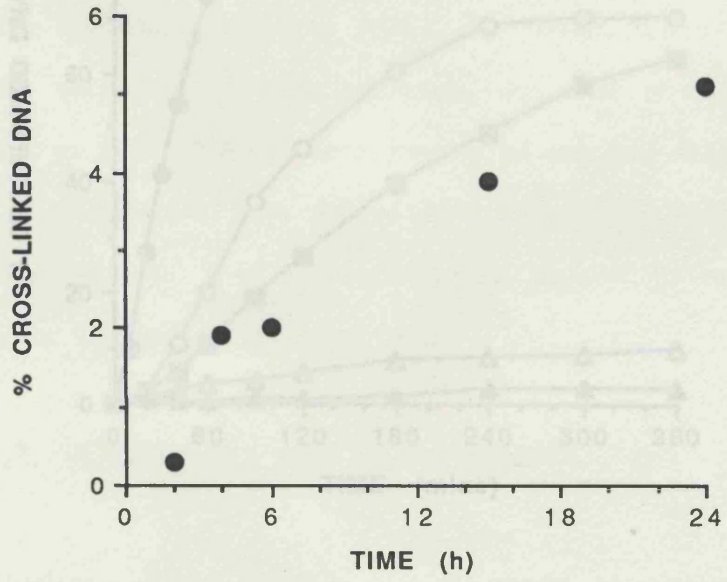


Figure 2.6 Time course for the formation of DNA cross-links induced by 1mM busulfan at 37° C.

2.4 Evaluation of DNA Cross-linking Induced by Bifunctional Agents

2.4.1. Nitrogen Mustards

A number of nitrogen mustard analogues were evaluated for their ability to induce cross-links in DNA using agarose gel electrophoresis. Figure 2.7 shows the time course for the formation of DNA cross-links induced by five nitrogen mustard compounds at equimolar concentrations. The initial rate and overall extent of cross-linking varied greatly for all five mustards with mechlorethamine clearly the most efficient cross-linking agent followed by uracil mustard, melphalan, phosphoramide mustard and isophosphoramide mustard.

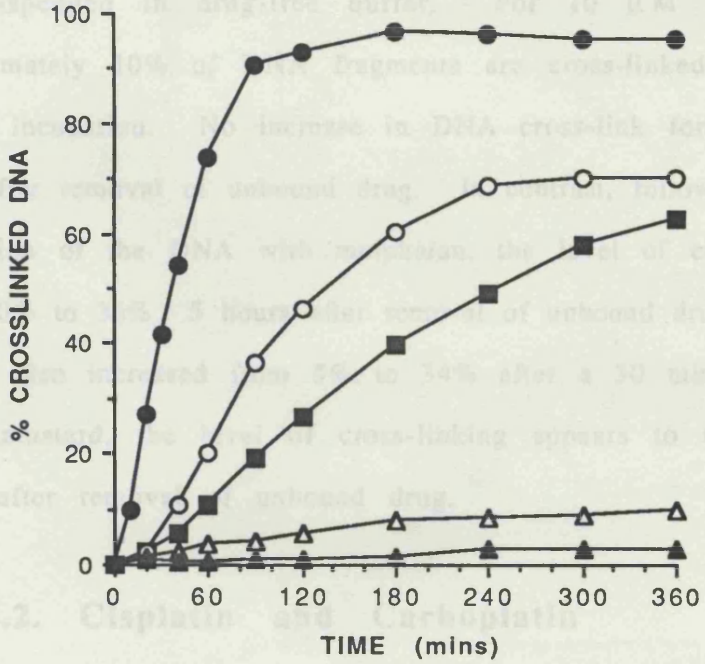


Figure 2.7. Time course for DNA cross-linking induced in DNA by five nitrogen mustard compounds at equimolar concentration (10 μM). Mechlorethamine (filled circles), uracil mustard (open circles), melphalan (filled squares), phosphoramide mustard (open triangles) and isophosphoramide mustard (filled triangles).

In addition, over 95% of DNA fragments were cross-linked by mechlorethamine at 2 hr while uracil mustard appeared to reach a plateau with roughly 70% of DNA cross-linked at 5 hr. In contrast, melphalan continues to form DNA cross-links at 6 hr. Clearly, the structure of the moiety attached to the chloroethylating portion of the drug influences both the rate and extent of DNA interstrand cross-link formation.

Time course reactions give information into the overall extent and rate of cross-link formation. However, DNA cross-link formation consists of two separate events. Figure 2.8 compares the rate of second arm cross-link formation in the absence of unbound drug together with the time course for cross-link formation for three mustard compounds. After a short initial incubation of excess drug with DNA, unbound drug is removed and the DNA is resuspended in drug-free buffer. For 10 μM mechlorethamine, approximately 10% of DNA fragments are cross-linked after an initial 10 minute incubation. No increase in DNA cross-link formation was observed 7 hr after removal of unbound drug. In contrast, following a 60 min initial incubation of the DNA with melphalan, the level of cross-linking increased from 10% to 36% 5 hours after removal of unbound drug. Although cross-linking also increased from 5% to 34% after a 30 min pre-incubation with uracil mustard, the level of cross-linking appears to reach a maximum 2 hours after removal of unbound drug.

2.4.2. Cisplatin and Carboplatin

A comparison of the cross-linking efficiency of cisplatin and carboplatin is shown in figure 2.9 for a range of concentrations. For cisplatin, cross-links could be detected down to 0.1 μM with 100% cross-linking occurring at 10 μM. In contrast, cross-linking with carboplatin was detected at 10 μM

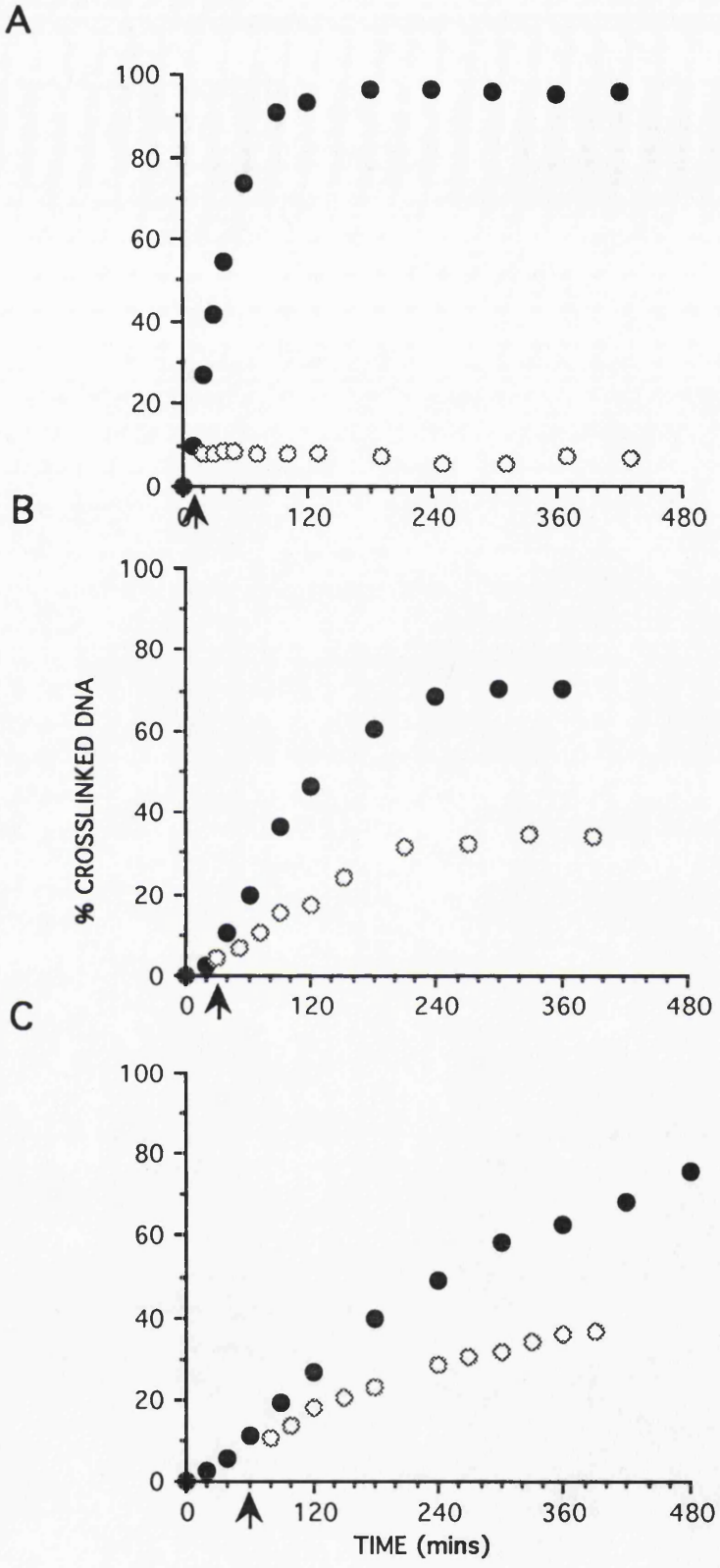


Figure 2.8 Time course for total and "second arm" cross-link reaction for three nitrogen mustards. Drug-DNA reactions were carried out in TEA (pH 7.2) in the presence of 10 μM drug (filled circles). Unbound drug was removed from the DNA at times indicated by the arrow and post incubated in drug-free buffer (open circles). A) nitrogen mustard. B) uracil mustard. C) melphalan.

with 100% of DNA cross-linked at 1000 μM. It is interesting to note that at increasing doses of drug that produce 100% of cross-linked DNA, the band migrates with an increased mobility through the gel. This effect may be due to the contortional bending of DNA induced by the formation of intrastrand cross-links by both compounds. More bending induced at higher concentrations may result in an increased mobility of the cross-linked DNA through the gel.

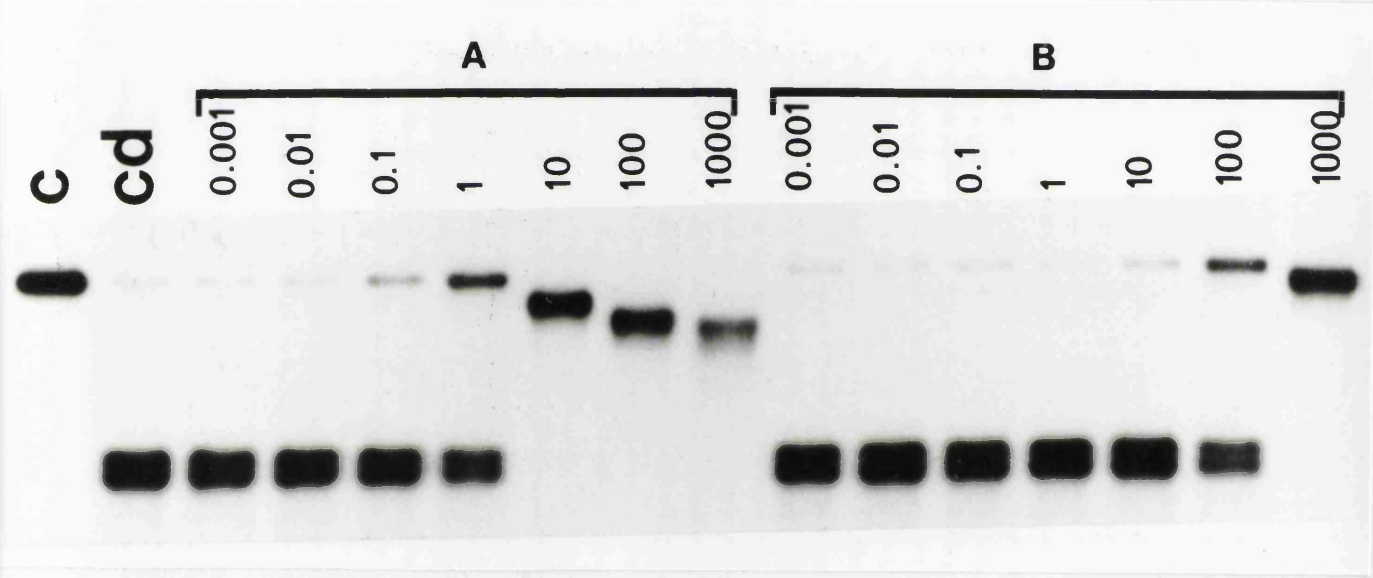


Figure 2.9 Cross-linking induced by cisplatin (A) and carboplatin (B) for a range of concentrations (μM) in 50 μl of TEA (pH 7.2) for 2 hr. CU is untreated double stranded DNA and CD is untreated denatured DNA.

The time course for DNA cross-link formation induced by 1 μM cisplatin and 50 μM carboplatin is shown in figure 2.10. After 2 hr at 37° C, 20% of the

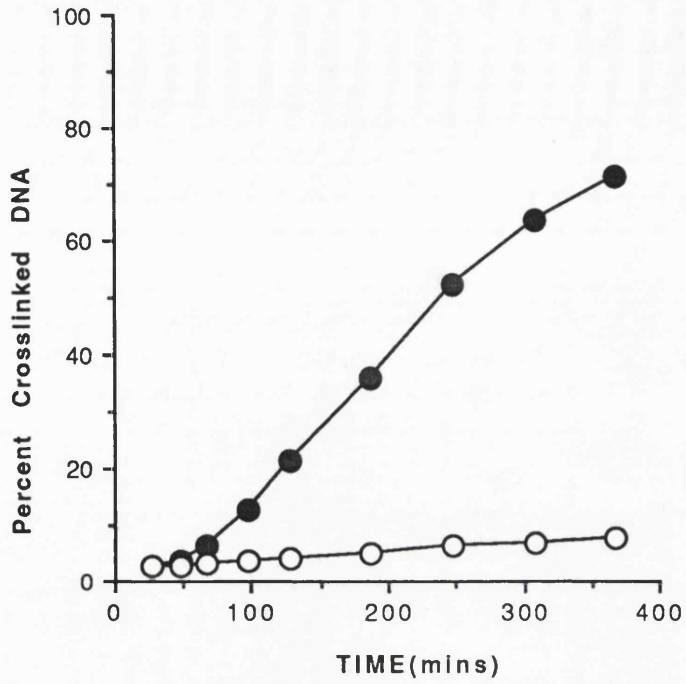


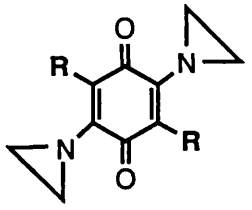
Figure 2.10. Time course for DNA cross-link formation induced by 1 μM cisplatin (filled circles) and 50 μM carboplatin (open circles) in TEA pH 7.2 at 37° C.

DNA was cross-linked in the presence of 1 μM cisplatin compared to 5% DNA cross-linked by 50 μM carboplatin. The level of cross-link formation observed with cisplatin continued to increase with almost 75% of the DNA cross-linked after 5 hours compared to only 9% for carboplatin.

2.4.3. Aziridinylbenzoquinones

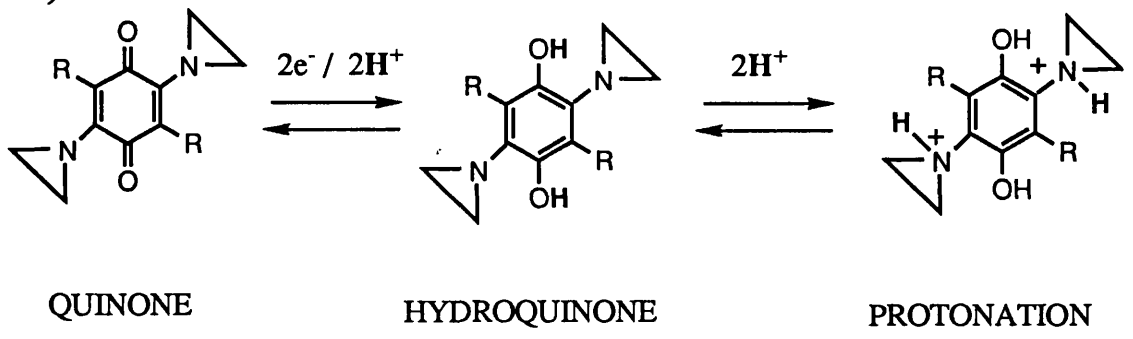
A series of structurally related bifunctional aziridinylbenzoquinones (figure 2.11A) have been evaluated for their ability to induce interstrand cross-links in isolated DNA. In general, enzymatic or chemical reduction of

A)



R	COMPOUND
$NHCH_2CH_2OH$	BZQ
H	DZQ
CH_3	MeDZQ
$NHCOOCH_3$	D1
$NHCOOC_2H_5$	D2 (AZQ)
$NHCOOC_3H_7$	D3 (n-propyl)
$NHCOOC_3H_7$	D4 (isopropyl)
$NHCOOC_4H_9$	D5 (n-butyl)
$NHCOOC_4H_9$	D6 (isobutyl)
$NHCOOC_4H_9$	D7 (sec-butyl)

B)



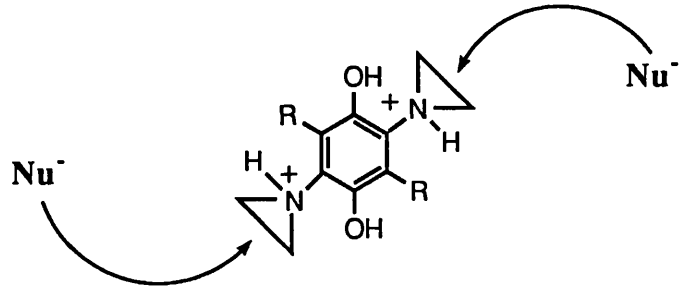


Figure 2.11 A) Structures of the symmetrically substituted analogues of AZQ. B) Schematic representation of the two electron reduction of a quinone to the hydroquinone form. The protonated aziridine ring is subject to nucleophilic attack.

the quinone moiety generates a reactive hydroquinone species which is capable of alkylating and cross-linking DNA via the aziridine rings (figure 2.11B). Interstrand cross-links induced in DNA by these compounds in their unreduced (quinone) form at equimolar concentrations is shown in figure 2.12A. After incubation at 37° C for 1 hour only BZQ and DZQ were capable of inducing interstrand cross-links at neutral pH. However, with the exception of BZQ_1 a significant increase in the level of DNA cross-linking was observed upon reduction of the quinones with NADH and DT-Diaphorase (figure 2.12B).

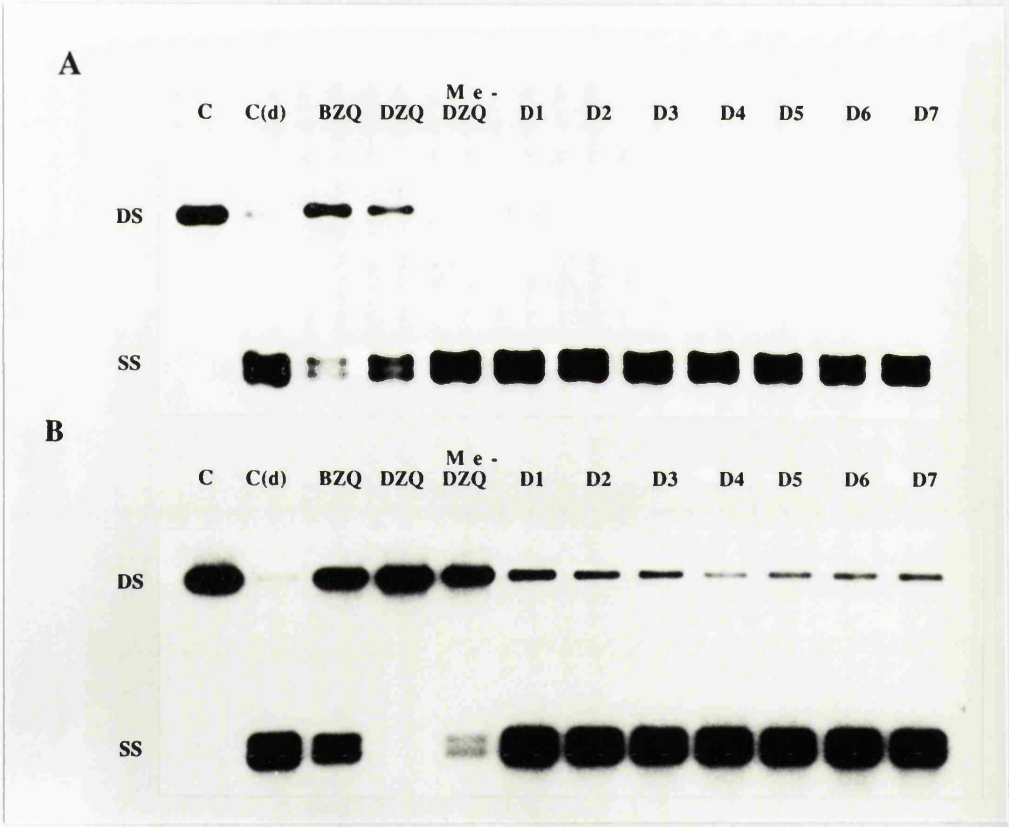


Figure 2.12 Interstrand cross-links induced in DNA by a series of structurally related quinones at equimolar concentrations (50 μM) for 1 hr at 37° C in 50 μl TEA buffer, pH 7.2. A) Cross-links induced in DNA by quinones in their unreduced form. B) Cross-linking induced in DNA by quinones in the presence of 100 mM NADH and 0.173 μg of DT-Diaphorase.

The level of cross-linking observed with BZQ in the presence or absence of DT-Diaphorase was 58% and 65% respectively suggesting that BZQ is a poor substrate for DT-Diaphorase. Moreover, the level of cross-linking induced in DNA by DZQ and MeDZQ was substantially enhanced upon reduction, suggesting these compounds are good substrates for DTD. In general, the extent of DNA cross-linking was reduced as the size of the groups attached to the 3 and 6 positions of the quinone ring increased.

In order to study in more detail the increase in the level of DNA cross-links induced by DZQ and MeDZQ upon reduction, DNA was reacted with either DZQ or MeDZQ in the presence or absence of DTD for a range of drug concentrations. Figure 2.13 shows that for unreduced DZQ 100% cross-

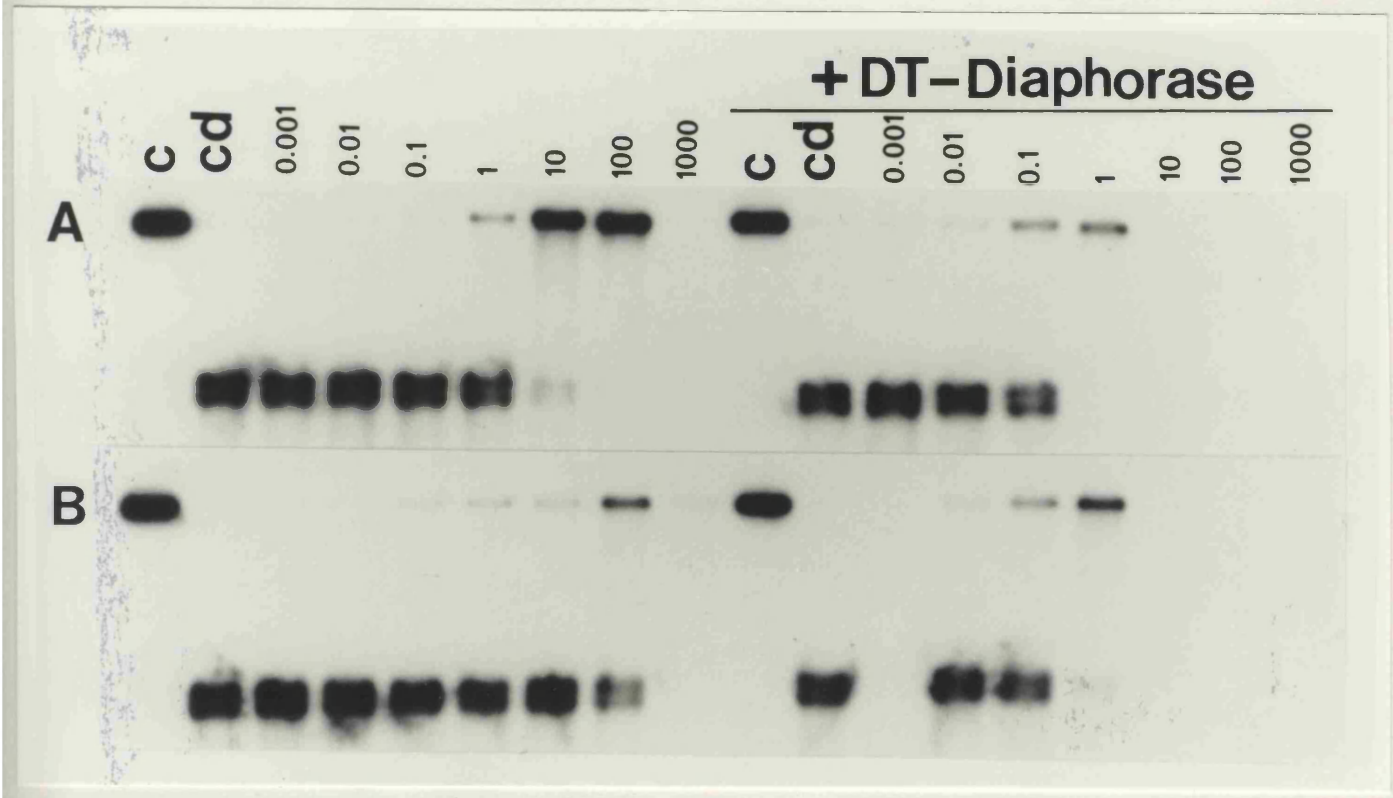


Figure 2.13 DNA Cross-linking induced by DZQ (panel A) and MeDZQ (panel B) for a range of concentrations (μM). Reactions were carried out at 37° C in 50 μl of TEA buffer (pH 5.8) in the presence or absence of DT-Diaphorase.

linking occurred at 100 μM under these conditions. However, upon reduction of DZQ with DTD, the same level of cross-linking was achieved at 1 μM. Similarly for MeDZQ, approximately 50% cross-linking was observed for the unreduced form of MeDZQ at 100 μM while only 0.1 μM was required to achieve a comparable level of cross-linking upon reduction. The complete loss of samples at the higher drug concentrations under both reducing and non-reducing conditions may be due in part to DNA strand scission. This is particularly evident for the reduced compounds and may be a result of the production of semiquinone radicals which are capable of causing DNA single strand breaks.

The time course for DNA cross-link formation induced by reduced DZQ and MeDZQ is shown in figure 2.14. After 2 hours, DZQ produced over two fold more DNA cross-links than MeDZQ at equimolar concentrations. No further increase in DNA cross-linking was detected after 2 hr for either compound.

Spectrophotometric analysis of the reduction of DZQ, MeDZQ, AZQ and BZQ by DT-diaphorase was carried out in order to assess the relationship between quinone reduction and the level of DNA cross-linking. The absorbance spectrum of a solution containing 100 μM quinone in the presence or absence of DT-diaphorase and NADH was monitored with time. Figure 2.15A and B shows the individual absorbance profile for both DT-diaphorase and NADH. Figure 2.15C shows that no change in absorbance could be detected upon incubation of 0.1 mM NADH with 173 ng of DT-diaphorase. Similarly, no change in absorbance was observed for a solution containing 0.1 mM DZQ and 0.1 mM NADH after 10 minutes in the absence of DT-diaphorase (figure 2-15E). However, addition of DT-diaphorase to a solution containing DZQ and NADH resulted in the immediate decrease in the absorbance profile of DZQ and NADH (figure 2.15F). It

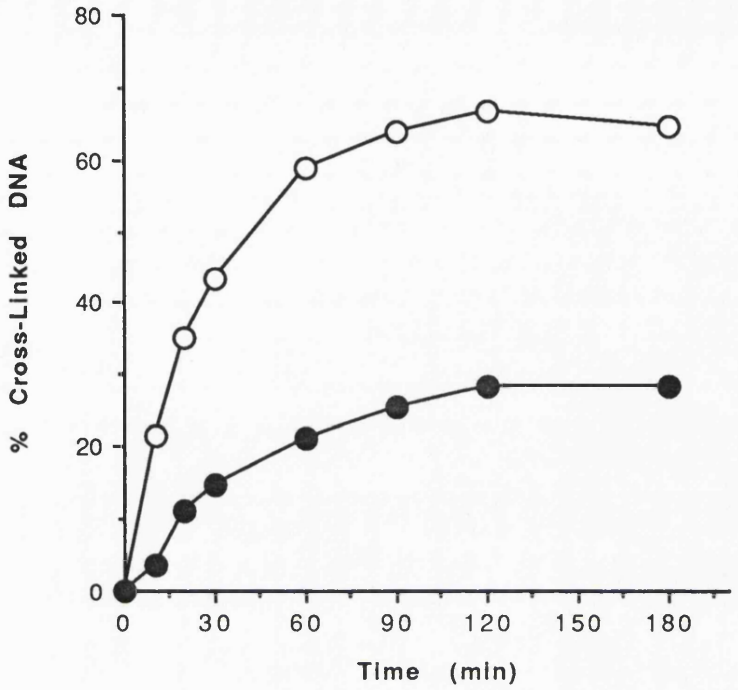
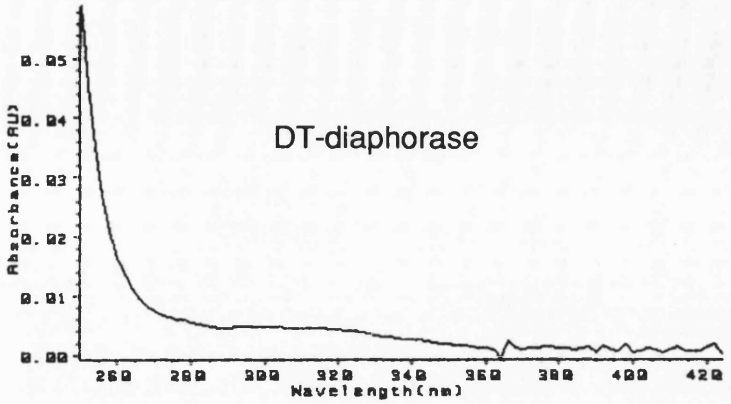


Figure 2.14 Time course of DNA cross-linking at 37° C with 0.5 µM DZQ (open circles) and 0.5 µM MeDZQ (filled circles) in 50 µ1 of TEA buffer (pH 5.8) containing 100 mM NADH and 0.173 µg DT-Diaphorase.

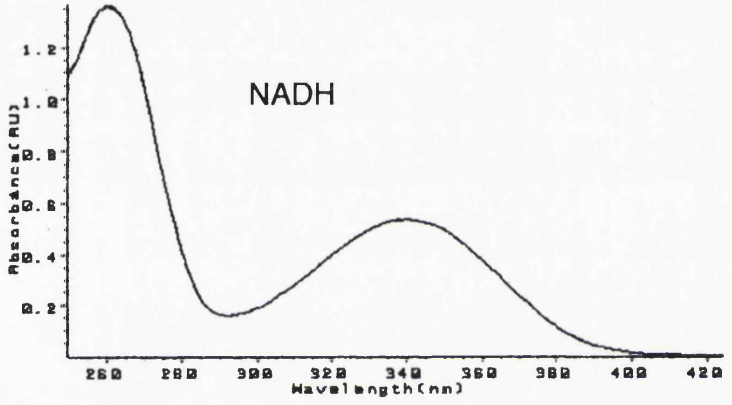
should be noted that no change in absorbance could be detected upon extended incubation of the enzyme with the quinone in the absence of NADH (data not shown)

The absorbance spectrum of NADH (2.15B) and DZQ (2.15D) is additive and their combined spectrum is shown in figure 2.15E. Thus a decrease in absorbance maximum for their combined spectrum upon addition of DT-diaphorase represents both NADH oxidation and a decrease in the concentration of the quinone form of DZQ. Hence, a decrease in the absorbance maximum for the quinone-NADH combined spectrum provides a reasonable measurement for the rate of DT-diaphorase mediated quinone reduction (Siegel et. al., 1990a).

A



B



C

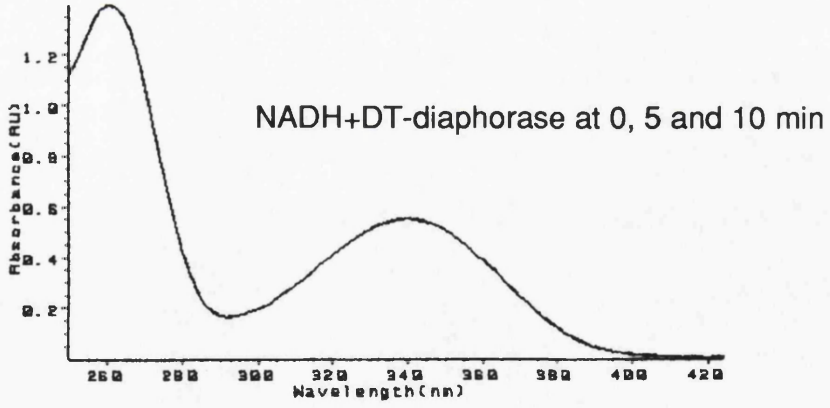
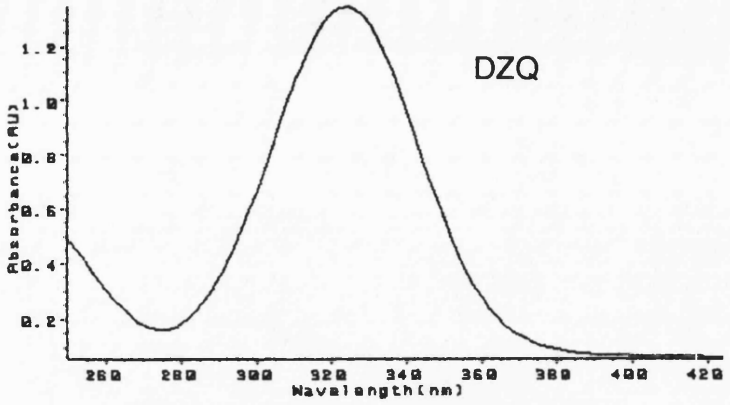
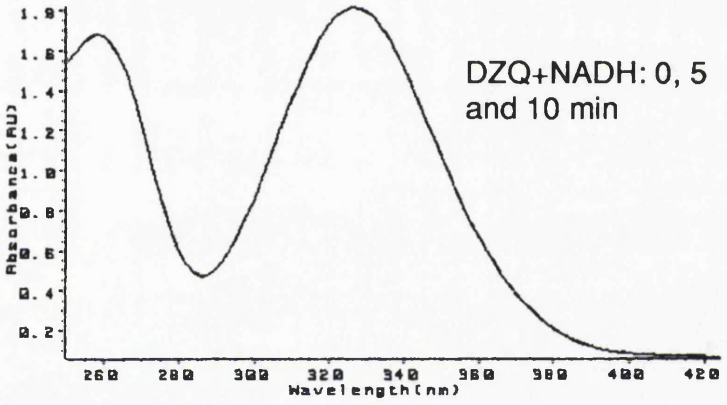


Figure 2.15 Spectrophotometric analysis of quinone reduction. Samples were scanned at 20 ^{o} C in 1 ml of TEA (pH 5.8) containing either 173 ng DT-diaphorase, 0.1 mM NADH or 0.1 mM quinone at the indicated times.

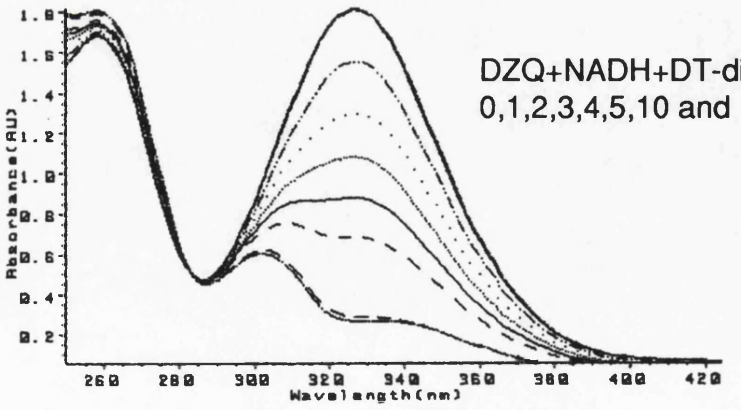
D


DZQ
Absorbance(AU)
Wavelength(nm)

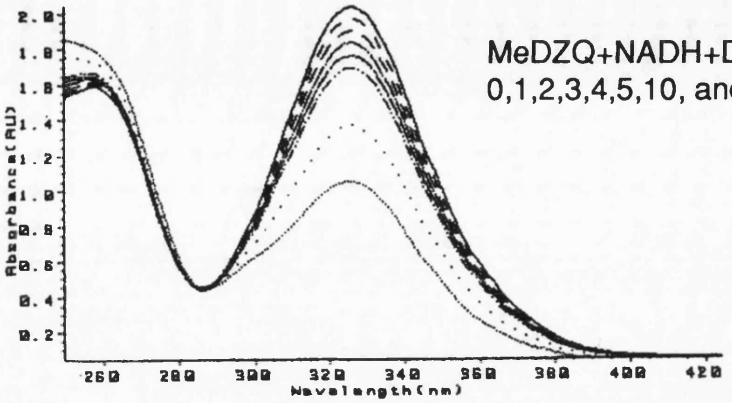
E


DZQ+NADH: 0, 5
and 10 min
Absorbance(AU)
Wavelength(nm)

F

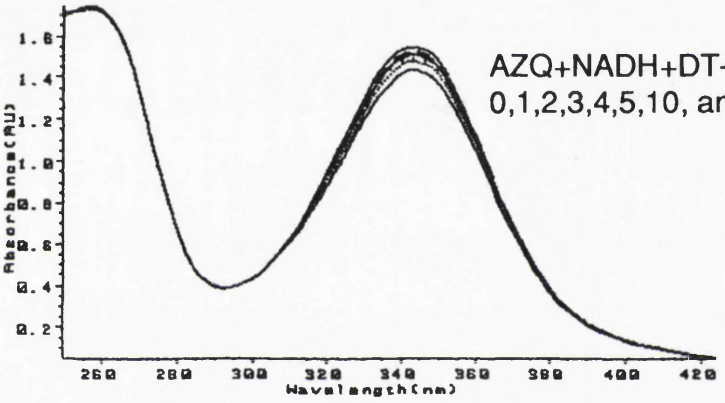

DZQ+NADH+DT-diaphorase
0,1,2,3,4,5,10 and 15 min.
Absorbance(AU)
Wavelength(nm)

H



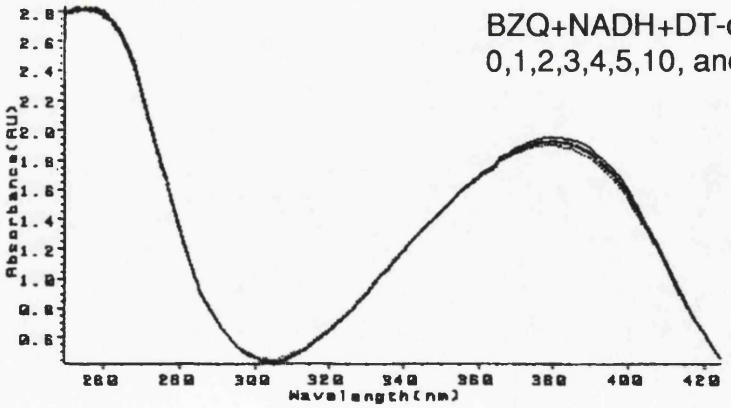
MeDZQ+NADH+DT-diaphorase:
0,1,2,3,4,5,10, and 15 min

I



AZQ+NADH+DT-diaphorase:
0,1,2,3,4,5,10, and 15 min

J



BZQ+NADH+DT-diaphorase:
0,1,2,3,4,5,10, and 15 min

Spectrophotometry was also used to examine the reduction of MeDZQ (2.15H), AZQ (2.15I) and BZQ (2.15J) by DT-diaphorase. The change in the absorbance maximum for each quinone-NADH sample was measured after the addition of DT-diaphorase and shown in figure 2.16. Clearly, DZQ is most readily reduced followed by MeDZQ and AZQ. Thus the initial rate of reduction for DZQ, MeDZQ and AZQ under aerobic conditions could be correlated with the level of cross-linking induced in isolated DNA (figure 2.12). However, very little change in the absorbance spectrum of BZQ was observed in the presence of NADH and DT-diaphorase suggesting that little or no reduction takes place after a 15 minute incubation. This result is consistent with the finding that the level of BZQ induced DNA cross-links was similar in the presence (58%) and absence (65%) of DT-diaphorase (figure 2.12 A,B).

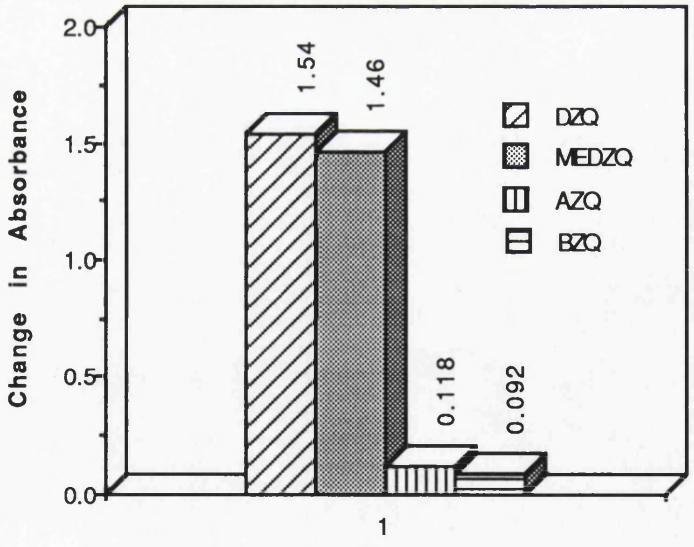
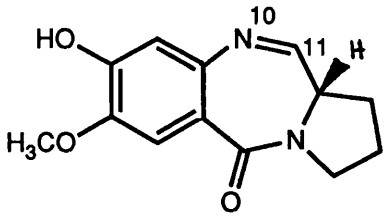


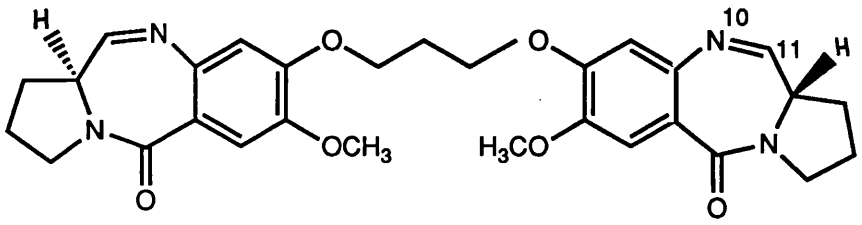
Figure 2.16 Change in the absorbance maximum of the indicated quinone 30 minutes after the addition of 0.173 µg of DT-Diaphorase. Absorbance values were taken at the absorbance maximum for each quinone/NADH complex.

2.4.4. Pyrrolobenzodiazepines

The pyrrolobenzodiazepine (PBD) compounds are a group of naturally occurring anitumour antibiotics. PBD compounds such as anthramycin and tomaymycin are alkylating agents capable of forming a covalent bond with the guanine-N2 position in the minor groove of DNA(Hertzberg et al., 1986). Previous studies have demonstrated that a number of monofunctional PBD compounds exhibit a pronounced DNA sequence specificity in which guanines located between purines are preferentially alkylated(Hertzberg et al., 1986; Hurley et al., 1988). In an effort to extend the sequence specificity of PBD agents, a bifunctional PBD compound has recently been synthesised (DSB-120) in which the two reactive units of the molecule consist of the monofunctional alkylating agent DC-81(figure 2.17) (Bose et al., 1992a).



DC-81



DSB-120

Figure 2.17 Structure of DC-81 and DSB-120.

The cross-linking efficiency of DSB-120 for a range of concentrations was determined and the results shown in figure 2.18. Cross-linking can be seen to occur with a concentration of DSB-120 as low as 10 nM. After 2 hr at 37° C, 90% of the DNA was shown to be cross-linked at 400 nM.

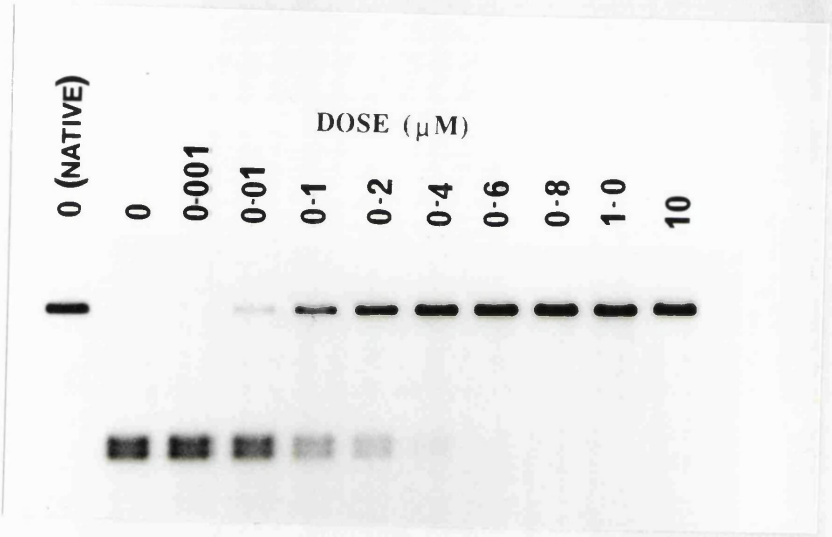
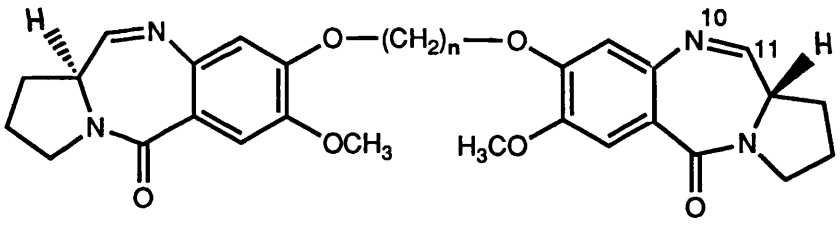


Figure 2.18 Cross-linking induced in DNA by DSB-120 for a range of concentrations. Reactions were carried out in 50 μl of TEA, pH 7.2, for 2 hr at 37° C.

As a result of the high efficiency of DNA cross-linking induced by DSB-120, a series of structurally related C8-linked bifunctional alkylating agents were synthesised (figure 2.19). These compounds varied in the length of the methylene chain (n=3 to n=6) connecting the two reactive portions of the molecule. In order to assess the effect of linker length on DNA cross-linking efficiency, these analogues were reacted with DNA over a range of concentrations. Figure 2.20 shows the effect of varied chain length on the



n = 3 : DSB-120

n = 4 : DSB-131

n = 5 : AT-286

n = 6 : AT-111

Figure 2.19 Structure of C-8 linked pyrrolobenzodiazepine compounds.

extent of DNA interstrand cross-link formation. Interestingly, the (n=3) and (n=5) compounds exhibited similar levels of cross-linking while the (n=4) and (n=6) analogues were approximately 18 and 14 fold less efficient, respectively (figure 2.21).

In order to assess the effect of increasing the concentration of the DNA substrate while keeping the drug concentration constant, an identical range of DSB-120 concentrations was reacted with either 10 or 50 ng of DNA and electrophoresed under identical conditions. Although a stronger signal was observed in samples containing high DNA concentration, quantitation of the cross-linked DNA for each dose revealed that increasing the DNA concentration five fold did not increase the level of DNA cross-linking to the same extent figure 2.22. These data show that DNA cross-linking was largely independent of the concentration of DNA substrate and depended primarily on the initial concentration DSB-120.

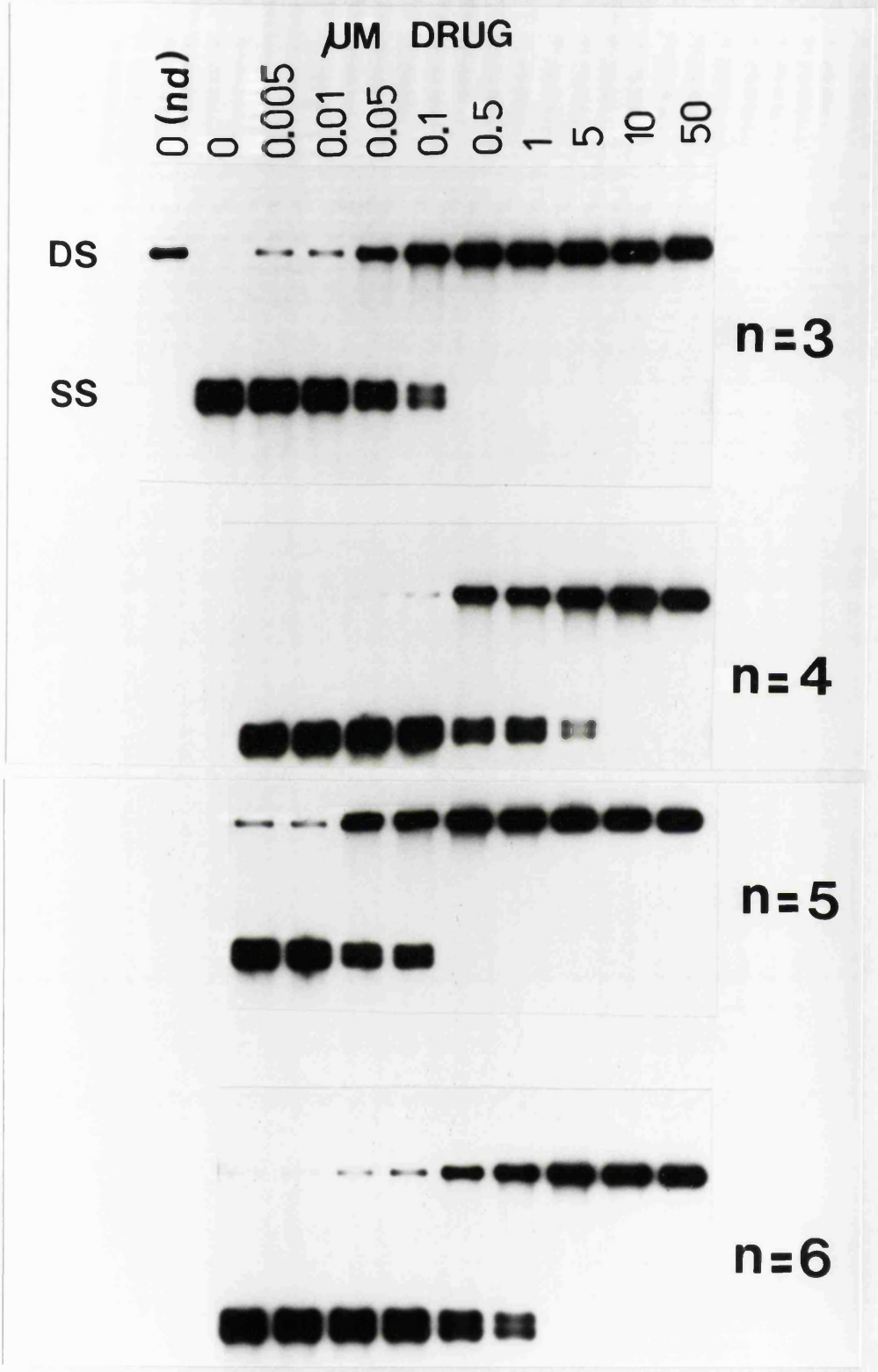


Figure 2.20. Extent of DNA cross-linking as a function of concentration for the various C-8 linked pyrrolobenzodiazepine compounds. Reactions were carried out in 50 μl of TEA, pH 7.2, at 37° C for 2 hr at the given concentrations (μM).

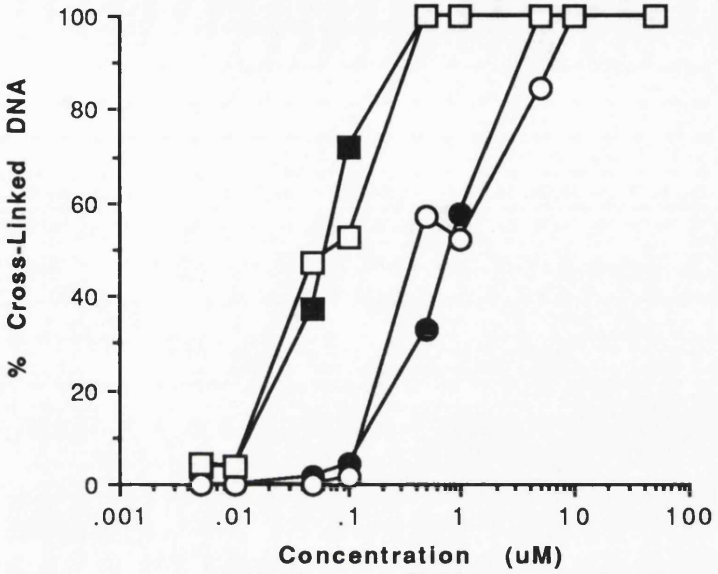


Figure 2.21 Extent of DNA cross-linking as a function of concentration for the various C-8 linked pyrrolobenzodiazepine compounds. Analogues containing an even number of methylene carbons in the linker, n=4 (open circles) and n=6 (filled circles), were less efficient DNA cross-linkers than the n=3 (filled squares) and the n=5 (open squares) compounds.

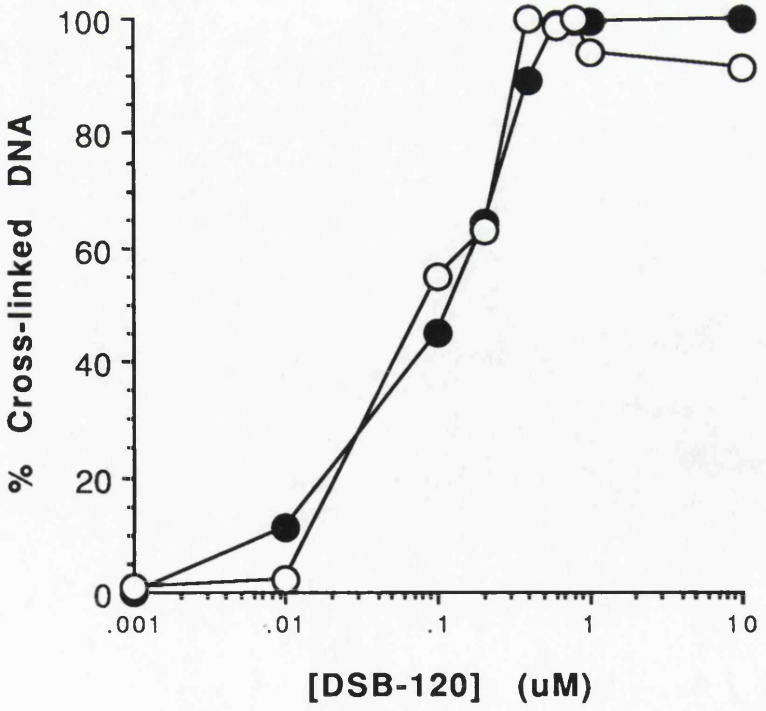


Figure 2.22 Extent of cross-linking on concentration of DSB-120 for two separate concentrations of DNA. Open circles, 1 ng DNA/μ1. Closed circles 0.2 ng DNA/μ1.

2.5 Discussion

These studies attempt to describe a simple, rapid and sensitive agarose gel based assay for the measurement of DNA interstrand cross-links. The method is based on the differential migration of double stranded and single stranded DNA through a neutral agarose gel. Native double stranded DNA migrates as a single band upon electrophoresis. DNA which has been denatured, either by heat or alkali, becomes single stranded and migrates with an increased electrophoretic mobility. A DNA interstrand cross-link prevents the complete separation of complementary strands under denaturing conditions and provides a point for the reassociation of the DNA. As a result, linear DNA fragments containing a cross-link will migrate as double stranded and can be separated from uncross-linked DNA upon electrophoresis. Thus it is possible to measure the extent of cross-linking in drug treated samples by measuring the amount of DNA migrating as double stranded. The assay is particularly applicable to the comparison of cross-links induced by structurally related compounds for a range of concentrations. In addition to measuring the extent of cross-linking induced by a given concentration of drug, the technique can be extended to measure the time course for the formation of DNA cross-links induced by a variety of cross-linking agents. The conversion of DNA monoadducts to cross-links can also be measured using a modification of the time course reaction.

Sensitivity of the method is enhanced by the use of a radiolabeled DNA substrate and allows routine detection of samples containing as little as 5-10 ng of DNA. Thus 1 μg of end labelled DNA is sufficient for five 20 lane experiments. Although conditions were optimised for 5' end labeling of the DNA to achieve a high level of specific activity, it is also possible to 3' end

label the DNA. It should be noted that highly purified DNA, preferably from a commercial source, should be used to obtain optimal labeling and gave the best results. Although purified plasmid pBR322 DNA was used in these studies, DNA from different sources and of sufficient quality could be substituted for pBR322 DNA for the analysis of in-vitro cross-linking.

Quantitation of cross-linked DNA and uncross-linked DNA within the same lane by laser densitometry of the autoradiograph allows the extent of cross-linking to be calculated directly without taking into account any variation in sample loading. Although analysis of the autoradiograph by densitometry is the method of choice for the quantitation of DNA cross-links, an alternative method involves exposing the gel to autoradiographic film, excising the bands and counting the amount of radioactivity in each band. Nonlabeled DNA can also be used as the substrate in cross-linking reactions but requires an increased amount of DNA and detection by ethidium bromide. This method requires photography of the stained gel and processing of the negative for densitometry. In addition, ethidium bromide binds less efficiently to single stranded DNA and requires a correction factor for quantitative analysis.

Experiments designed to determine the optimal time and temperature for thermal denaturation revealed that linear pBR322 DNA becomes completely denatured after 2 minutes at 90^{o} C in strand separation buffer followed by snap cooling in an ice-water bath. These denaturing conditions consistently produced greater than 98% single stranded DNA upon electrophoresis. In addition, no degradation of the DNA was observed following a 5 minute incubation of the DNA at 90^{o} C in strand separation buffer. Denaturation could also be achieved by treatment of the DNA with at least 100 mM sodium hydroxide for two minutes at room temperature.

However, degradation of the DNA was observed at higher concentration of alkali and at longer times. Although thermal treatment was the method of choice for denaturing DNA, alkaline treatment of DNA containing alkylations at the guanine-N7 and adenine-N3 positions has been shown to form imidazole ring opened products which are more stable to thermal treatment (Hemminki et. al., 1989). Extensive heat treatment of alkylated purines leads to the loss of guanine and adenine residues from DNA and is the basis for the G>A sequencing reactions described by Maxam and Gilbert (1977). At 100^{o} C, the half life for depurination of DNA containing 7-methylguanine and 7-(2-aminoethyl)guanine at neutral pH was reported to be 4.1 and 1.5 minutes respectively (Hemminki et. al., 1989). The half lives were increased to 69 and 34 minutes upon pretreatment of the alkylated DNA with 0.25 M NaOH to form ring opened products. Hence, extensive thermal treatment of DNA cross-linked through purine residues might lead to depurination reactions and an underestimation of the extent of cross-linking induced by major groove alkylating agents such as the nitrogen mustards. Although thermal depurination was not observed under the conditions used, a decrease in the total autoradiographic signal was detected in some cases at very high doses of major groove alkylating agents which may reflect, in part, a depurination and strand scission reaction. Treatment of DNA containing N7-alkylguanines with dilute alkali to generate a more stable ring opened adduct may be a viable option if the heat denaturation step is believed to cause significant depurination.

All reactions were carried out in triethanolamine buffers principally because no adjustment for positive ion (sodium, potassium, etc.) concentration was required upon ethanol precipitation of the DNA. Cross-linking reactions can be carried out in sodium or potassium phosphate buffers provided their presence is taken into account in the precipitation

step. If the concentration of salt is too high in the reaction mixture then some salt will precipitate along with the DNA which will subsequently interfere with gel loading and electrophoresis.

The method described in these studies provides a useful tool for the direct analysis of DNA cross-links induced by structurally distinct bifunctional agents. Data obtained with this assay may complement other techniques such as alkaline elution by giving basic information on the time course for chemical cross-linking without the influence of complicating cellular factors such as DNA repair.

CHAPTER 3: NUCLEOTIDE SEQUENCE PREFERENCES FOR THE FORMATION OF DNA CROSS-LINKS

DNA interstrand cross-linking agents are among the most effective compounds used in the treatment of human cancers. In many cases, the atomic sites on DNA which are covalently modified by these bifunctional drugs have already been well characterised (e.g. guanine N-7). In addition, the monoalkylation sequence specificity of many chemotherapeutic alkylating agents including the nitrogen mustards, chloroethylnitrosoureas, and triazenes has been established (Hartley, et. al., 1986, 1988a,b). For nitrogen mustard compounds, the guanine-N7 sequence specificity was found to be dependent on the structure of the group attached to the non-reactive portion of the molecule with mechlorethamine preferring guanines located in the middle of 5'-GGG sequences (Hartley et. al., 1986). The isolation of guanines cross-linked through the N7 positions in hydrolysates of nitrogen mustard treated DNA together with experiments on static models of DNA first led to the reasonable suggestion that nitrogen mustards would preferentially cross-link DNA through guanine-N7 positions in a 5'-GC sequence(Brooks and Lawley, 1961). Later studies, based on monoalkylation sequence specificity, also proposed that nitrogen mustard compounds might form cross-links in 5'-GC sequences (Mattes et al., 1986a)(Kohn et. al., 1987).

More recently, the guanine N-7 sequence specificity of a series of structurally related analogues of AZQ was investigated upon chemical (Hartley et. al., 1991) or enzymatic reduction(Lee, et. al., 1992). While the quinone and hydroquinone forms of several structurally similar aziridines exhibited relatively non-specific alkylation of guanine-N7 positions, the

reduced form of DZQ reacted preferentially at guanines in 5'-GC sequences and more specifically at 5'-TGC sequences (Lee et al., 1992). A model was proposed to account for the unique reaction of the hydroquinone form of DZQ at 5'-GC sites (Hartley et al., 1991a), and suggested that the unique selectivity may result in an altered sequence preference for DNA interstrand cross-linking.

Thus although the sequence specificity of monoalkylation for the nitrogen mustard and aziridinylbenzoquinone compounds has previously been described, the sequence preferences for the formation of DNA cross-links induced by these compounds remained unclear. In the following studies the nucleotide sequence preferences for the formation of DNA interstrand cross-links induced by nitrogen mustard, DZQ and MeDZQ was investigated using synthetic oligonucleotides containing defined sequences. Analysis of preferential cross-link formation in oligonucleotides containing several potential cross-linking sites was carried out by isolating electrophoretically distinct cross-linked products and examining the pattern of guanine-N7 alkylation upon piperidine fragmentation.

3.1. Materials

Oligonucleotides:

Oligomers were synthesised on a 1.0 micromole scale using cyanoethyl chemistry and fully deprotected with hydroxyl groups on both 5' and 3' ends. The crude synthetic DNA was supplied as a precipitate under 1 ml of 80% ethanol and stored at -20°C.

Enzymes:

DT-Diaphorase (DTD): specific activity 660 nmol/min/mg protein was obtained from Dr. Dave Ross of the University of Colorado.

T4 polynucleotide kinase: (see section 2.1)

Radioisotope: (See section 2.1)

General buffers: (See section 2.1)

Sequencing gel buffers:

TBE (running buffer): 90 mM Tris, 90 mM boric acid and 2 mM EDTA, pH 8.3.

Loading buffer: 98% deionised formamide, 10 mM EDTA (pH 8.0), 0.025% xylene cyanol and 0.025% bromophenol blue.

Gel slice elution buffer: 150 mM NaCl, 10 mM Tris (pH 8.0), and 1 mM EDTA

Chromatography columns:

Sephadex spin columns (Bio-Rad, P6: 5 base pair exclusion limit) consisted of 0.8 ml of P-6 polyacrylamide and were stored in SSC, pH 7.0 (0.15 M sodium chloride, 17.5 mM sodium citrate, 0.02% sodium azide)

Sep-Pak C_{18} desalting columns were obtained from Waters (Millipore).

Chemicals:

Premixed electrophoresis grade acrylamide:bis acrylamide (19:1) was obtained from Sigma and used as a 40% stock solution. Piperidine, NADH (grade III), ammonium persulphate, sodium ascorbate, boric acid and TEMED were also from Sigma. Dimethylsulphate and iron ammonium sulphate hexahydrate were obtained from Aldrich.

3.2. Methods

3.2.1. Preparation of Sequencing gels

Denaturing DNA sequencing gels were 20% acrylamide:bis acrylamide (19:1), and 7 M urea in 90 mM Tris, 90 mM boric acid and 2 mM EDTA, pH 8.3. Electrophoresis was carried out on a 0.4 mm x 50 cm x 20 cm (Bio-Rad) sequencing apparatus. Gels were prepared by combining 40 ml of 40% acrylamide stock solution, 8 ml of 10x TBE, and 33.63 g of urea and adjusting the final volume to 80 ml with distilled deionised water. The solution was gently heated to dissolve the urea and vacuum filtered through Whatman 3 MM filter paper. The solution was then placed on ice in order to slow the

polymerisation reaction. 50 µl of TEMED and 70 µl of 25% aqueous ammonium persulphate (w/v) were added to 10 ml of acrylamide solution, mixed and poured onto a thin strip of filter paper. The sequencing apparatus was then pressed down on top of the filter paper so that the solution would rise into the space between the plates at the bottom edge of the apparatus by capillary action. Once the sealing gel had polymerised, 91 µl of 25% aqueous APS and 70 µl of TEMED were added to the remaining 70 ml of acrylamide solution. The solution was mixed by swirling and carefully poured into the space between the two glass plates of the apparatus using a 50 ml syringe fitted with a stopcock. Gels were allowed to polymerise for at least 1.5 hours or were left overnight for use in the morning. Following polymerisation the gel was pre-electrophoresed at 3000 V for 1 hour. The sample wells were flushed with electrophoresis buffer to remove leeched urea and unpolymerised acrylamide which can interfere with sample loading and electrophoresis.

3.2.2. Purification of Synthetic Oligonucleotides

This step in the procedure was necessary due to the fact that crude synthetic oligonucleotides contain a small percentage of failure sequences which represent a significant proportion of the available 5' ends. These oligomers are thus disproportionately radiolabeled if not removed from full length sequences. Synthetic oligonucleotides were purified through a 20% denaturing sequencing gel. Ethanol was removed from the crude oligomers following centrifugation for 15 minutes at 4° C. The pellets were dried briefly under vacuum and resuspended at 1µg/µl in deionised water. Approximately 100 µg of the crude oligomer was removed and lyophilized. The DNA was subsequently resuspended in 20 µl of loading buffer, heated to 90° C for 2 minutes and immersed in an ice water bath. Approximately 50

μg (10 μl) of DNA was loaded per well (15 mm x 10 mm x 0.4 mm wells; 8 well comb) using a 10 μl Hamilton syringe. Samples were electrophoresed at 3000 V (plate temperature of 45-50° C) until the xylene cyanol dye marker had migrated approximately 8 cm. Following electrophoresis, the gel was transferred to Saran wrap and placed over a fluorescent TLC plate (Merck HPTLC silica gel, 60 F254 pre-coated, 10 cm x 20 cm). Bands were visualised by illuminating the gel from above with short wave UV light and excised from the gel with a scalpel. The gel slices were crushed and soaked in 1 ml of elution buffer (150 mM NaCl, 10 mM Tris, pH 8.0, and 1 mM EDTA) in a 1.5 ml microfuge tube overnight at 37°C.

The DNA solution was desalted using a Sep-pak C-18 cartridge. The cartridge was prepared for desalting by flushing it with 10 ml of acetonitrile (in a fume hood) followed by 10 ml of distilled deionised water. The buffer containing DNA (1.0 ml) was subsequently loaded onto the column and flushed with 10 ml of distilled deionised water to remove salt. The DNA was eluted from the column as one 1.5 ml fraction with 60% aqueous methanol into a 1.5 ml microfuge tube.

The oligonucleotide concentration was estimated by measuring the absorbance of the oligonucleotide solution at 260 nm using 60% aqueous methanol as a blank. According to suppliers specification, an absorbance of 1.0 corresponded to a DNA concentration of 33 μg/ml. The methanol was evaporated from the DNA containing solution by heating the sample at 45° C for 2 h and the remaining aqueous sample was lyophilized and resuspended at 0.5 μg/μl in ultrapure water. The purified oligonucleotide was stored at -20° C. Typical recovery of full length synthetic oligomer using this method was between 50-65%.

3.2.3. 5' End Labeling

3.2.3.1. Single site oligomers

Approximately 5.0 µg of the self complementary oligomers and 2.5 µg of the non-self complementary oligomers were independently labeled in 25 µl of 1x forward reaction containing 20 µCi of [γ-^{32}P] ATP and 5 units of T4 polynucleotide kinase. Reactions were carried out for 30 min at 37^{o} C.

3.2.3.2. Multiple site oligomers

Approximately 5.0 µg of the purified single stranded DNA oligomer was 5' end-labelled in 25 µl of 1x forward reaction buffer containing 50 µCi of [γ -^{32}P] ATP and 5 units of T4 polynucleotide kinase. The reaction was carried out for 30 minutes at 37^{o} C.

3.2.3.3. Removal of unincorporated ATP

Removal of unincorporated ATP and the recovery of labeled oligonucleotide was achieved using sephadex spin columns (Bio Rad). The column was prepared during the radioactive labeling reaction by inverting it several times to resuspend settled gel matrix and allowing the storage buffer to drain out through the bottom of the column by gravity. Any remaining storage buffer was removed by placing the column in a 1.5 ml microfuge tube and centrifuging the column for 2 minutes in a swinging bucket rotor at 1100xg. Subsequently, 300 µl aliquots of distilled deionised water were carefully added to the centre of the column and centrifuged for 2 minutes at 1100xg. This procedure was repeated four times to equilibrate the column.

Following the labeling reaction, the sample was adjusted to 20 mM EDTA in a final volume of 50 µl. The sample was then carefully added to the centre of the spin column behind a safety screen. The column was subsequently placed in a clean 1.5 ml microfuge tube and centrifuged at 1100xg for 4 min (100% recovery of the oligomer from the column was assumed). The column containing the unincorporated ATP was then discarded in a proper radioactive disposal container. The microfuge tube containing labeled DNA oligomer was then capped and immersed in an ethanol dry ice bath. The cap to the tube was then perforated prior to lyophilization.

The use of sephadex spin columns was preferred due both to the extreme inefficiency of ethanol precipitation of short DNA fragments and the efficient removal of unincorporated ATP.

3.2.4. Annealing conditions

Single site oligomers

Following removal of unincorporated ATP, the self complementary oligomers were concentrated by lyophilization. The non-self complementary single stranded DNAs were combined, mixed by vortexing and briefly centrifuged prior to lyophilization. The samples were then resuspended in 20 µl of distilled deionised water and annealed by placing the tubes containing the samples in 200 ml of water at 65^{o} C and allowing the solution to cool slowly to 4^{o} C in a refrigerator.

Multiple site oligomers

In order to produce 5' singly end-labeled DNA duplexes, 5 µg of the unlabeled single stranded oligonucleotide was combined with 5 µg of its complementary strand. The solution was mixed by vortex and briefly centrifuged. The sample was then placed into 200 ml of water at 90o C and allowed to cool slowly to room temperature.

3.2.5. Drug Reactions

All drug-DNA reactions were carried out in 50 µl of TEA buffer (pH 5.8 or 7.2) containing approximately 2.5 µg of 5' end-labeled duplex DNA. All reactions were terminated by addition of an equal volume of alkylation stop solution and ethanol precipitated. Following careful removal of the supernatant, samples were briefly dried under vacuum.

For the multiple site oligomers, reactions were carried out for 2 hours at 37^{o} C. After the incubation, a small aliquot (2 µl) of each reaction mixture was removed prior to separation of the reaction products in order to assess the total alkylation pattern (both monoadducts and cross-links). The 2 µl aliquot was added to 8 µl of distilled deionised water in a clean microfuge tube prior to precipitation with 10 µl of alkylation stop solution and 3 volumes of ethanol. The DNA pellets were dried briefly under vacuum and stored at -20^{o} C for later analysis. The remaining reaction mixtures were terminated by addition of an equal volume of alkylation stop solution, ethanol precipitated and dried briefly under vacuum.

3.2.6. Separation and Isolation of Cross-Linked DNA

For single site oligomers, the drug treated DNA was resuspended in 5 µl of loading buffer and loaded into a 20% denaturing DNA sequencing gel. Samples were electrophoresed at 3000 V until the xylene cyanol dye front had migrated approximately 10 cm. Following electrophoresis, the gel was carefully transferred to a processed piece of autoradiographic film. This was necessary due to the difficulty in attaching filter paper to high percentage polyacrylamide gels. Once the gel was transferred to the film it was immediately covered with food wrap (Saran) to prevent the gel from dehydration. The wet gel was exposed to autoradiographic film at room temperature for 15 minutes to 2 hours.

For multiple site oligomers, drug treated DNA was resuspended in 10 µl of loading buffer and loaded into a 20% denaturing sequencing gel. Samples were electrophoresed at 3000 V until the xylene cyanol marker dye had migrated approximately 15 cm. The gel was carefully transferred to a piece of processed film and covered with Saran wrap. The gel was fixed into place in the film cassette and a piece of autoradiographic film was placed on top of the gel. The film was exposed to the wet gel at room temperature for 15 to 30 minutes. The image from the resulting processed autoradiograph was used as a template and placed behind the wet gel to visualise the radiolabeled DNA. Cross-linked DNA as well as untreated control DNA was excised from the gel (ca. 0.5 cm x 1.0 cm gel slices, except for controls which were roughly 1.0 cm x 2.0 cm.). Gel slices were crushed and placed in 1.5 ml microfuge tubes containing 1.0 ml of DNA elution buffer. The gel slices containing DNA were vigorously mixed by vortex and incubated overnight at 37^{o} C prior to desalting with Sep Pak C-18 columns as described for purification of unlabeled DNA.

All desalted DNA samples were Cerenkov counted using a Beckman LS1800 liquid scintillation counter. Cross-linked and alkylated DNA samples were counted as dry pellets. Control untreated samples were resuspended in 50 µl of 5 mM Tris-HCl (pH 7.5) and a 5 µl aliquot was removed and counted for radioactivity.

3.2.7. Chemical Cleavage Reactions

3.2.7.1. Guanine specific sequencing reactions

The guanine specific sequencing reaction was carried out on a 5 µl aliquot of the control end labeled, gel purified DNA. The reaction was carried out in 200 µl of 5 mM Tris-HCl, pH 7.5 in a 1.5 ml screw cap microfuge tube. In a fume hood, 1 µl of dimethyl sulphate was added to the DNA sample and the samples were incubated for 10 min at 25° C. The reaction was stopped by addition of 100 µl of 7.5 M ammonium acetate and 1 µl of (10 mg/ml) yeast tRNA. The DNA was then ethanol precipitated and the supernatant inactivated by combining it with 0.5 M NaOH. The DNA pellet was dried briefly under vacuum prior to adding 100 µl of freshly diluted 10% (1.0 M) aqueous piperidine. The solution was heated to 90° C in a tightly sealed microfuge tube for 30 min. During the course of these studies it was noted that piperidine reactions carried out in snap-cap microfuge tubes were less than ideal for this step in the procedure. This was thought to be due to the escape of the volatile piperidine component at elevated temperatures through incomplete sealing of the snap-cap microfuge tube which resulted in the incomplete strand cleavage at guanine residues. This inefficient piperidine cleavage resulted in "shadowing" bands migrating close to and with a slightly higher apparent molecular weight than efficiently cleaved

guanine fragments upon separation of the sequencing products in a denaturing sequencing gel. The use of tightly sealed screw capped microfuge tubes during the piperidine step was found to eliminate these shadowing secondary bands. The hot piperidine solution was immersed into a dry ice/ethanol bath and subsequently lyophilized. The DNA pellet was resuspended in 15 µl of distilled deionised water and re-lyophilized. This step was repeated once more to remove any last traces of piperidine.

3.2.7.2. Hydroxyl radical chemical cleavage reactions

Non specific hydroxyl radical cleavage reactions were performed using 7 µl of the gel purified, end labeled DNA in 5 mM Tris, pH 7.5. Reactions were carried out in 10 µl of 5 mM Tris-HCl (pH 7.5) containing 0.05 mM $Fe(NH_4)_2(SO_4)$/0.1 mM EDTA, 10 mM H_2O_2, and 1 mM sodium ascorbate. A 10x solution of iron(II)/EDTA was prepared immediately before use by combining equal volumes of 1 mM $Fe(NH_4)_2(SO_4)\cdot 6(H_2O)$ and 2 mM EDTA. Iron (II) solutions will oxidise to iron (III) in aerated solutions and should be discarded if an orange colour is observed. All stock solutions were prepared immediately before use. The iron(II)/EDTA solution (1 µl), 100 mM H_2O_2 (1 µl), and 10 mM sodium ascorbate (1 µl) were added separately to the walls of the microfuge tube containing the DNA sample. The reaction was initiated by a brief centrifugation step and the sample was incubated for 1 minute at 20° C. The reaction was terminated by immersing the sample in a dry ice/ethanol bath and lyophilized.

3.2.7.3. Piperidine treatment of cross-linked and alkylated DNA

All purified cross-linked DNA samples were resuspended in 100 µl of freshly diluted 10% aqueous piperidine and heated to 90° C for 15 minutes to

quantitatively convert sites of guanine-N7 alkylation to strand breaks (Mattes et al., 1986b). The samples were briefly centrifuged, immersed in a dry ice ethanol bath and lyophilized. The DNA was resuspended in 15 µl of distilled deionised water and re-lyophilized. This procedure was repeated once more to remove any traces of piperidine.

Identical treatments were carried out on alkylated DNA samples removed from the drug-DNA reaction mixtures prior to separation of the reaction products.

3.2.8. Electrophoresis of Fragmentation products

DNA samples treated with dimethyl sulphate were resuspended in electrophoresis loading at a concentration of approximately 8.0 x 10^3 cpm/µl. Products resulting from hydroxyl radical cleavage and piperidine treated cross-linked DNA samples were resuspended in loading buffer at approximately 2.0 x 10^4 cpm/µl and 2.0 x 10^3 cpm/µl respectively. All samples were heated to 90° C for 2 minutes prior to immersion in an ice/water bath. Equal volumes of each sample (5 µl) were loaded into a 20% denaturing sequencing gel and electrophoresed at 3000 V until the xylene cyanol dye front had migrated approximately 15 cm. Following electrophoresis, the gel was transferred to a piece of processed film and exposed to autoradiographic film overnight at -70° C.

3.2.9. Scintillation counting

Bands excised from the sequencing gel containing both native and cross-linked DNA were placed into a 1.5 ml microfuge tube which was then placed

into a 20 ml scintillation vial. The Samples were counted in a Beckman LS100 liquid scintillation counter.

3.2.10. Densitometry

Autoradiographs were scanned using a Pharmacia LKB enhanced Ultrascan-XL laser densitometer and plots were normalised to the highest absorbance value in each lane.

3.3 Results

3.3.1. Nitrogen Mustards

Mechlorethamine and melphalan (1mM) were reacted with four duplex oligonucleotides containing a single potential cross-linking site (A-D, figure 3.1).

A 5'-A T A T A T C A T A T A T
T A T A T A **G** T A T A T A

B 5'-A T A T A T C **G** A T A T A T
T A T A T A **G** C T A T A T A

C 5'-A T A T A T **G** C A T A T A T
T A T A T A C **G** T A T A T A

D 5'-A T A T A T **G** A C A T A T A T
T A T A T A C T **G** T A T A T A

Figure 3.1 Sequence of duplex oligonucleotides (A-D) containing a single potential cross-linking site.

The DNA was ethanol precipitated and the reaction products separated on a 20% polyacrylamide denaturing sequencing gel (figure 3.2). Under these conditions DNA strands that are covalently cross-linked by the drug are unable to denature completely and migrate with reduced mobility through

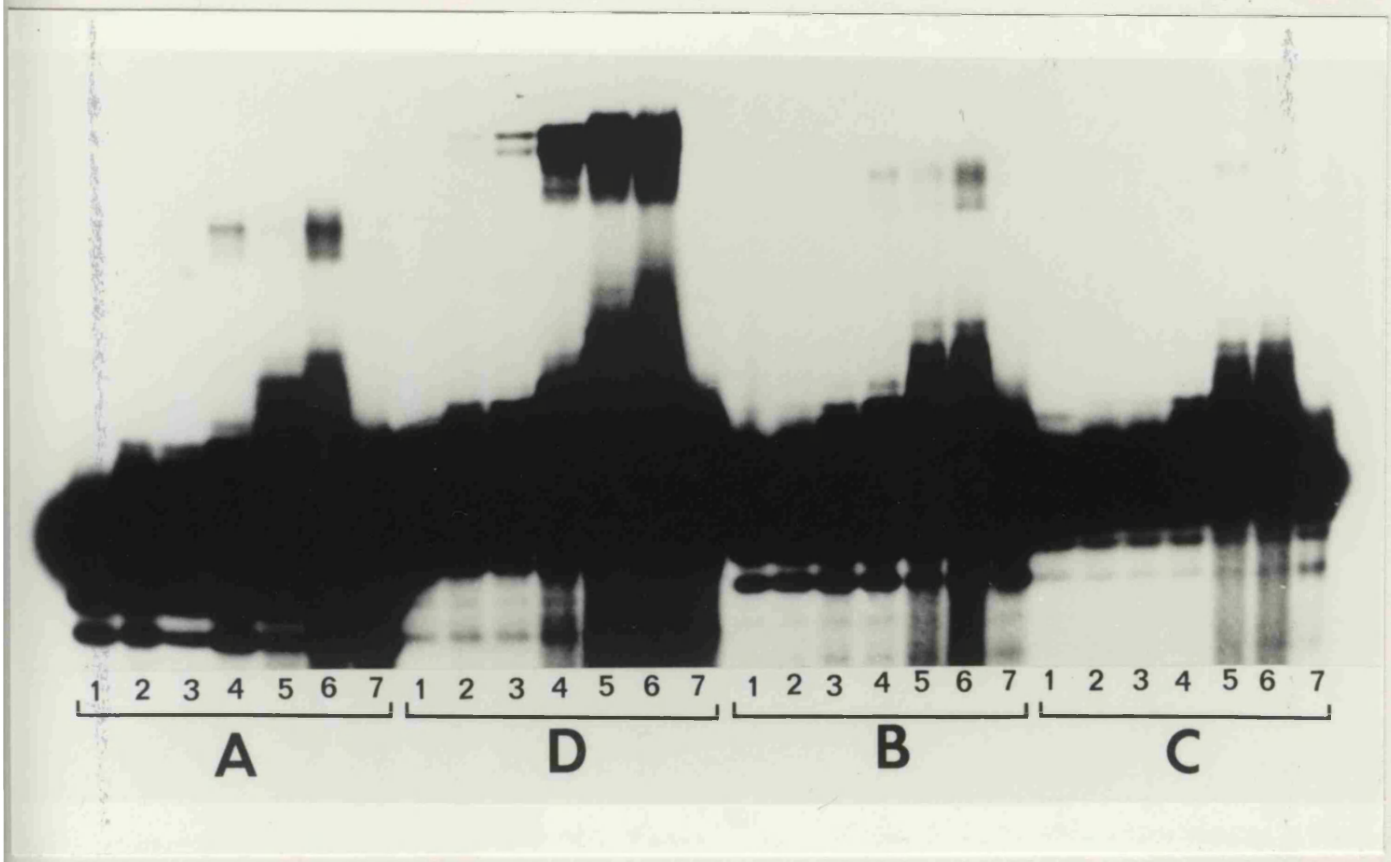


Figure 3.2 Separation of products generated upon reaction of nitrogen mustard and melphalan with DNA duplexes A-D. Drug-DNA reactions were carried out in 20 µ1 of TEA (pH 7.2) at 20° C. Products shown in lane 6 were a result of reactions carried out at 37° C. Duplexes were treated with 1 mM HN2 for 0.5 hr (lane 2); 1 hr (lane 3); 2 hr (lane 4) and 6hr (lane 5). Lane 7 shows reaction products produced after incubation of duplexes with 1 mM melphalan for 6 hr. Lane 1, control untreated DNA incubated for 6 hr.

the gel compared to unmodified DNA and DNA containing monoadducts. Mechlorethamine was found to preferentially cross-link the DNA duplex containing the 5'-GNC sequence (duplex D). An increasing amount of cross-linked product was observed with time upon reaction of HN2 with duplex D although a low level of cross-linking could also be detected with duplexes A and B at elevated temperature (lane 6, figure 3.2). High doses of drug (1 mM) were required due to the fact that the efficiency of cross-linking in short oligomers was extremely inefficient. No cross-link products were observed upon reaction of 1 mM melphalan with duplexes A-D after 6 hr at 20° C.

3.3.2. DZQ and MeDZQ

A 23 base pair duplex oligonucleotide (M1, figure 3.3) labeled either on the top strand (duplex I) or the bottom strand (duplex II) was treated with DZQ and MeDZQ for 2 h at 37° C under reducing or non-reducing conditions as described in materials and methods. Product mixtures were ethanol precipitated and analysed using 20% denaturing PAGE. Figure 3.4 shows several cross-linked products of different electrophoretic mobilities produced upon treatment of I and II with DZQ and MeDZQ. No cross-linked products could be detected for unreduced MeDZQ under these conditions (data not shown). Cross-linked products, numbered in order of migration, were excised from the gel and the relative amount of DNA in each band was determined by Cerenkov counting (Table 1). Cross-linked DNA constituted less than 1% of total DNA in each reaction mixture. Cross-linked DNAs were treated with piperidine for 15 minutes at 90° C to quantitatively cleave at sites of guanine-N7 alkylation. The resulting fragments were separated using 20% denaturing PAGE (figure 3.5 A,B) and

M1

(duplexI)

```
          3          8   10          15          20 21
5'-*C T G A C T C G T G T C A T G C A C A G G A T
      C T G A G C A C A G T A C G T G T C C T A G A-5'
        21' 19'       14'       10'  8'          2'
```

(duplexII)

```
          3          8   10          15          20 21
5'-C T G A C T C G T G T C A T G C A C A G G A T
      C T G A G C A C A G T A C G T G T C C T A G A*-5'
        21' 19'       14'       10'  8'          2'
```

Figure 3.3 Sequence of duplex oligonucleotide M1 which was 5' end labeled on the top strand (duplex I) or the bottom strand (duplex II). Guanine residues are numbered from the 5' end with guanines on the bottom strand denoted by a (').

the autoradiograms analysed by linear densitometry (figure 3.6 A,B and C). No trace of the initial cross-linked product could be detected after aqueous piperidine treatment suggesting the drug was covalently linked between two guanine-N7 positions on opposite strands of the DNA duplex. The position of guanine-N7 alkylation on cross-linked DNA was determined by comparison with guanine specific chemical cleavage of the corresponding singly end-labeled, unmodified DNA (figures 3.5 and 3.6, G lanes). To determine the site of cross-linking for each drug treatment, fragmentation patterns generated from duplex I cross-linked DNAs were compared to duplex II cross-linked DNAs of corresponding electrophoretic mobility.

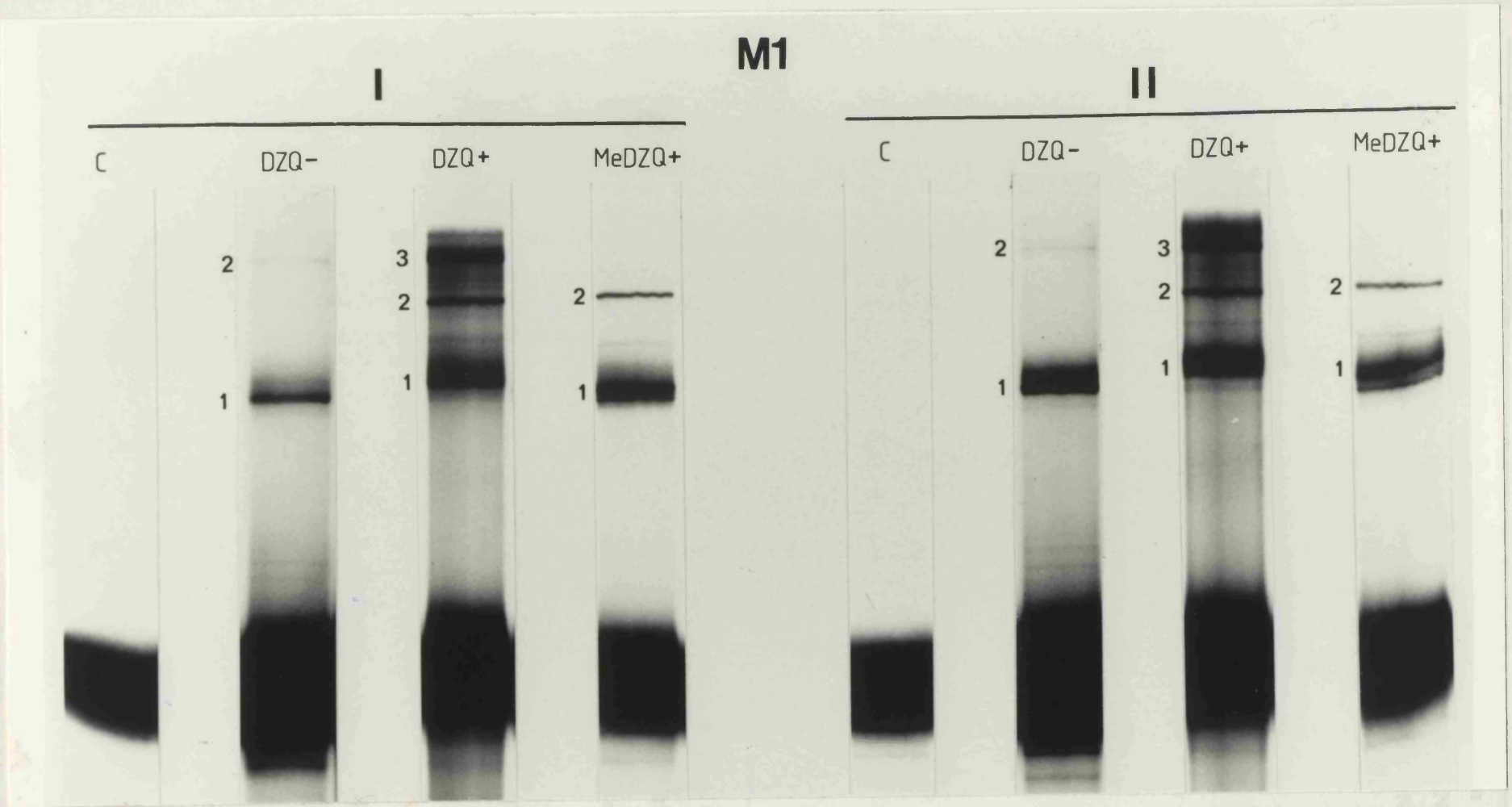


Figure 3.4. Autoradiograph of a 20% denaturing sequencing gel showing cross-links formed in M1 duplexes (I) and (II) by DZQ and MeDZQ, C lanes are control untreated DNA. DZQ- lanes are 500 μM DZQ, pH 7.2 under non reducing conditions. DZQ+ lanes are 100 μM DZQ, pH 5.8 + DT-diaphorase. MeDZQ lanes are 100 μM MeDZQ, pH 5.8 + DT-diaphorase. Numbers to the left of the lanes identify the position of the band excised from the gel.

Table 1: Amount of M1 cross-linked DNA in bands excised from the gel in figure 3.4 as determined by Cerenkov counting. Quantity of DNA in each band is expressed as a percentage of the total cross-linked DNA for the indicated drug treatment and represents the average from duplex I and II.

Treatment	Band	% contribution
DZQ-	1	88
	2	12
DZQ+	1	38
	2	28
	3	34
MeDZQ+	1	63
	2	37

In order to assess the guanine-N7 sequence specificity of total alkylations, a fraction of the drug treated DNA samples was removed from the reaction mixture prior to separation of the reaction products and treated with piperidine. The resulting fragmentation patterns were therefore representative of both monoalkylated and cross-linked DNA (figures 3.5 and 3.6, T lanes).

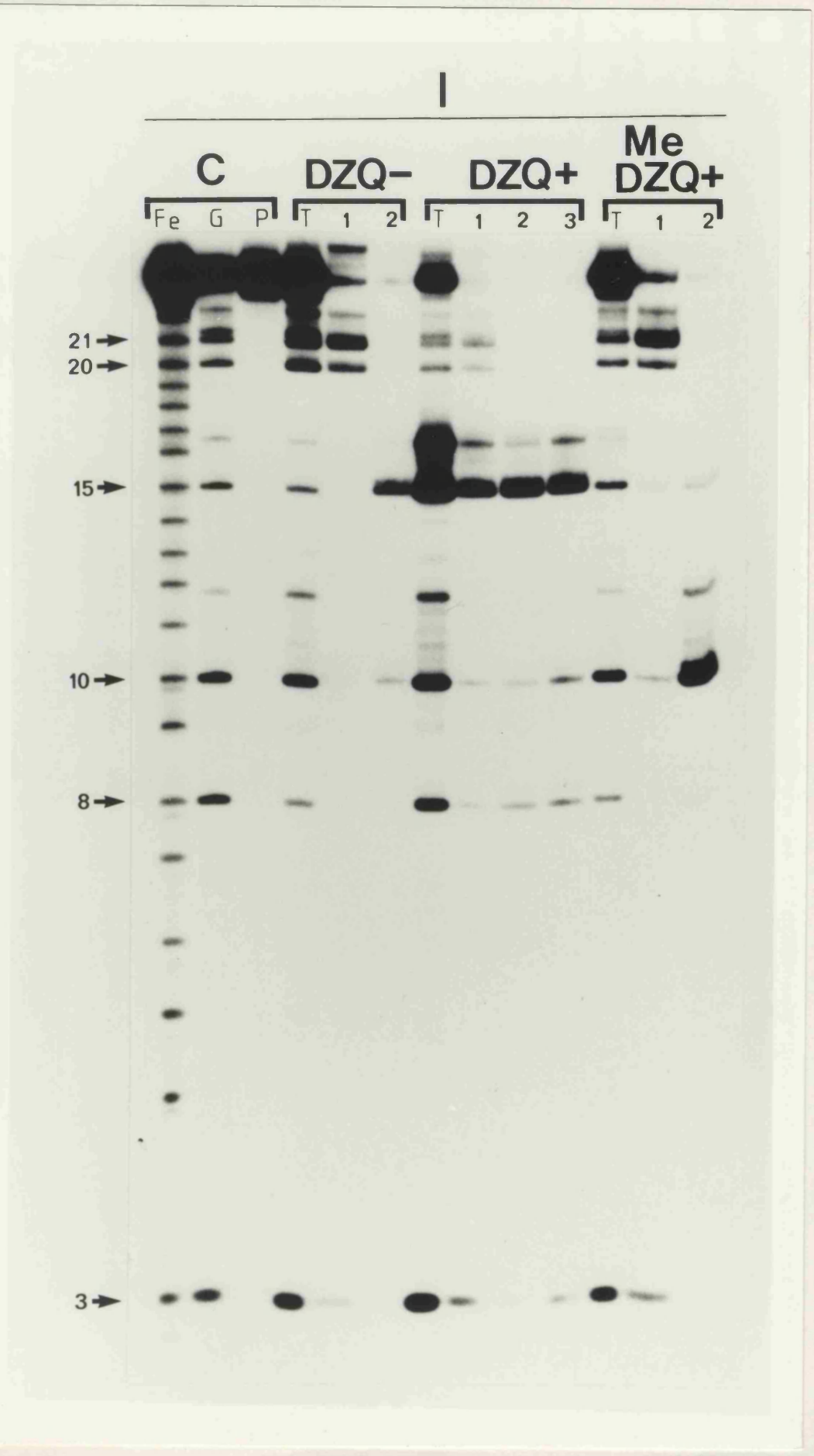
Figure 3.4 shows that each drug treatment produced significant levels of a cross-linked product migrating with increased electrophoretic mobility through the sequencing gel (band 1 DNAs). In general, piperidine treatment of cross-linked DNAs migrating in band 1 resulted in the cleavage of guanines located at or near the ends of the cross-linked duplex. For example, reaction of unreduced DZQ with M1 at neutral pH produced two cross-linked products of different electrophoretic mobility (figure 3.4,

bands 1 and 2). Piperidine treatment of DNA in band 1 produced fragments resulting from cleavage of G3, G20, and G21 on duplex I and G2' and G21' on duplex II (figures 3.5 A,B and 3.6A). Similar fragmentation patterns were observed for band 1 DNAs in the other drug treatments. The structure of these cross-linked products is not well understood and although their significance cannot be discounted, previous studies have demonstrated that cross-linked DNA duplexes migrating with maximum electrophoretic mobility are terminally cross-linked and are therefore not representative of the double stranded portion of the DNA duplex (Weidner et. al., 1990; Millard et. al., 1991).

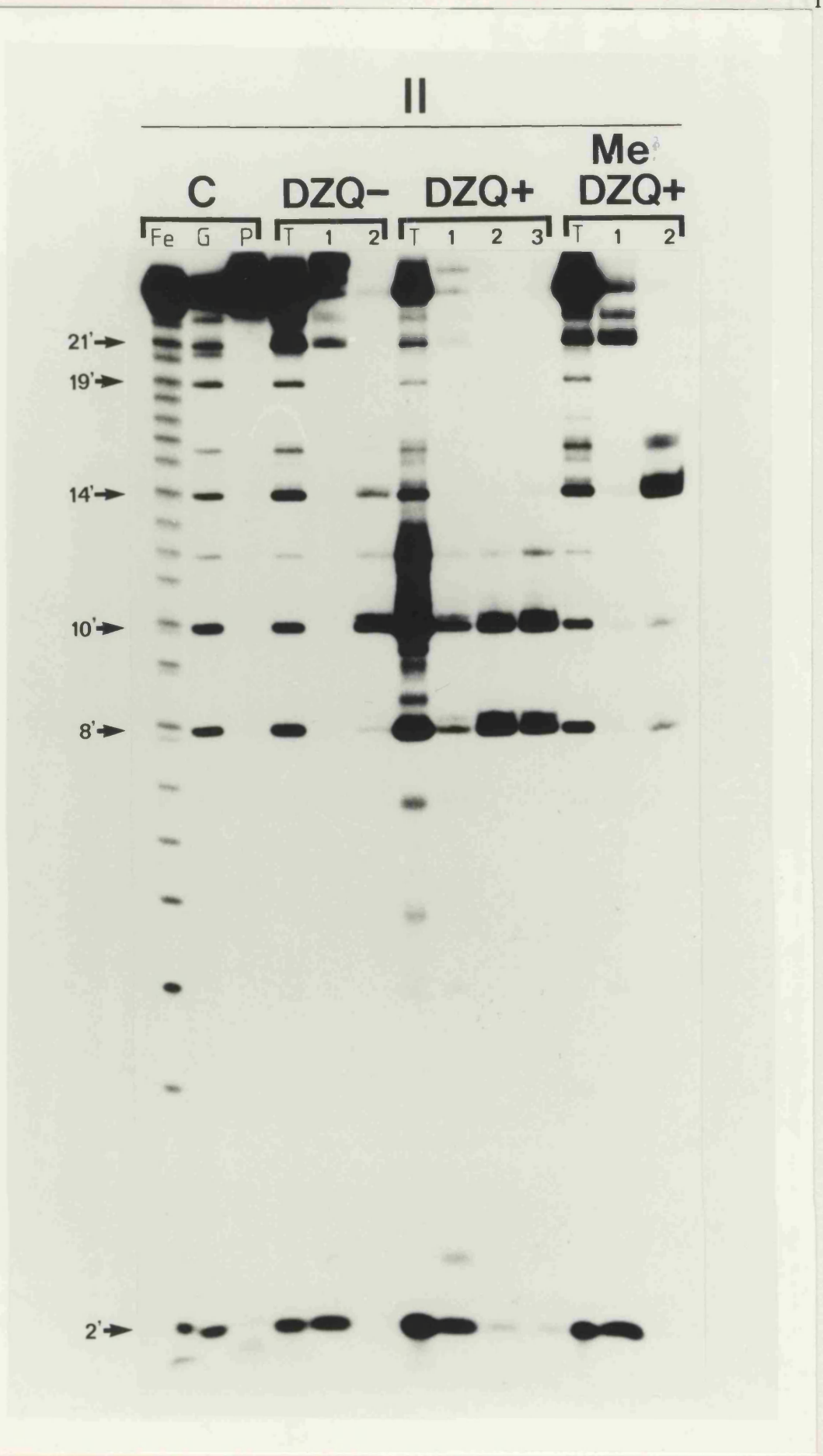
For the case of unreduced DZQ, piperidine treatment of cross-linked DNA migrating in band 2 revealed almost exclusive alkylation of G15 on duplex I and G10' on duplex II suggesting a dG to dG DNA interstrand cross-link at the 5'-GC sequence (figures 3.5 A,B and 3.6 A).

Figure 3.5 (overleaf) Electrophoretic separation of M1 fragments produced upon piperidine treatment of DNA cross-linked by DZQ and MeDZQ. Panels A and B show fragments produced upon fragmentation of duplex I and duplex II cross-linked DNAs respectively. Numbers at the top of the gel indicate cross-linked DNAs with different mobilities and correspond to the numbered bands in figure 3.4. C indicates control lanes, where Fe is control DNA treated with Fe/EDTA/H_2O_2/Ascorbic acid showing the position of each base. G is guanine specific cleavage of control DNA, and P is piperidine treatment of unalkylated DNA. T shows fragments produced upon piperidine treatment of total reaction products prior to separation by denaturing PAGE. DZQ- shows fragmentation patterns of isolated DNAs cross-linked with 500 μM DZQ under non reducing conditions. DZQ+ and MeDZQ+ show fragmentation patterns produced by isolated DNAs cross-linked by 100 μM DZQ and 100 μM MeDZQ in the presence of DT-Diaphorase. Numbers to the left of the gel indicate the position of the guanine from the 5' end label. Guanines in II are distinguished by a (') after the number.

A



B



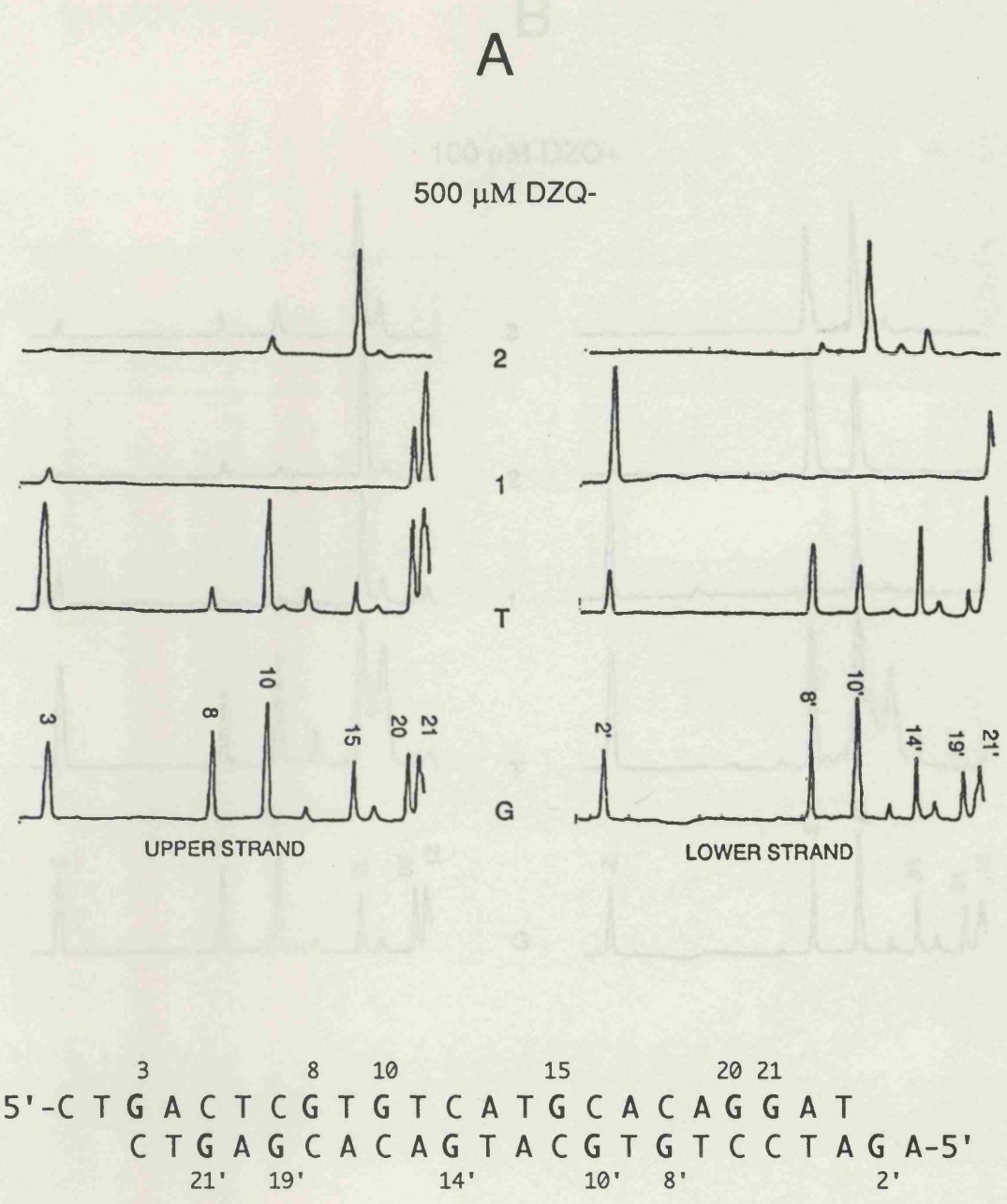
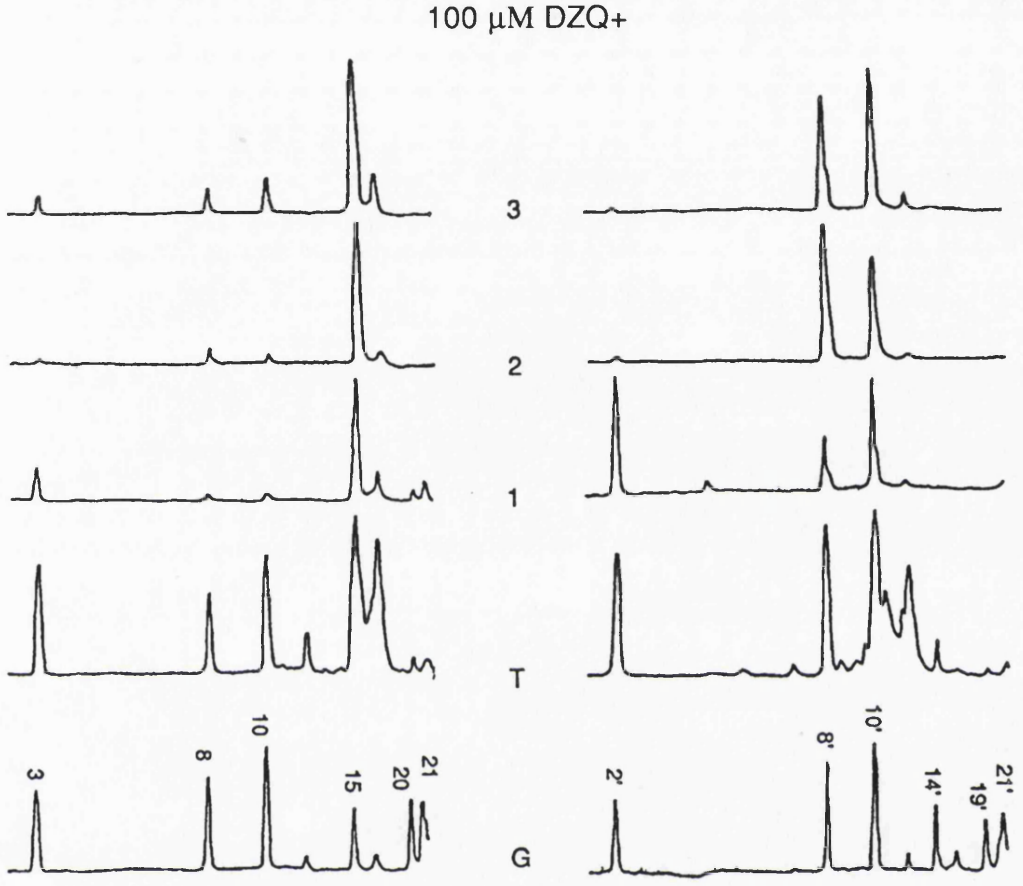


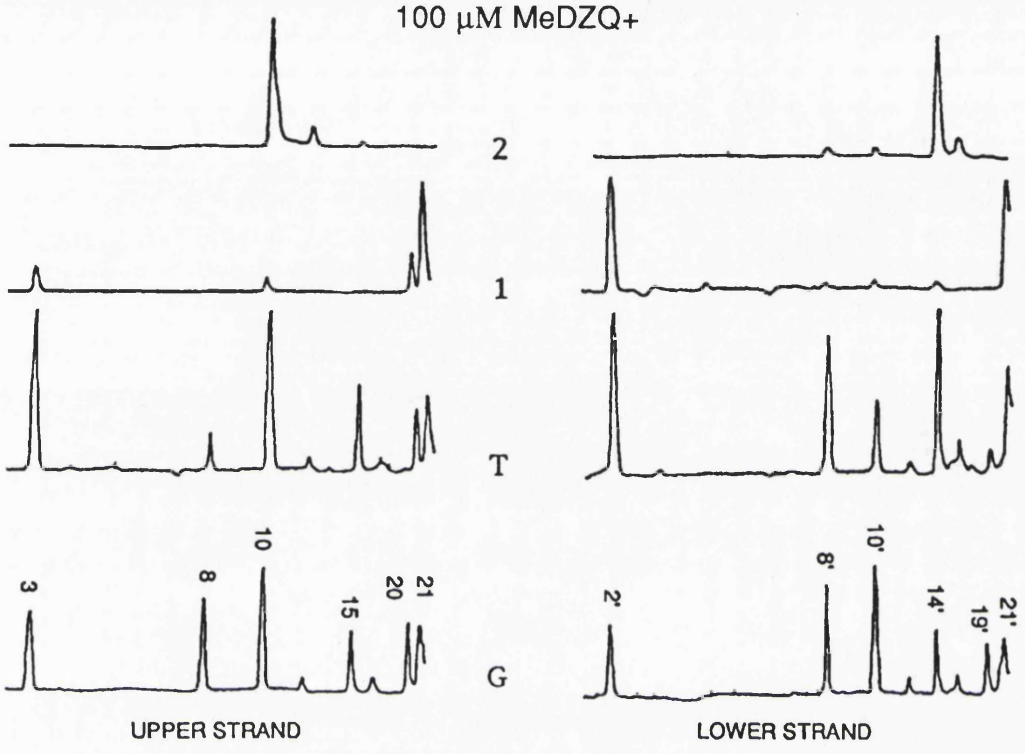
Figure 3.6. Densitometry representing M1 fragmentation patterns produced upon piperidine treatment of duplex I cross-linked DNAs (upper strand) and duplex II cross-linked DNAs (lower strand) of corresponding electrophoretic mobility. Numbers between traces indicate the mobility of the cross-linked product as shown in figure 3.4. T indicates total alkylations. G shows guanine specific sequencing lanes of control DNA. Numbered peaks show the position of guanine from the 5' end label. Panel A compares I and II DNAs cross-linked with 500 μM DZQ under non reducing conditions. Panels B and C show I and II DNAs cross-linked with 100 μM DZQ and 100 μM MeDZQ respectively under reducing conditions.

B




```
          3          8   10          15          20 21
5'-C T G A C T C G T G T C A T G C A C A G G A T
      C T G A G C A C A G T A C G T G T C C T A G A-5'
         21' 19'       14'       10' 8'          2'
```

C




```
          3          8   10          15          20 21
5'-C T G A C T C G T G T C A T G C A C A G G A T
      C T G A G C A C A G T A C G T G T C C T A G A-5'
         21' 19'        14'       10' 8'          2'
```

However, reduction of DZQ to the hydroquinone produced 3 distinct cross-linked products of different electrophoretic mobility (figure 3.4). Piperidine treatment of the cross-linked DNA in band 3 showed alkylation of G15 on duplex I and G8' and G10' on duplex II (figures 3.5 A,B and 3.6B). A similar piperidine fragmentation pattern was observed for cross-linked DNA in band 2. The sequence preference for the formation of the DNA cross-link in bands 2 and 3 is not immediately clear due to the presence of more than one fragment from duplex II (G8' and G10'). However, it is possible that the position of the cross-link may differ from one duplex to the next with a proportion of the DNA cross-linked through the 5'-GC sequence (G15 through G10') and the remaining DNA cross-linked through the 5'-GNNC sequence (G15 through G8').

In contrast to reduced DZQ, reaction of reduced MeDZQ with M1 produced only two cross-linked products of different electrophoretic mobility. Fragmentation of the cross-linked DNA in band 2 produced almost exclusive alkylation of G10 on duplex I and G14' on duplex II suggesting a dG to dG cross-link in a 5'-GNC sequence (figure 3.5 A,B and 3.6C). Thus reduced MeDZQ cross-links the 5'-GNC sequence in M1, a preference that is not observed with reduced DZQ.

Analysis of total alkylations (both cross-links and monoadducts) showed that unreduced DZQ exhibited relatively little preferential alkylation of guanine-N7 positions on duplexes I and II (figure 3.5 A,B and 3.6A). However, reduction of DZQ to the hydroquinone by DT-Diaphorase produced strong preferential alkylation of G15 on duplex I and G10' on duplex II. In contrast, relatively non-specific guanine-N7 alkylation was observed upon reduction of MeDZQ. Figures 3.5A and B (T lanes) show substantial amounts

of full length single stranded DNA indicating that many duplexes are not alkylated and that most strands contain only one alkylation.

Fragmentation patterns produced from all cross-linked DNA duplexes were analysed by linear densitometry and shown in figure 3.6A-C. In general, cross-linked DNAs of different electrophoretic mobility displayed unique fragmentation patterns upon treatment with piperidine. Interestingly, preferential alkylation of guanines located in 5'-TGC sequences (G15, G8' and G10') was observed for each DNA duplex cross-linked by the reduced form of DZQ (figure 3.6B). This effect was not detected for either unreduced DZQ or reduced MeDZQ. In addition, DNA cross-linked by reduced DZQ and migrating in bands 2 and 3 produced two fragments upon piperidine treatment of duplex II (figure 3.6B). Since it was not clear whether M1 was cross-linked at the 5'-GC sequence or the 5'-GNNC sequence or both, a second duplex oligonucleotide was synthesised (M2) in which these two sequences were separated by several nucleotides within the central portion of the duplex (figure 3.7)

Duplex M2, labeled either on the top strand (duplex I) or the bottom strand (duplex II), was reacted with DZQ and MeDZQ in the presence or absence of NADH and DT-Diaphorase for 2 hours at 37^{o} C. The reaction products were ethanol precipitated and separated through a 20% denaturing sequencing gel. Figure 3.8 shows several cross-linked products of different electrophoretic mobilities were produced upon reaction of DZQ and MeDZQ with M2. These cross-linked products, numbered in order of migration, were excised from the gel and the relative amount of DNA in each band was determined Cerenkov counting (table 2). Cross-linked products were treated with piperidine and the resulting fragments were separated using 20% denaturing sequencing gel electrophoresis(figure 3.9).

M2

duplex I

```
          4                 10                14                21
5'-*T A T G A C T A T G C A T G A T C T A C G A T
    A T A C T G A T A C G T A C T A G A T G C T A-5'
              18'               13'               7'      4'
```

duplex II

```
          4                 10                14                21
5'-T A T G A C T A T G C A T G A T C T A C G A T
   A T A C T G A T A C G T A C T A G A T G C T A*-5'
             18'               13'               7'      4'
```

Figure 3.7 Sequence of duplex oligonucleotide M2 which was 5' end labeled either on the top strand (duplex I) or the bottom strand (duplex II). Guanine residues are numbered from the 5' end with guanines on the bottom strand denoted by a (').

Band 2 was one of the principal cross-linked products resulting from the reaction of unreduced DZQ with I and II (figure 3.8, table 2). Fragmentation of the cross-linked DNA migrating in band 2 revealed almost exclusive alkylation of G4 on duplex I and G18' on duplex II indicating a cross-link at the 5'-GNC sequence (figure 3.9). However, upon reduction of DZQ to the hydroquinone, a decrease in the electrophoretic mobility of the major cross-linked products was observed (bands 3 and 4, figure 3.8). Close examination of the autoradiograph revealed that band 3 was comprised of a number of closely spaced bands while band 4 was the major cross-linked product and migrated as a single band through the gel. Piperidine cleavage of the cross-linked DNA in band 4 showed extensive alkylation of

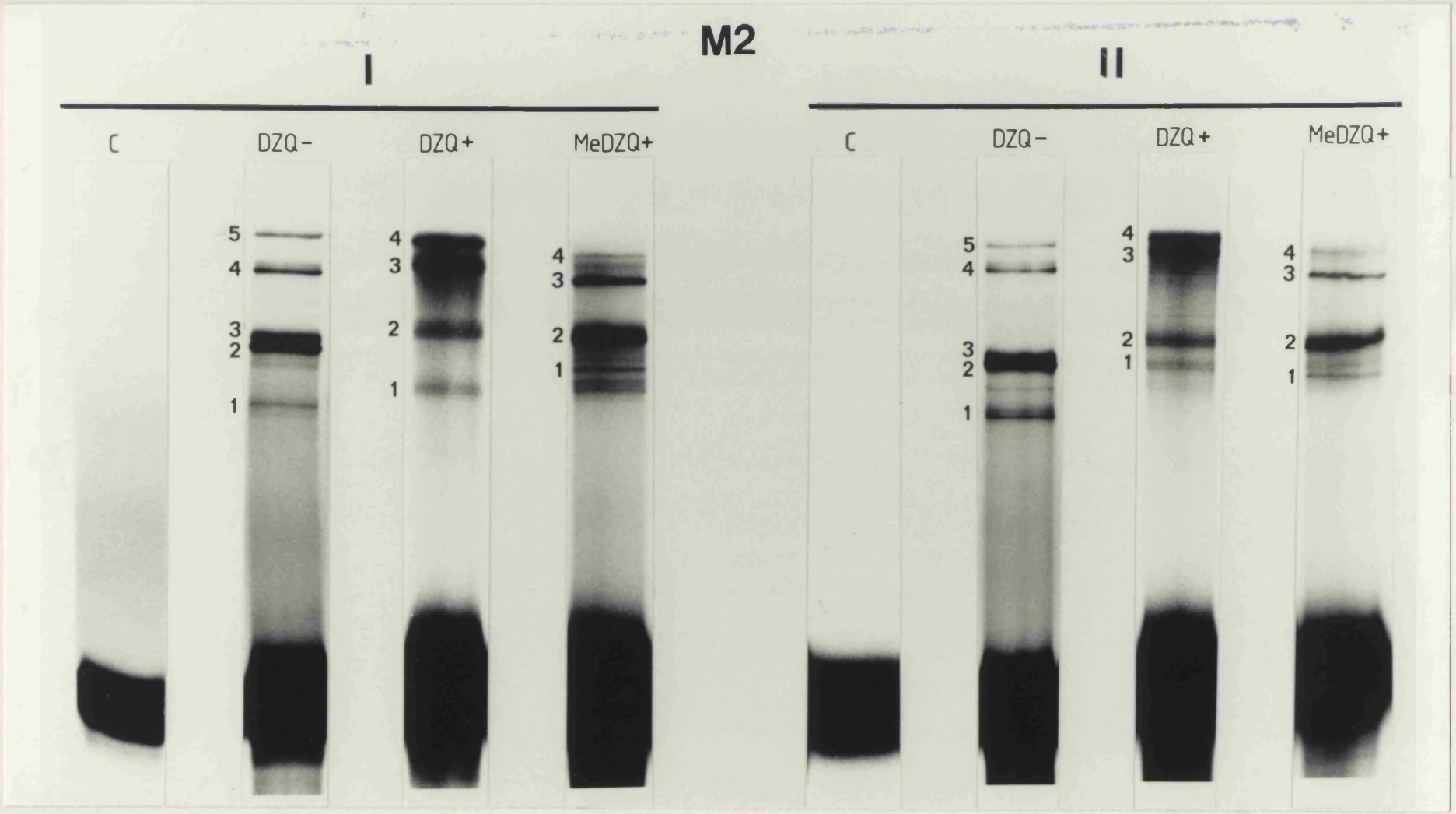


Figure 3.8. Autoradiograph of a 20% denaturing sequencing gel showing cross-links formed in M2 duplexes (I) and (II) by DZQ and MeDZQ, C lanes are control untreated DNA. DZQ- lanes are 500 μM DZQ, pH 5.8 under non reducing conditions. DZQ+ lanes are 100 μM DZQ, pH 5.8 + DT-diaphorase. MeDZQ lanes are 100 μM MeDZQ, pH 5.8 + DT-diaphorase. Numbers to the left of the lanes identify the position of the band excised from the gel.

Table 2: Amount of M2 cross-linked DNA in bands excised from the gel in figure 3.8 as determined by densitometry. Amount of DNA in each band is expressed as a percentage of the total cross-linked DNA for the indicated treatment. Data represents the average from duplex I and II from a single experiment.

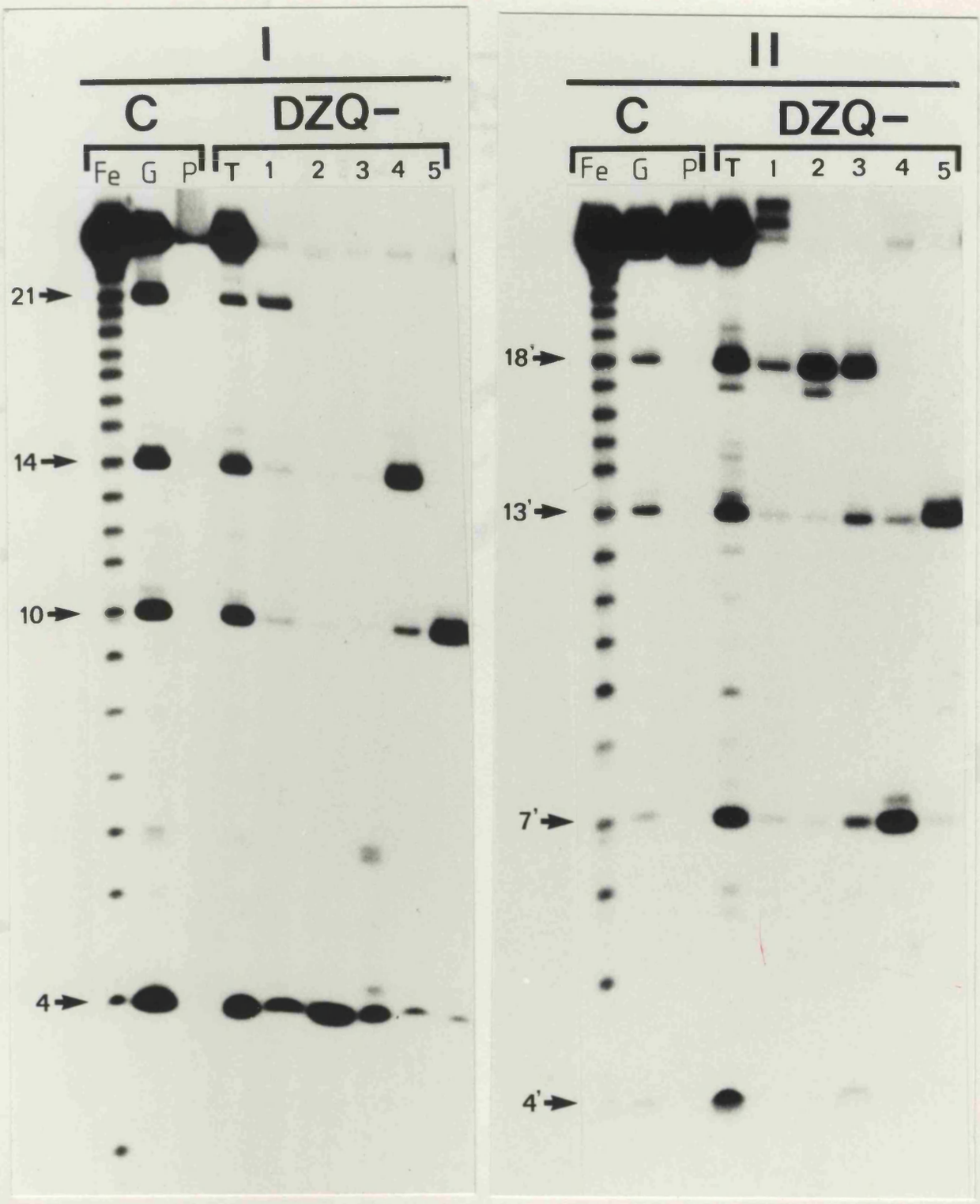
Treatment	Band	% contribution
DZQ-	1	15.7
	2	31.4
	3	23.8
	4	18.6
	5	10.5
DZQ+	1	18.2
	2	26.5
	3	27.2
	4	28.1
MeDZQ+	1	16.0
	2	49.8
	3	21.2
	4	13.0

G10 on duplex I and G13' on duplex II indicating preferential cross-linking at the 5'-GC sequence (figures 3.9). In contrast to reduced DZQ, the major cross-linked product formed upon reaction of reduced MeDZQ with I and II (band 2) showed a clear majority of alkylations at G4 and G18' implicating cross-link formation at the 5'-GNC site within the sequence (figures 3.9)

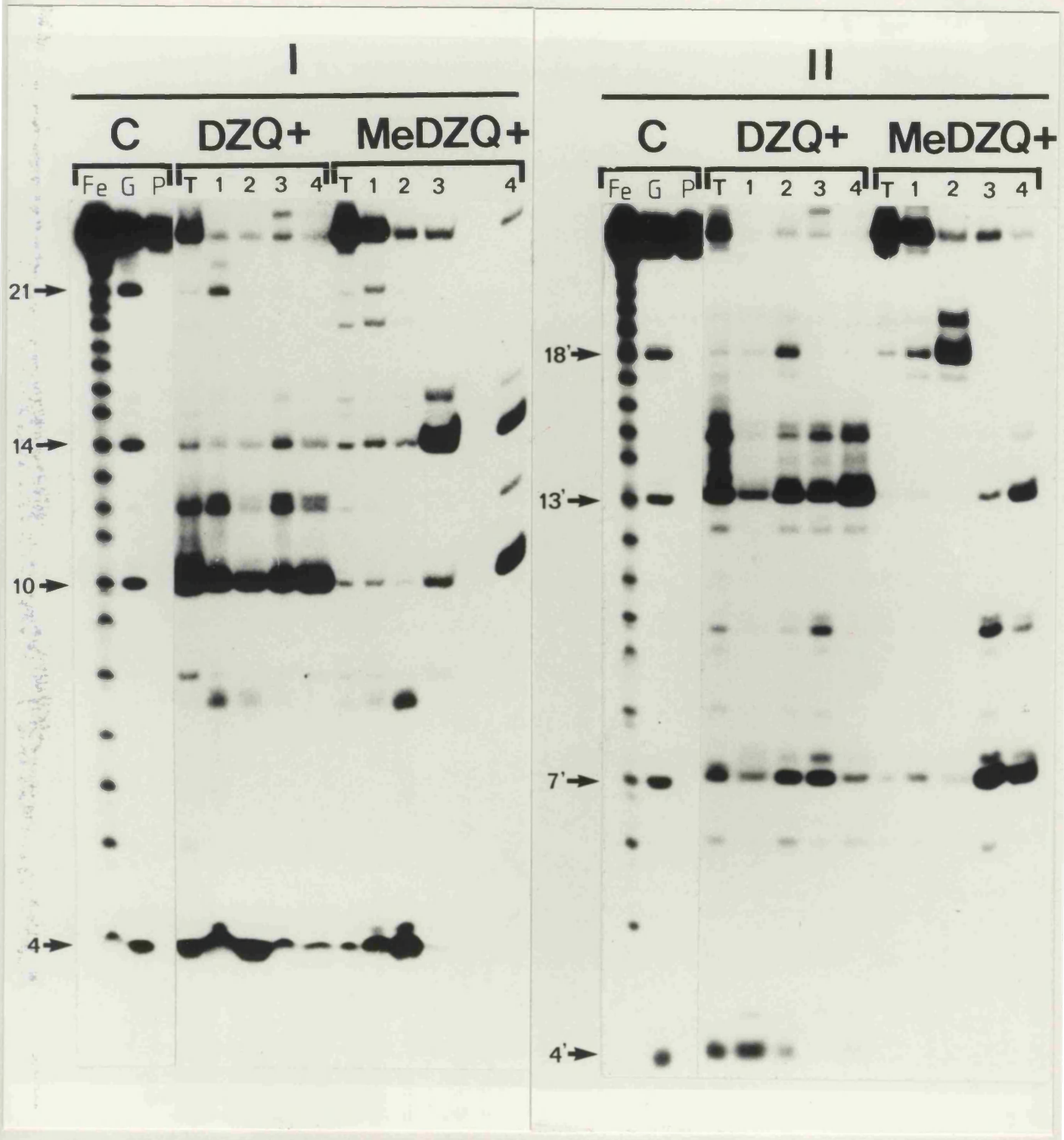
As with M1, the total alkylation products formed upon reaction of unreduced DZQ with M2 showed little preferential alkylation of guanines on either I or II (figures 3.9). However, upon reduction of DZQ to the hydroquinone form a strong preferential alkylation of G10 and G13' was observed. No such preferential alkylation of guanines was detected with reduced MeDZQ. Significant amounts of the single stranded DNA remained intact upon treatment of alkylated DNA with piperidine (figure 3.9) indicating that a majority of the DNA molecules were not alkylated.

Figure 3.9 (Overleaf) Portion of a 20% denaturing polyacrylamide gel showing piperidine induced fragmentation of M2 cross-linked DNAs from duplex I and II (figure 3.8). C indicates control lanes, where Fe is control DNA treated with Fe/EDTA/H_2O_2/ascorbic acid showing the position of each base. G is guanine specific cleavage of control DNA, and P is piperidine treatment of unalkylated DNA. T shows fragments produced upon piperidine treatment of total reaction products prior to separation by denaturing PAGE. Numbered lanes are cross-linked DNAs with different mobilities and correspond to the numbered bands in figure 3.8 Panel A shows patterns produced from I and II DNAs cross-linked by 500 μM DZQ pH 5.8 under non reducing conditions (DZQ-). Panel B shows patterns produced from I and II DNAs cross-linked with 100 μM DZQ (DZQ+), and 100 μM MeDZQ (MeDZQ+) at pH 5.8 in the presence of DT-Diaphorase. Numbers to the left of the gel indicate the position of the guanine from the 5' end label. Guanines in II are distinguished by a (') after the number.

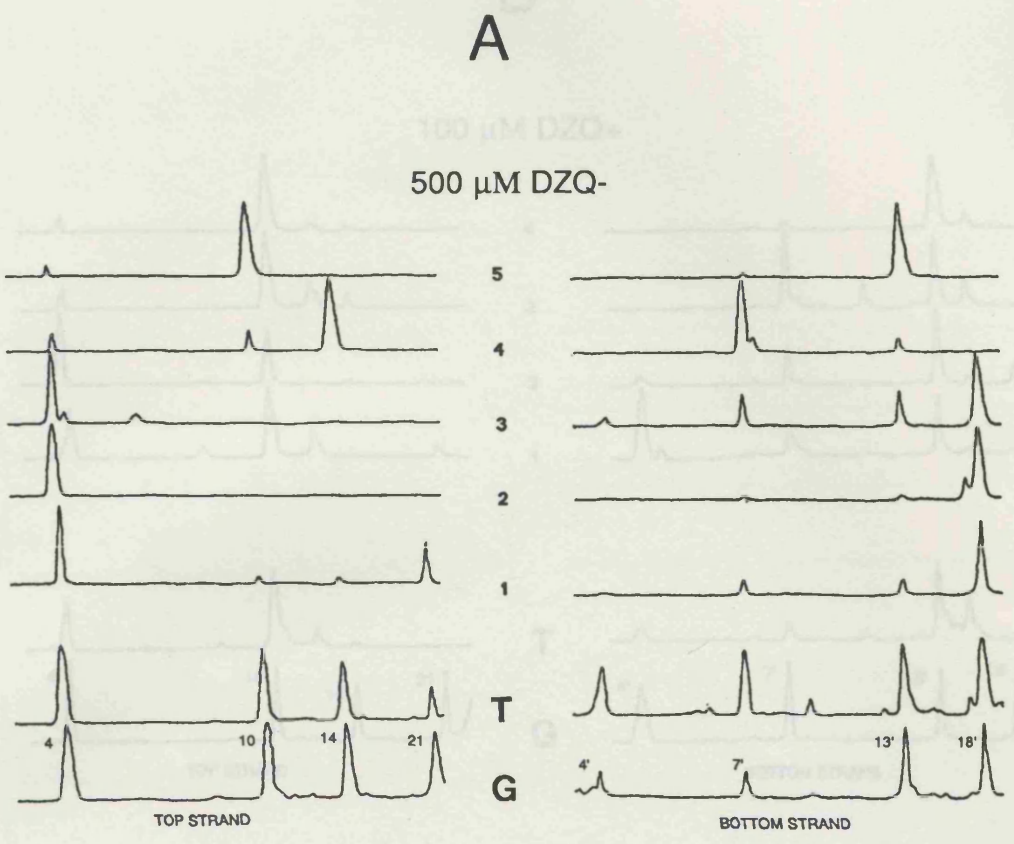
A



B


I
C
DZQ+
MeDZQ+
Fe G P T 1 2 3 4 T 1 2 3 4
21
14
10
4
II
C
DZQ+
MeDZQ+
Fe G P T 1 2 3 4 T 1 2 3 4
18'
13'
7'
4'

The fragmentation patterns produced by all cross-linked DNAs were analysed by linear densitometry and compared in figure 3.10A-C. As observed with M1, cross-linked DNAs of different electrophoretic mobility displayed unique fragmentation patterns upon piperidine treatment. Those cross-linked DNAs having increased mobility through a denaturing gel (band 1 DNAs) showed prominent alkylations at guanines near the ends of the duplex while cross-linked DNA having diminished electrophoretic mobility (bands 4 and 5 DNAs) displayed alkylations from centrally located guanines. The occurrence of more than one fragment upon treatment of some fractions of cross-linked DNAs with piperidine indicates heterogeneity with respect to the position of the guanine alkylation and thus precludes the exact assignment of the cross-link site. This is particularly evident in DNA duplexes cross-linked by reduced DZQ where alkylations at G10 and G13' are present in all cross-linked DNAs and may be a result of the strong preferential alkylation at 5'-TGC sites. In addition, complex fragmentation patterns could result in more than one cross-linked product with the same migration through the denaturing polyacrylamide gel. Nevertheless, analysis of the alkylation patterns produced by less abundant cross-linked products clearly indicated that unreduced DZQ could produce a cross-link at the 5'-GC site (band 5, figure 3.10A) but in a lower yield. More interestingly, however was the finding that unreduced DZQ (band 4, figure 3.10A) and reduced MeDZQ (band 3, figure 3.10C) exhibited significant alkylation of G14 and G7' suggesting the formation of a cross-link across four base pairs at the 5'-GNNC sequence.



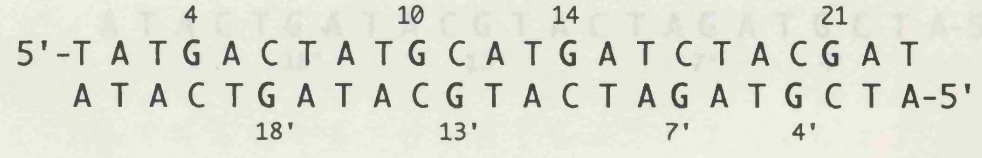
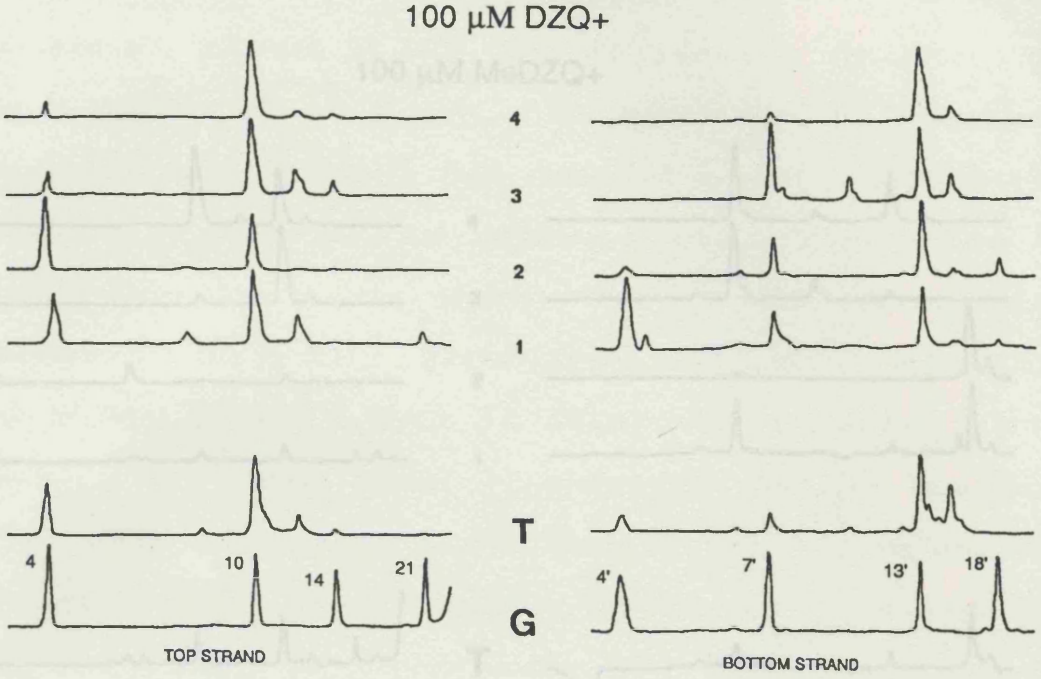


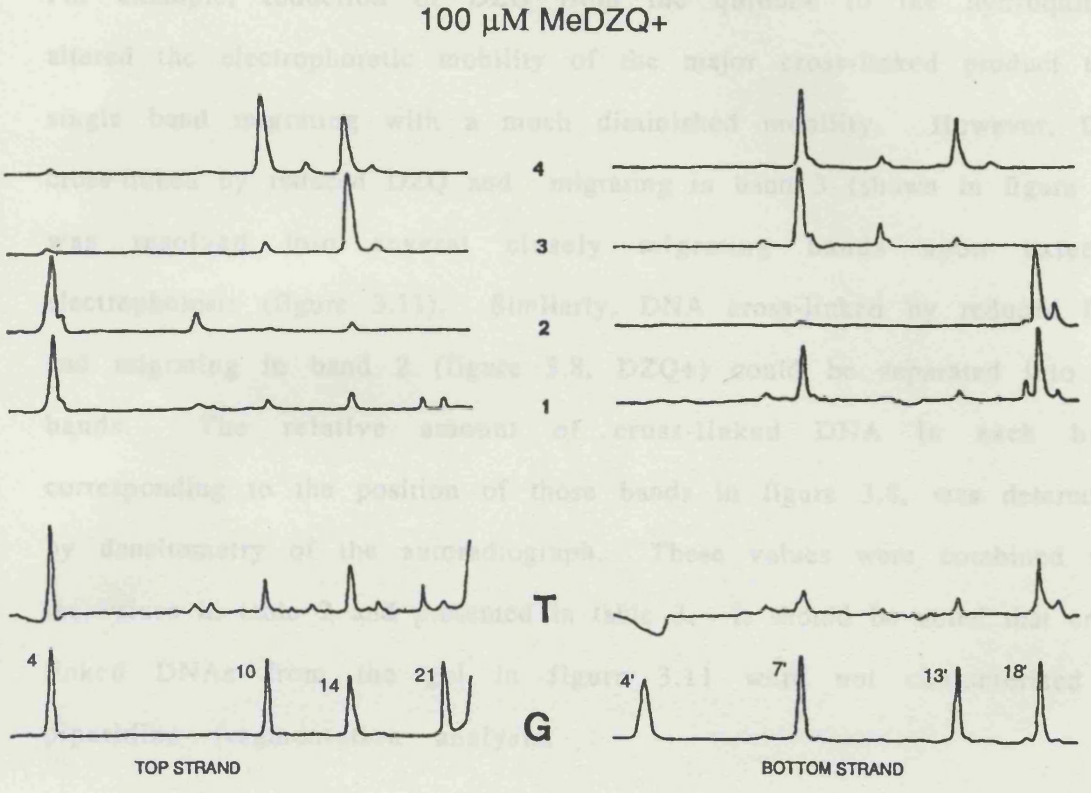
Figure 3.10 Densitometry representing M2 fragmentation patterns produced upon piperidine treatment of duplex I cross-linked DNAs (top strand) and duplex (II) cross-linked DNAs (bottom strand) of corresponding electrophoretic mobility. Numbers between the traces indicate the mobility of the cross-linked product through the sequencing gel as shown in figure 3.8. T shows total alkylations and G shows guanine specific sequencing lanes of control DNA. Numbered peaks show the position of the guanine residue from the 5' phosphate. Panel A compares I and II DNAs cross-linked by 500 μM DZQ under non reducing conditions. Panels B and C show I and II DNAs cross-linked by 100 μM DZQ and 100 μM MeDZQ in the presence of DT-diaphorase.

B




```
          4          10         14          21
5'-T A T G A C T A T G C A T G A T C T A C G A T
   A T A C T G A T A C G T A C T A G A T G C T A-5'
            18'        13'         7'      4'
```

C




```
          4              10        14              21
5'-T A T G A C T A T G C A T G A T C T A C G A T
   A T A C T G A T A C G T A C T A G A T G C T A-5'
                18'        13'            7'    4'
```

The distribution and reproducibility of cross-linked products induced in M2 by DZQ and MeDZQ was investigated in more detail by extended electrophoretic separation of the drug-DNA reaction mixtures as shown in figure 3.11. Although the basic distribution of major cross-linked products was retained, the resolution of many more bands was detected. For example, reduction of DZQ from the quinone to the hydroquinone altered the electrophoretic mobility of the major cross-linked product to a single band migrating with a much diminished mobility. However, DNA cross-linked by reduced DZQ and migrating in band 3 (shown in figure 3.8) was resolved into several closely migrating bands upon extended electrophoresis (figure 3.11). Similarly, DNA cross-linked by reduced DZQ and migrating in band 2 (figure 3.8, DZQ+) could be separated into two bands. The relative amount of cross-linked DNA in each band, corresponding to the position of those bands in figure 3.8, was determined by densitometry of the autoradiograph. These values were combined with the values in table 2 and presented in table 3. It should be noted that cross-linked DNAs from the gel in figure 3.11 were not characterised by piperidine fragmentation analysis.

The sequence preferences for the formation of DNA cross-links induced by DZQ and MeDZQ were also investigated in synthetic duplex oligonucleotides containing a single potential cross-linking site (figure 3.1). Figure 3.12 shows the separation of products generated from the reaction of DZQ and MeDZQ with oligomers B, C and D. Unreduced DZQ produced a cross-linked product in the 5'-GNC containing oligomer (D) but not in the 5'-CG (B) or the 5'-GC (C) containing duplexes. However, reduction of DZQ to the hydroquinone produced a cross-linked product in the 5'-GC duplex (C) and also to a lesser extent in the 5'-GNC containing duplex (D). Cross-linking

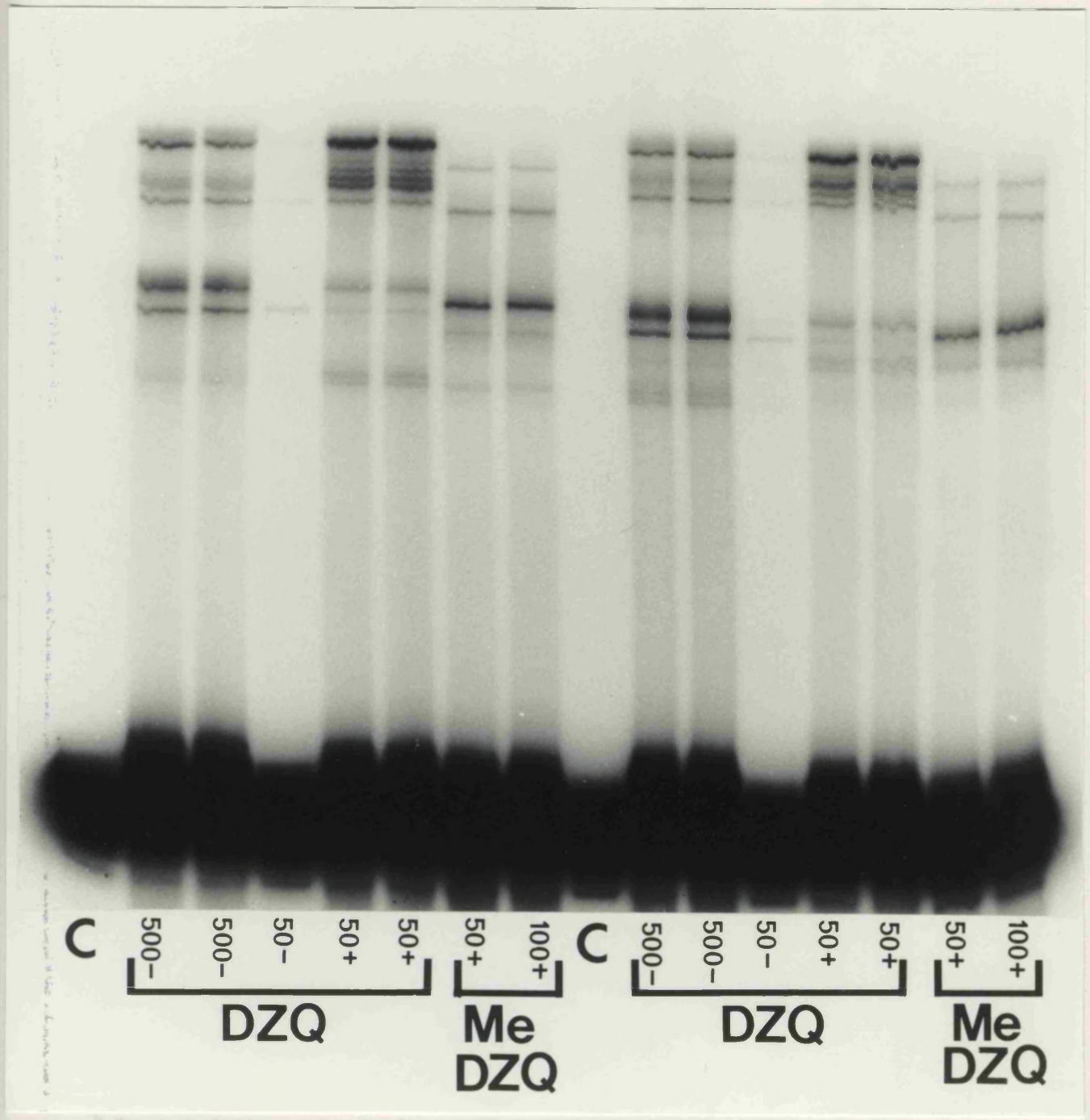


Figure 3.11 Separation of products resulting from the reaction of DZQ and MeDZQ with M2. Drug-DNA reactions were carried out in 50 µl of TEA (pH 5.8) in the presence (+) or absence (-) of 0.173 mg of DT-Diaphorase and 0.1 mM NADH. Left hand lanes, duplex I; right hand lanes, duplex II. C indicates control untreated DNA. Numbers below lanes are concentrations in µM.

Table 3. Summary of the cross-linking sites of DZQ and MeDZQ in M2 listed in order of preference. Percent cross-linked DNA in each band was determined by densitometry and represents the average from duplex I and duplex II. Band 1 DNAs which were minor products produced with M2 and which may be the result of terminal cross-links were excluded.

Treatment	Band	%[a]	Cross-link site
DZQ-	3	28.5 (4.7)	5'-GNC
	2	22.5 (7.0)	5'-GNC
	5	18.2 (6.4)	5'-GC
	4	17.1 (3.6)	5'-GNNC
DZQ+	4	32.5 (3.8)	5'-GC
	3[b]	30.5 (3.3)	?[c]
	2	16.3 (1.6)	?
MeDZQ+	2	52.5 (3.1)	5'-GNC
	3	21.9 (2.1)	5'-GNNC
	4	11.7 (3.1)	?

[a] Average value obtained from duplex I and II. Numbers in parentheses indicate +/- standard deviation from three independent experiments.

[b] Band 3 DNA consisted of a number of tightly spaced bands upon close examination of the autoradiograph.

[c] ? Denotes that exact assignment of cross-link site was not possible.

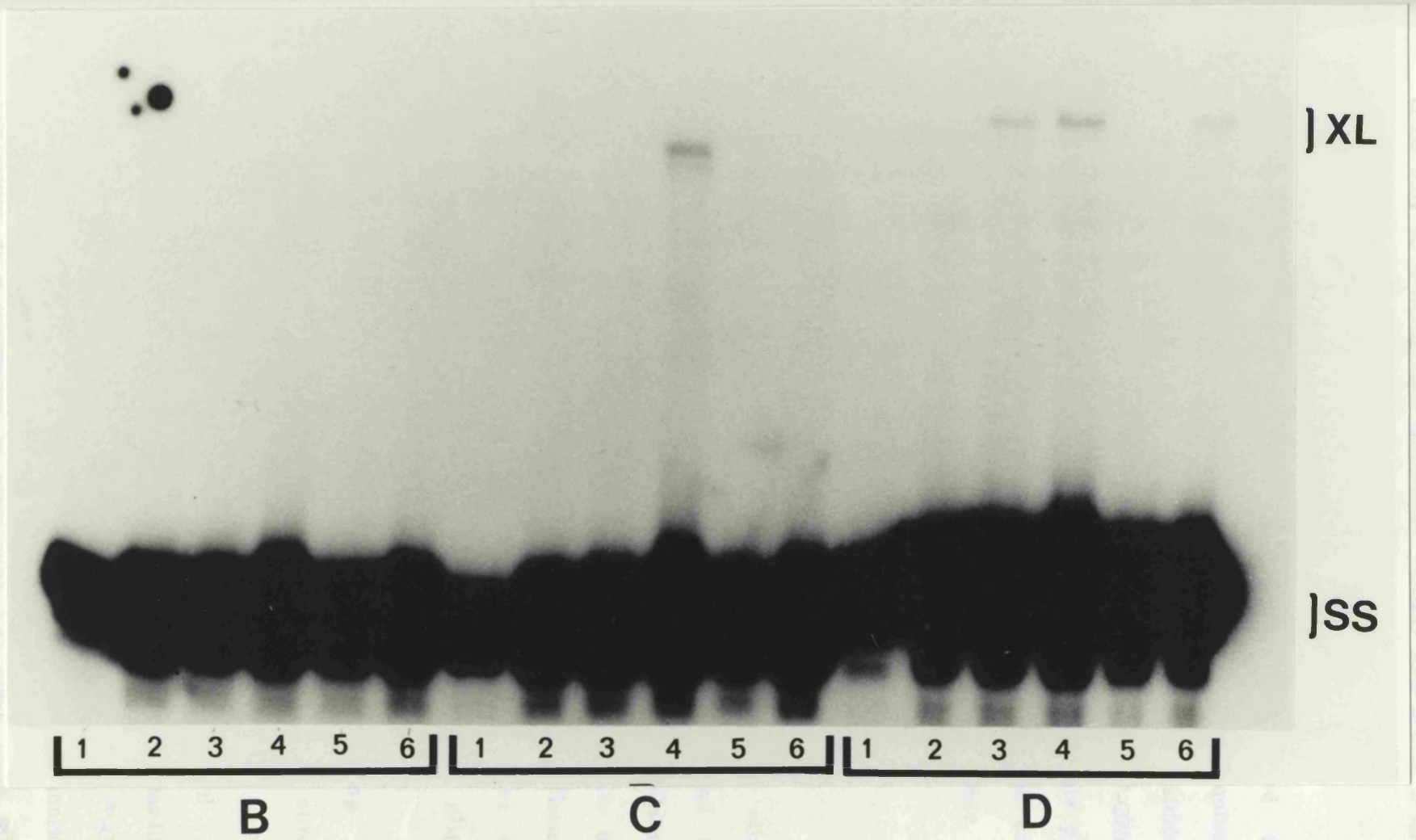


Figure 3.12 Autoradiogram of a 20% denaturing polyacrylamide gel showing the cross-linking induced in duplex oligonucleotides B, C, and D by DZQ and MeDZQ under reducing and non-reducing conditions.

Duplexes were treated for 2 hr at 8 ^{o} C. In each case lane 3 is 500 μM unreduced DZQ, lane 4 is 100 μM reduced DZQ, lane 5 is 500 μM unreduced MeDZQ, lane 6 is 100 μM reduced MeDZQ. Lanes 1 and 2 are untreated DNA.

could only be detected upon reaction of MeDZQ with duplex D (5'-GNC) under reducing conditions.

Moreover, unreduced DZQ preferentially cross-linked the 5'-GNC containing duplex while reduction of DZQ to the hydroquinone produced preferential cross-linking at the 5'-GC containing duplex although a lower level of cross-linking could also be detected in the 5'-GNC containing duplex. In contrast, the reduced form of MeDZQ could only produce cross-links across the 5'-GNC containing duplex. These data are consistent with preferential cross-link formation induced in the 23 base pair duplex oligonucleotides containing several cross-linking sites.

3.4. Discussion

Reaction of mechlorethamine with a panel of duplex oligonucleotides containing single potential cross-linking sites was found to result in preferential cross-link formation in a DNA duplex containing a 5'-GNC sequence. In addition, a low level of cross-linking could also be detected in the duplex containing a 5'-CG sequence (figure 3.2). These results are consistent with the findings of other workers who have reported that mechlorethamine preferentially cross-linked duplex oligonucleotides containing a 5'-GNC sequence (Millard et. al., 1990). It was also demonstrated that although the 5'-GNC containing duplex was preferentially cross-linked, it was not the sole site for nitrogen mustard induced DNA cross-links (Millard et. al., 1990). In a related study, piperidine fragmentation of a nitrogen mustard cross-linked oligonucleotide showed that 44% of DNA duplexes were cross-linked at a 5'-GNC sequence while only 2% and 8% were cross-linked at 5'-GC and 5'-CG sequences respectively (Hopkins et. al., 1991). It was demonstrated that the conversion of monoadducts to cross-links was much more efficient at 5'-

GNC sites than at 5'-GC sites and that this may account for the preferential cross-linking observed with nitrogen mustard. In a separate study, a duplex oligonucleotide containing a nitrogen mustard cross-link was purified by denaturing PAGE and shown to be cross-linked through guanine N7 positions in a 5'-GNC sequence (Ojwang et. al., 1989). In the present study, a low level of cross-linking was also observed upon reaction of nitrogen mustard with a DNA duplex in which only one strand contained a guanine (duplex A, and 3.2). The structure of this cross-linked product is not known and is as yet uncharacterised.

The appearance of two closely spaced cross-linked product bands generated from the reaction of nitrogen mustard with duplex D (figure 3.2) was unexpected due to the fact that there is only one potential cross-linking site within the duplex containing guanine residues (e.g. 5'-GNC, duplex D). However, previous studies have proposed that the differential migration of duplexes cross-linked through guanine-N7 positions may be due in part to the formation of FAPY imidazole ring opened products (Ojwang et. al., 1989). It was found that heating a cross-linked DNA duplex oligonucleotide at 37^{o} C in the presence of dilute alkali stabilised the cross-link by forming a FAPY ring opened product. However, the ring opened products, although more stable, were electrophoretically distinct from the ring closed species since FAPY adducts exist as a mixture of structural and rotational isomers (Ojwang et. al., 1989).

The finding that nitrogen mustard preferentially cross-links duplex oligonucleotides in a 5'-GNC sequence was not predicted from molecular modelling studies. The limited molecular reach of nitrogen mustard when fully extended (7.5 Å) was anticipated to form cross-links between guanines located in 5'-GC sequences in which the spanned distance between

guanines on opposing strands is 7.7 A in B-DNA. (Brooks and Lawley, 1961; Kohn et. al., 1987; Ojwang et. al., 1989(Arnott et al., 1974). Although other factors such as the structure of the transition state involved in cross-link formation, flanking base sequence, and conformation of DNA are likely to influence cross-link formation, the preference for nitrogen mustard to form cross-links at 5'-GNC sequences appears to be general (Hopkins et. al., 1991; Ojwang et. al., 1989; Millard et. al., 1990).

The reaction of DZQ and MeDZQ with duplex oligonucleotides containing several potential cross-linking sites resulted in the formation of a number of cross-linked products with different electrophoretic mobilities. Reduction of DZQ from the quinone to the hydroquinone form by DT-Diaphorase was found to alter both the extent and distribution of cross-linked products. Analysis of purified cross-linked DNA products by piperidine fragmentation indicated that reduction of DZQ alters the sequence preference for interstrand cross-link formation between guanine-N7 positions from a 5'-GNC to a 5'-GC site. In contrast, the reduced form of MeDZQ was shown to preferentially cross-link DNA at 5'-GNC sites. These results, which were confirmed in oligomers containing single sites, lend support to molecular modelling studies that have suggested intercalation of the hydroquinone form of DZQ at 5'-GC sites is facilitated by hydrogen bonding of the hydroxyl groups with the O2 and C4-NH_2 groups of cytosine. This orientation allows the reactive aziridine carbon atoms to be in a favourable position above the plane of the aromatic ring for covalent bond formation to the guanine-N7 position (Hartley et al., 1991a). Stabilisation of the intercalated hydroquinone may account for the preferential monoalkylation of guanines in 5'-GC sequences and subsequent cross-link formation at these sites. Indeed, as was demonstrated for nitrogen mustard, the efficiency of the conversion of monoadducts to

cross-links may influence to a large extent the sequence specificity for cross-link formation. In addition, previous studies have suggested that preferential cross-link formation takes place at locations that will result in the minimum distortion of B-DNA (Hopkins et al., 1991).

A general feature of the present study was the finding that cross-linked DNAs of different electrophoretic mobility resulted in unique fragmentation patterns upon treatment with aqueous piperidine. Cross-linked DNA migrating with increased mobility through a sequencing gel showed alkylation of guanines at or near the ends of the duplex. These results are consistent with the findings of Weidner et. al., (1991) in which the mobility of synthetic DNA oligomers cross-linked by mitomycin C analogues was shown to be related to the position of the cross-link from the terminal 5' phosphate group. Using a Fe/EDTA fragmentation protocol it was demonstrated that cross-linked DNA migrating with a diminished mobility through a denaturing sequencing gel was cross-linked near the middle of the duplex while terminally cross-linked DNA migrated with an increased mobility (Weidner et. al., 1991). Later studies confirmed that the mobility of cross-linked DNA increased as the position of the cross-link was moved toward either end of the duplex (Millard et. al., 1991). It was hypothesised that the reactivity of residues at the ends of duplex oligonucleotides is higher than in duplex regions. For this reason, terminally cross-linked DNAs were discounted as not being representative of cross-linking reactions taking place in the double stranded region of DNA.

Reaction of the quinone form of DZQ with duplex M1 produced two cross-linked products with different electrophoretic mobility (figure 3.4). Analysis of the cross-linked products by piperidine fragmentation showed that 12% of

the cross-links in M1 DNA were located at the 5'-GC sequence (band 2) with the remaining DNA apparently terminally cross-linked (figure 3.6A). In contrast, reaction of the hydroquinone form of DZQ with M1 resulted in three electrophoretically distinct cross-linked products (figure 3.4). Piperidine treatment of the non-terminally cross-linked DNA, band 2 (28%) and band 3 (34%), produced a similar fragmentation pattern to that of unreduced DZQ with the exception of an additional alkylation at G8' (figure 3.6B). The appearance of two or more fragments resulting from piperidine treatment of singly end-labeled cross-linked DNA indicates heterogeneity with respect to the position of alkylation and precludes the exact assignment of a cross-link site.

One possible explanation for the pattern obtained for non-terminal cross-links induced in M1 DNA by reduced DZQ (band 3, figure 3.6B) is that a fraction of the DNA duplexes are cross-linked at the 5'-GC sequence while the remaining fraction are cross-linked at the 5'-GNNC sequence. An equally feasible possibility is that this pattern arises from a population of DNAs which are all cross-linked at the 5'-GC sequence but in which a fraction of these cross-linked molecules contain a monoalkylation at G8'. If the former explanation is also true for band 2 DNA (i.e. 5'-GC + 5'-GNNC cross-links), then it is not immediately clear why the cross-linked DNA in band 2 migrates with a different electrophoretic mobility from the cross-linked DNA in band 3, since DNA cross-linked at the same sequence would presumably have the same electrophoretic mobility. It is not unreasonable to assume that some M1 DNA molecules cross-linked by reduced DZQ (in band 3) could contain accompanying monoalkylations given that G8' and in particular G10' are preferred sites for monoalkylation. In addition, alkylations at G8' and G10' are present in all DNAs cross-linked by reduced DZQ (figure 3.6 B). Although the site of non-terminal cross-links induced

in M1 DNA by reduced DZQ could not be determined with any degree of certainty, it was clear that either a 5'-GC or a 5'-GNNC cross-link was involved.

In contrast to reduced DZQ, reduction of MeDZQ to the hydroquinone form produced two electrophoretically distinct cross-linked products in M1 DNA (figure 3.4). Fragmentation of band 2 DNA resulted in almost exclusive alkylation of G10 and G14' indicating a cross-link in a 5'-GNC sequence. Thus it became clear that the two structurally similar compounds, DZQ and MeDZQ, preferentially cross-linked duplex M1 at different sites within the same DNA sequence.

In order to define more precisely the nature of the interstrand cross-link formed by reduced DZQ and MeDZQ in DNA, a second duplex oligonucleotide (M2) was designed so that the 5'-GNC, 5'-GC and 5'-GNNC sites were separated within the duplex portion of the molecule. Reaction of unreduced DZQ with M2 produced a number of electrophoretically distinct cross-linked products (figure 3.8). The sequence preference for the formation of non-terminally cross-linked DNAs induced by unreduced DZQ was 5'-GNC > 5'-GC > 5'-GNNC as shown in table 3. However, upon reduction of DZQ to the hydroquinone by DT-diaphorase, a decrease in the electrophoretic mobility of the major cross-linked products (bands 3 and 4, figure 3.8) was observed. Although a similar level of cross-linked product was detected in band 3 and band 4 (table 2), extended electrophoretic separation of the cross-linked products revealed band 3 to consist of a number of closely migrating bands while band 4 remained intact and constituted the major product. Piperidine cleavage of the cross-linked DNA in band 4 clearly indicated a cross-link at the 5'-GC sequence. As with M1, reduction of DZQ to the hydroquinone significantly enhanced total alkylation of guanines at 5'-TGC sequences

and has been observed previously (Hartley et. al., 1991). It is interesting to note that G10 and G13', both at 5'-TGC sites, are alkylated in all M2 DNAs cross-linked by reduced DZQ (figure 3.10, bands 1-4). In contrast to reduced DZQ, reduced MeDZQ preferentially cross-linked DNA at the 5'-GNC sequence.

Although the nucleotide sequence preferences for the formation of cross-links induced in M2 generally support the findings in M1, there were some important differences. For example, the two major products formed upon reaction of unreduced DZQ with M2 (bands 2 and 3) showed cross-linking at the 5'-GNC sequence. In contrast, no 5'-GNC cross-links were detected in M1 even though M1 contains two 5'-GNC sequences. The reason for this apparent disparity is not clear. It is possible that since duplex M1 is not blunt ended, the 5' overhanging end may serve as a particularly accessible site for alkylation and cross-linking. This is especially evident for G2' in band 2 of M1 (figure 3.6A). Another contributing factor to the lack of 5'-GNC cross-links in M1 may be due to the fact that the reaction with unreduced DZQ was carried out at neutral pH while the reaction with M2 was performed at pH 5.8.

In order to confirm the nucleotide sequence specificity for cross-linking, DZQ and MeDZQ were reacted with duplex oligonucleotides containing a single potential cross-linking site. Results showed that unreduced DZQ preferentially cross-linked the 5'GNC containing oligomer while reduced DZQ preferentially cross-linked across the 5'-GC sequence with some cross-linking detected in the 5'-GNC sequence, although to a lesser extent. In addition, cross-linking was only observed for MeDZQ+ in the duplex containing the 5'-GNC site (figure 3.12).

A limiting factor involved in using piperidine fragmentation of cross-linked DNA to determine cross-linking specificity is that only compounds reacting at the N7-position of guanine can be evaluated. In addition, the dose of cross-linking agent must be carefully selected so that so called "single hit" kinetics are achieved in which a majority of labeled DNA strands are not alkylated. Another weakness of piperidine cleavage of cross-linked DNA is that the presence of more than one fragment produced from a cross-linked DNA molecule complicates the interpretation of the site of cross-linking. The strengths of the method include the ability to evaluate even minor products of a cross-linking reaction and to evaluate the pattern of guanine-N7 alkylation of cross-linked DNA in detail. For example, analysis of the minor cross-linked products using non-specific hydroxyl radical chemical cleavage (Fe/EDTA) is difficult due to the extreme inefficiency of the cleavage reaction. In contrast, piperidine cleavage is quantitative for DNA cross-linked through guanine-N7 positions. In addition, the presence of monoadducts on cross-linked DNA molecules may be overlooked using Fe/EDTA chemical cleavage since fragments produced are diagnostic only for the cross-link. The use of oligonucleotides containing a number of cross-linking sites has the advantage over single site oligomers in that the cross-linking preferences of a single drug can be compared within the same DNA sequence. Nevertheless, single site oligonucleotides together with oligomers containing a number of potential cross-linking sites can be used to effectively elucidate the nucleotide preference for the formation of DNA cross-links.

The DNA interstrand cross-linking sequence specificity of a number of bifunctional alkylating agents has been investigated in other laboratories. It has been shown that reductively activated mitomycin C cross-links were

formed predominantly at 5'-CG sites, while cisplatin was found to preferentially cross-link guanines at 5'-GC sequences using an Fe(II)/EDTA/hydrogen peroxide/ascorbic acid analysis (Hopkins et al., 1991). In the present study, the unreduced form of DZQ and the reduced form of MeDZQ were found to preferentially cross-link at the 5'-GNC site of a duplex oligonucleotide in a manner similar to that of mechlorethamine. However, the cross-link distance in DZQ and MeDZQ is much greater (10 atoms) than in mechlorethamine which allows the formation of cross-links spanning four base pairs in a 5'-GNNC sequence which is not observed with cisplatin or mitomycin C. A low level of crosslinking at a 5'-GNNC sequence has been suggested for mechlorethamine (Hopkins et. al., 1991)

Clearly, reduction of DZQ to the hydroquinone by DT-Diaphorase results not only in an altered sequence preference for guanine-N7 monoalkylations but also influences the ultimate formation of DNA interstrand cross-links between guanine-N7 sites. This is not the case with other 2,5-diaziridinyl-1,4-benzoquinones which contain more bulky side groups in the 3 and 6 positions. It is not clear whether the properties of DZQ would be maintained if one side group remains as hydrogen while the other is modified. Compounds of this type would have the advantage that, depending on the structure of the side groups, the redox capability of the quinone, and hence the ease of formation of the hydroquinone, could be modified. Alternatively the DZQ molecule could be attached to sequence specific ligands to produce alkylating agents with enhanced or altered sequence selectivities. Nonsymmetrical compounds of both types are currently being investigated.

CHAPTER 4. GENERAL DISCUSSION

Bifunctional alkylating agents play a critical role in the effective treatment of human cancer and efforts continue to be made toward understanding their mechanism of action at the molecular level. It has become increasingly clear in recent years that DNA is the relevant cellular target for these agents and that cytotoxicity has often been correlated with the formation of DNA interstrand cross-links. A basic understanding of the biochemical nature of drug-DNA interactions has generated considerable interest in the development of new cross-linking compounds together with agents capable of reacting with DNA in a sequence specific manner.

The studies outlined in the first part of this thesis describe a simple, sensitive and rapid agarose gel based assay for measuring the formation of DNA interstrand cross-links. In addition to measuring the extent and rate of cross-link formation, the technique can be extended to measure the rate at which DNA monoadducts are converted to interstrand cross-links. The assay is particularly well suited for evaluating the effect of structure on the ability of a given agent to cross-link DNA. In these studies a number of structurally diverse bifunctional agents have been evaluated for their ability to produce cross-links in isolated DNA.

For the nitrogen mustard compounds, both the extent and rate of DNA cross-linking was found to be influenced to a large extent by the nature of the group attached to the reactive portion of the molecule. The rate of DNA cross-link formation at equimolar concentration was mechlorethamine > uracil mustard > melphalan > phosphoramide mustard > isophosphoramide mustard. Previous studies have examined the kinetics of DNA interstrand

cross-link formation induced in L1210 cells by several nitrogen mustard compounds using the technique of alkaline elution. It was recently shown that nitrogen mustard produced the highest initial level of DNA interstrand cross-links immediately after a 30 minute incubation followed by uracil mustard and melphalan (O'Connor et. al., 1990). In addition, the production of DNA interstrand cross-links, expressed either as the kinetics of formation or area under the curve, could be correlated with loss of colony forming ability while DNA protein cross-link formation and strand break production could not. In a separate study, a comparison between DNA cross-linking ability and cytotoxicity induced in LS174T human colonic adenocarcinoma and leukaemic K562 cells by melphalan, chlorambucil, and 4[bis(2-chloroethyl) amino]benzoic acid was carried out (Sunters et al., 1992). The results showed that the ranking order of cytotoxicities could be correlated with the ability of each compound to form cross-links in isolated DNA using the agarose gel based technique. This was different than that predicted by the rate of hydrolysis of the compounds, a factor which was previously used as a crude predictor of biological activity for aromatic nitrogen mustards (Bardos et al., 1965).

With the agarose gel based assay, it was possible to measure an increase in DNA cross-links induced by uracil mustard and melphalan after removal of unbound drug while no such increase was observed with nitrogen mustard. In alkaline elution studies, very little additional increase in cross-linking could be detected in the DNA of L1210 cells treated with nitrogen mustard after removal of the drug from the culture media (Ross et al., 1978). In contrast, a significant increase in melphalan induced DNA cross-links was observed up to 6 hours after drug removal. Although differential kinetics of cross-link formation might be attributed to differences in the rate of repair of nitrogen mustard and melphalan cross-links, results presented in

this thesis indicate that an increase in cross-linking over time in the absence of unbound drug is due to the rate of second arm cross-link formation which is also likely to occur in the DNA of intact cells. In addition, delayed cross-linking has also been observed in the DNA of L1210 cells treated with CCNU and BCNU (Erickson et al., 1980b; Ewig & Kohn, 1978).

The clinically useful platinum coordination compounds cisplatin and carboplatin also displayed significant differences in the extent and rate of DNA cross-link formation. In addition to DNA interstrand cross-links, cisplatin and carboplatin are capable of forming DNA intrastrand cross-links. The increased mobility of interstrand cross-linked DNAs at increasing doses of drug (see figure 2.9) may be associated with the formation of intrastrand cross-links in these fragments. Cisplatin at 10 μM was found to cross-link 100% of DNA after 2 hr while 1000 μM carboplatin was required to produce an equivalent level of cross-linking. These results are supported by the study of Knox et al, (1986) in which it was shown that approximately 100 fold larger doses of carboplatin than cisplatin was required to produce a similar level of binding to plasmid DNA and that this difference could be attributed to the much faster rate of aquation of cisplatin. However, once cisplatin and carboplatin were bound to DNA in equivalent amounts, a similar level of interstrand cross-links and cytotoxicity was observed (Knox et al., 1986). In animal tumour models, 3- to 4-fold more carboplatin than cisplatin was required to obtain an equivalent frequency of DNA interstrand cross-links as measured by alkaline elution. Interestingly, a 13 fold higher dose of carboplatin was required to achieve equitoxicity (DeNeve et al., 1990).

Results presented in this thesis have shown that the enzyme DT-diaphorase was capable of reducing a number of structurally related analogues of AZQ to a species capable of alkylating and cross-linking DNA. At equimolar concentrations, only the DZQ and BZQ analogues were capable of producing DNA interstrand cross-links under nonreducing conditions. All other quinone analogues required the presence of DT-Diaphorase before DNA cross-linking could be detected. In general, the level of DNA cross-linking observed with these agents in their reduced form decreased with an increase in the size of the alkyl substituents at the 3 and 6 position of the quinone ring. In addition, following reduction with DT-diaphorase, the level of DNA interstrand cross-links induced by DZQ and MeDZQ was increased to a much greater degree than that of any other quinone including AZQ. In contrast, cross-linking was not increased with BZQ in the presence of DT-diaphorase. Spectrophotometric analysis showed that the absorption spectrum of DZQ and NADH in the region between 300-400 nm remained unchanged after a fifteen minute incubation. In contrast, a dramatic decrease in the absorption maximum of DZQ and NADH was observed with time upon the addition of DT-diaphorase which was taken as a measure of the initial rate of DZQ reduction. DZQ was the most readily reduced quinone in the presence of DT-diaphorase followed by MeDZQ, AZQ and BZQ. This rate of reduction correlated well with the increase in the level of DNA cross-linking observed upon reduction by DT-diaphorase. The finding that the level of BZQ cross-links induced in DNA was similar under reducing and non-reducing conditions suggests that this compound is a poor substrate for DT-diaphorase .

In addition to enzymatic reduction, aziridinylbenzoquinones have also exhibited the potential to undergo chemical reduction to a species capable of alkylating and cross-linking DNA (Hartley et al., 1991a). Quinone

induced cross-linking in isolated DNA for a number of AZQ analogues at equimolar concentration was shown to increase upon reduction with ascorbic acid and was pH dependent. The effect was particularly pronounced for DZQ in which cross-linking was increased from 8% at neutral pH to 100% at neutral pH in the presence of 2 mM ascorbic acid. In contrast, cross-linking could only be detected for AZQ and its analogues at low pH in the presence of ascorbic acid. As with enzymatic reduction, a general decrease in the level of cross-linking was observed as the size of the substituent at the 3 and 6 position of the quinone ring increased. Related studies have demonstrated that DZQ, upon reduction by glutathione, was able to inhibit the action of bacteriophage M13-DNA which was shown to be due to reductive alkylation (Lusthof et al., 1990; Lusthof et al., 1988) Interestingly, DZQ was shown to form a conjugate with glutathione which was not associated with the aziridine ring but through a substitution reaction at the 3 or 6 position of the quinone ring. In contrast, AZQ did not form a conjugate with glutathione.

Enzymatic or chemical reduction of anitumour quinones to more reactive metabolites is thought to be the basis for the cytotoxic effects observed with these compounds (Butler & Hoey, 1987; Butler et al., 1987; Dzielendziak et al., 1990; Powis, 1989). Antitumour aziridinylbenzoquinones such as AZQ and BZQ have been used clinically despite the fact that the mechanism of action of this class of compounds is not fully understood. Quinone containing aziridine compounds such as AZQ have the potential to undergo both one and two electron reduction to form more reactive metabolites. One electron reduction of the quinone can lead to the formation of semiquinone radicals. Interaction of the semiquinone with oxygen can lead to the production of superoxide and other species capable of damaging DNA (Gutierrez, 1989). A detailed study of the relationship between the

reduction of AZQ analogues, DNA damage and toxicity recently demonstrated that DZQ was most easily reduced by DT-diaphorase followed by MeDZQ, D5, D7, D3, D1, AZQ, D6 and D4 (figure 2.11) while BZQ was not a substrate (Gibson et al., 1992). Interestingly, the order of toxicity to the human HT-29 cell line, which is rich in DT-diaphorase, was reported to be MeDZQ followed by DZQ, BZQ, D1, D5, AZQ, D7, D3, D6, and D4. In addition, the cytotoxicity induced by these compounds could be inhibited by dicoumarol, a known inhibitor of DT-diaphorase. DZQ and MeDZQ were also found to be 5-6 fold less toxic to a cell line deficient in DT-diaphorase. In the same study, analysis of DNA damage in HT-29 cells by alkaline elution showed that DZQ induced primarily DNA strand breaks while MeDZQ produced DNA interstrand cross-links. The formation of both types of lesions could be inhibited by dicoumarol. Dzielendziak and co-workers have also observed a correlation between DNA cross-linking, ease of quinone reduction and cytotoxicity in two human cell lines (Dzielendziak et al., 1990).

Although a single mechanism cannot account for all the observed cytotoxic effects of these compounds, it is clear that DNA interstrand cross-linking is important in this respect and that the DT-diaphorase mediated two electron reduction of the quinone group plays an important role in the activation of a number of aziridinylbenzoquinones (Gibson et al., 1992; Pacheco et al., 1991; Ross et al., 1990; Siegel et al., 1990a). Based upon these findings, MeDZQ would be a model compound to be tested in tumours containing high levels of DT-diaphorase relative to surrounding tissues, while BZQ would be predicted to be a good candidate for tumours lacking any detectable level of DT-diaphorase activity.

The pyrrolo(1,4) benzodiazepines (PBD s) are a group of antitumour antibiotics produced by various actinomycetes and include such compounds

as anthramycin and tomaymycin. The biological potency of these compounds is generally believed to be associated with their ability to covalently bind to DNA (Hurley et al., 1988). The reactive species has been proposed to be the N10-C11 imine form of the drug which undergoes nucleophilic attack from the N2 position of guanine to form the minor groove adduct covering 2 to 3 base pairs (Hertzberg et al., 1986). A study designed to examine various PBD monofunctional analogues for their ability to react with DNA in a sequence specific manner has revealed preferential binding at PuGPu sites (Hurley et al., 1988).

The C8 linked PBD compounds are a novel class of agent based on the naturally occurring monofunctional antitumour antibiotic DC-81. The bifunctional alkylating agent DSB-120, containing two DC-81 units linked via a 1,3-propanediyldioxy bridge, was recently synthesised in an effort to extend the number of base pairs recognised and thereby enhance the sequence preference of DC-81. Molecular modelling and NMR studies have shown that DSB-120 spans six base pairs and recognises a central 5'-GATC sequence. Cross-linking observed with DSB-120 was remarkably efficient with 100% DNA cross-linked at doses as low as 500 nM. These results demonstrate that DSB-120 is 50 fold more efficient than the major groove cross-linking agent nitrogen mustard and approximately 300 fold more effective than melphalan using the same assay (Hartley et al., 1991b). In vitro cytotoxicity data have shown that DSB-120 was up to 660 fold more cytotoxic to the ADJ/PC6 cell strain than the parent monofunctional agent DC-81. DSB-120 was also between 30 and 40 fold more cytotoxic to L1210 and K562 cells than DC-81(Bose et al., 1992a).

In light of the cytotoxicity of DSB-120 together with its high DNA cross-linking efficiency, three analogues, which varied with respect to the

length of the methylene chain connecting the two reactive portions of the molecule, were synthesised and evaluated for their ability to cross-link DNA (Bose et. al., 1992b). The n=3 and n=5 compounds showed a similar level of cross-linking and were more efficient cross-linkers than the n=4 and n=6 analogues. The striking cross-linking efficiency of these agents is illustrated by the fact that the least efficient of the C8-linked analogues, the n=4 compound, was still 20 fold more effective at cross-linking DNA than melphalan while the n=3 compound was 300 fold more effective than melphalan in this respect.

Interestingly, a correlation was observed between the efficiency of DNA cross-linking induced by the bifunctional PBD compounds and cytotoxicity in a number of cell strains including the ADJ/PC6 and L1210 lines. For example, the n=3 analogue was 700 fold more cytotoxic than the n=4 compound and the n=5 analogue was 5 fold more cytotoxic than the n=6 compound to the ADJ/PC6 cell line (Bose et al., 1992b). Similarly, the n=3 and the n=5 compounds were 120 and 75 fold more cytotoxic to the L1210 cell line than the n=4 and n=6 analogues respectively. IC50 values in both CH1 and K562 cell lines showed similar patterns of cytotoxicity. Although the n=3 and n=5 analogues were similar with respect to cross-linking efficiency, the increase in the helix melting temperature for the n=3 compound was reported to be nearly twice that of the n=5 analogue (Bose et al., 1992b). This may be due to differences in their non-covalent binding properties or the possibility that monoadducts or intrastrand cross-links influence thermal denaturation but not the efficiency of covalent cross-linking.

Moreover, DNA cross-linking could be evaluated for a wide range of bifunctional alkylating agents using an agarose gel based technique. The

assay provides a rapid and sensitive tool for the measurement of cross-links produced in isolated DNA and can serve as a basis for the comparison of drug-DNA cross-linking reactions in cells without the additional factors of drug uptake, intracellular metabolism, or DNA repair.

The second aspect of cross-linking described in this thesis was the nucleotide sequence preferences for the formation of DNA interstrand cross-links in synthetic oligonucleotides. Previous studies have utilised base modified synthetic oligonucleotides to examine both the carcinogenic and mutagenic effects of many DNA interacting drugs (Basu & Essigmann, 1988; Ferguson et al., 1989). In addition, a number of chemotherapeutic alkylating agents have exhibited varying degrees of DNA sequence specificity in relatively short fragments of DNA using variations in DNA sequencing methodology (Hartley et al., 1986; Hartley et al., 1988a; Hartley et al., 1988b). However, a great majority of these studies have focused primarily on the specificity of DNA monoalkylation while the sequence specificity of DNA interstrand cross-linking has been largely unexplored.

The finding that nitrogen mustard preferentially cross-links synthetic duplex oligonucleotides in 5'-GNC sequences is consistent with the findings of Millard et al., (1990), and Ojwang et al.,(1989) who have also reported this preferential cross-linking in synthetic oligonucleotides. These results are not consistent with previous molecular modelling and DNA hydrolysate studies which have suggested preferential cross-linking across 5'-CG or 5'-GC sequences (Brooks & Lawley, 1961; Hausheer et al., 1989).

The sequence preference for DNA cross-linking induced by the bioreductive agents DZQ and MeDZQ were prompted by earlier studies which demonstrated two important findings. The first was that the level of cross-

links induced in isolated DNA by DZQ and MeDZQ was increased to a much greater extent in the presence of DT-Diaphorase compared to other quinone compounds including AZQ. In addition, while the quinone and hydroquinone forms of several structurally similar aziridines exhibited relatively non-specific alkylation of guanine-N7 positions, the reduced form of DZQ reacted preferentially at guanines in 5'-GC sequences and more specifically at 5'TGC sequences (Hartley et al., 1991a; Lee et al., 1992). It was subsequently found that DZQ and MeDZQ preferentially cross-link at 5'-GC and 5'-GNC sequences respectively.

Using this methodology it is possible to evaluate how subtle changes in the structure of a given bifunctional agent affect the sequence preferences for DNA cross-linking. In addition, automated synthetic nucleotide chemistry makes it possible to study the influence of any DNA sequence, including the effect of flanking base sequences, on drug-DNA cross-linking reactions. Separation of the cross-linked product by denaturing PAGE can afford highly purified cross-linked products which can subsequently be isolated and characterised by various chemical cleavage reactions. The DNA sequence cross-linked by several bifunctional agents has now been reported using synthetic DNA oligonucleotides and polyacrylamide gel electrophoresis (Hopkins et al., 1991; Weidner et al., 1990).

Although it is not clear whether the sequence selective cross-linking observed in synthetic duplex oligonucleotides is maintained within the cell, a recent study has demonstrated that the pattern of alkylation induced in isolated DNA by a number of nitrogen mustard compounds was preserved in intact cells (Hartley et al., 1991c). However, a number of factors is likely to influence the formation, location and repair of cross-links induced in

cellular DNA. In addition to the tertiary arrangement of DNA organised into tightly packed chromatin structures, there is evidence to suggest that DNA sequence influences both the conformation of the helix and biological function (Fujimoto & Schurr, 1990; Nielsen, 1991).

It is now becoming increasingly clear that repair of DNA damage (including interstrand cross-links) induced in cellular DNA by chemically reactive compounds differs in transcriptionally active and inactive regions of the genome. For example, (Madhani et al., 1986) has shown that thymidine dimers induced in cellular DNA by UV light were preferentially repaired in the transcriptionally active c-abl gene compared to the inactive c-mos gene in 3T3 fibroblasts. Vos and Hanawalt (1987) have demonstrated that 80% of cross-links formed in the active dihidrofolate reductase gene by psoralen were repaired after 24 hours while only 45% of monoadducts were removed (Vos & Hanawalt, 1987). Nitrogen mustard cross-links induced in cellular DNA also appear to be formed and repaired in a heterogeneous manner in the genome. These and other studies appear to suggest that differential repair occurs not only in different regions of the genome but in many instances is compound specific (Futscher et al., 1992; Hartley et al., 1993).

Although non random DNA repair at the gene level is becoming better understood, much less is known about how DNA interstrand cross-links induced at specific sequences are processed and repaired by cellular enzymes. In a recent study by Van Houten (1986), a synthetic duplex DNA molecule containing a psoralen cross-link at a precisely defined sequence was purified and characterised. The cross-linked duplex was inserted into a long DNA fragment and subsequently subjected to the action of ABC excinuclease to unambiguously examine mechanism of enzymatic repair.

In addition, the construction of a psoralen cross-linked DNA allowed the determination of the contact sites between the repair enzyme and the DNA fragment (Van et al., 1986a; Van et al., 1986b). In a similar study, a synthetic duplex oligonucleotide containing a single nitrogen mustard cross-link was purified and ligated into a human expression vector (Ojwang et al., 1989). This method has the advantage of being generally applicable to duplex oligonucleotides cross-linked with other bifunctional agents (Grueneberg et al., 1991). These techniques may be of particular interest in elucidating the repair of structurally distinct DNA cross-links as DNA repair has been implicated as an important mechanism in drug resistance (Hartley et al., 1993).

A basic understanding of the mechanism by which bifunctional agents recognise and cross-link DNA may also have important implications for the rational design and development of antitumour agents which recognise specific DNA sequences. It has been suggested that certain tumours which are extremely sensitive to cytotoxic drug action may contain essential genes which are unusually susceptible to damage by DNA damaging agents (Hartley et al., 1993). For example, recent studies have suggested that GC rich regions in genes could be preferred sites for damage by such agents as the nitrogen mustards and the chloroethylnitrosoureas. The human genome contains regions of unexpectedly high GC content (>80%) including some oncogene sequences (Zerial et al., 1986). Both the nitrogen mustards and the chloroethylnitrosoureas have been shown to preferentially alkylate the 5' flanking region of the high GC containing sequences of the c-Ha-ras oncogene in-vitro (Mattes et al., 1988). Sequences of DNA rich in GC sequences are often associated with the control of gene expression such as the SPI transcription factor binding sites of the c-Ha-ras gene. In addition to the number of oncogenes rich in

GC sequences, a number of viral sequences, in particular the Epstein Barr virus (EBV), have been identified as being unusually high in GC content. It has been suggested that the GC rich regions within the EBV genome may play an important role in control function (Karlin, 1986).

The studies outlined above have shown that even small changes in the structure of a bifunctional agent can significantly alter both the extent of DNA interstrand cross-link formation and the sequence at which the cross-linking reaction takes place. In a sense, it is remarkable that small molecules such as the nitrogen mustards and aziridinylbenzoquinones can distinguish between different sequences within DNA. On the other hand, the length of the DNA sequence cross-linked by these agents is disappointingly short, being 2 to 3 base pairs. It has been estimated that a unique sequence within the genome of a human cell consists of between 15 and 19 base pairs (Dervan, 1986; Nielsen, 1991). However, it may not be necessary to achieve such a level of specificity and even a modest increase in the length of the recognition site could lead to substantial increase in biological effect (Hartley & Souhami, 1993). In addition, as the structure of the binding ligand becomes more complex, pharmacological problems may arise involving distribution and metabolism of the drug within the cell..

An understanding of the mechanism by which small molecules interact with and cross-link DNA can serve as a basis for the design and development of novel compounds which are capable of recognising an increased number of base pairs. Indeed, novel bifunctional compounds capable of recognising and cross-linking across 4 to 6 base pairs at specific DNA sequences have already been reported (Bose et al., 1992a; Bose et al., 1992b; Ding & Hurley, 1991). As the knowledge of drug-DNA interstrand cross-linking reactions continues to expand, the prospect increases for a

new generation of compounds which are more effective in the treatment of neoplastic disease .

REFERENCES

Ahmed, M. and Hadi, S. M. Sequence preference in alkylation by N-methyl-N-nitrosourea and N-ethyl-N-nitrosourea. Med. Sci. Res. 18 (1988): 1173-1174.

Arnott, S., Campbell-Smith, P. J., and Chandrasekaran, R. "Atomic coordinates and molecular conformations for DNA-DNA, RNA-RNA and DNA-RNA helices." In Handbook of Biochemistry and Molecular Biology, ed. Sober. 411-422. Cleveland: Chemical Rubber Co. Press, 1974.

Bachur, N. R., Gordon, S. L., and Gee, M. V. A general mechanism for microsomal activation of quinone anticancer agents to free radicals. Cancer Res. 38 (1978): 1745-1750.

Bachur, N. R., Gordon, S. L., and Gee, M. V. NADPH cytochrome P-450 reductase activation of quinone anticancer agents to free radicals. Proc. Natl. Acad. Sci. USA 76 (1979): 954-957.

Ball, C. R. and Roberts, J. J. Estimation of interstrand DNA cross-linking resulting from mustard gas alkylation of HeLa cells. Chem. Biol. Interactions 4 (1971/72): 297-303.

Bannon, P. and Verly, W. Alkylation of phosphates and stability of phosphate triesters in DNA. Eur. J. Biochem. 31 (1972): 103.

Barcelo, F. and Miro, A. Equilibrium binding of four anthracyclines to nucleic acids: thermodynamic properties and sequence selectivity. Int. J. Biol. Macromol. 13 (1991): 235-240.

Bardos, T. J., Datta-Gupta, N., Hebborn, P., and Triggle, D. J. A study of comparative chemical and biological activities of alkylating agents. J. Med. Chem. 8 (1965): 167-174.

Basu, A. K. and Essigmann, J. M. Site specifically modified oligodeoxynucleotides as probes for the structural and biological effects of DNA-damaging agents. Chem. Res. Toxicol. 1 (1988): 1-18.

Baterman, J. C. Chemotherapy of solid tumors with triethylene thiophosphoramide. N. Engl. J. Med. 252 (1955): 879.

Bedford, P. and Fox, B.W. DNA-DNA interstrand crosslinking by dimethanesulphonic acid esters. Biochem. Pharmacol. 32 (1983): 2297-2301.

Begleiter, A. and Leith, M. K. Activity of quinone alkylating agents in quinone-resistant cells. Cancer Res 50 (10 1990): 2872-6.

Berardini, M. D., Souhami, R. L., Lee, C-S, Gibson, N. W., Butler, J., and Hartley, J. A. Two structurally related diaziridinylbenzoquinones preferentially cross-link DNA at different sites upon reduction with DT-diaphorase. Biochemistry In Press (1993):

Bernges, F. and Holler, E. The reaction of platinum (II) complexes with DNA. Kinetics of intrastrand cross-link formation in-vitro. Nucleic Acids Research 19 (7 1991): 1483-1489.

Booth, B. A., Creasey, W. A., and Sartorelli, A. C. Alterations in cellular metabolism associated with cell death induced by uracil mustard and 6-thiguanine. Proc. Natl. Acad. Sci. USA 52 (1964): 1396.

Borowy-Borowski, H., Lipman, R., Chowdary, D., and Tomasz, M. Duplex oligodeoxyribonucleotides cross-linked by mitomycin C at a single site: synthesis, properties, and cross-link reversibility. Biochemistry 29 (12 1990a): 2993-2999.

Borowy-Borowski, H., Lipman, R., and Tomasz, M. Recognition between Mitomycin C and Specific DNA Sequences for Cross-Link Formation. Biochemistry 29 (1990b): 2999-3006.

Bose, D. S., Thompson, A. S., Ching, J., Hartley, J. A., Berardini, M. D., Jenkins, T. C., Neidle, S., Hurley, L. H., and Thurston, D. E. Rational design of a highly efficient irreversible DNA interstrand cross-Linking agent based on the pyrrolobenzodiazepine ring system. J. Am. Chem. Soc. 114 (12 1992a): 4939-4941.

Bose, S. D., Thompson, A. S., Smellie, M., Berardini, M. B., Hartley, J. A., Jenkins, T. C., Neidle, S., and Thurston, D. E. Effect of linker length on DNA

binding affinity, cross-linking efficiency and cytotoxicity of C8-linked pyrrolobenzodiazepine dimers. J. Chem. Soc., Chem. Commun. (1992b): 1518-1520.

Boyer, V., Moustacchi, E., and Sage, E. Sequence specificity of various psoralen derivatives with DNA: role in biological activity. Biochemistry 27 (1988): 3011-3018.

Brewer, H. B., Comstock, J. P., and Aronow, L. Effects of nitrogen mustard on protein and nucleic acid synthesis in mouse fibroblasts growing in vitro. Biochem. Pharmacol. 8 (1961): 281.

Briscoe, W. T. and Cotter, L. The effects of neighboring bases on N-methyl-N-nitrosourea alkylation of DNA. Chem.-Biol. Interact. 52 (1984): 103-110.

Briscoe, W. T. and Cotter, L. DNA sequence has an effect on the extent and kinds of alkylation of DNA by a potent carcinogen. Chem.-Biol. Interact. 56 (1985): 321-331.

Brooks, P. and Lawley, P. D. The reaction of mustard gas with nucleic acids in-vitro and in-vivo. Biochem. J. 77 (1960): 478-484.

Brooks, P. and Lawley, P. D. The reaction of mono- and di-functional alkylating agents with nucleic acids. Bichem. J. 80 (1961): 496-503.

Brooks, P. and Lawley, P. D. The action of alkylating agents on DNA in relation to biological effects of the alkylating agents. Exptl. Cell Res. 9 (supp) (1963): 512-520.

Burchenal, J. H., Crossley, M. L., and Stock, C. C. The action of certain ethylenimine (aziridine) derivatives on mouse leukemia. Arch. Biochem. 26 (1950): 321-323.

Butler, J. and Hoey, B. M. Are reduced quinones necessarily involved in the antitumour activity of quinone drugs? Br J Cancer Suppl 8 (53 1987): 53-9.

Butler, J., Hoey, B. M., and Lea, J. S. The reduction of anti-tumour diaziridinyl benzoquinones. Biochim Biophys Acta 925 (2 1987): 144-9.

Cera, C., Egbertson, M., Teng, S. P., Crothers, D. M., and Danishefsky, S. J. DNA cross-linking by intermediates in the mitomycin activation cascade. Biochemistry 28 (13 1989): 5665-9.

Cole, R. S. Repair of DNA containing interstrand cross-links in escherichia coli: sequential excision and recombination. Proc. Nat. Acad. Sci. U.S.A. 70 (4 1973): 1064-1068.

Cole, R. S., Levitan, D., and Sinden, R. R. Removal of psoralen interstrand cross-links from DNA of escherichia coli: mechanism and genetic control. J. Mol. Biol. 103 (1976): 39-59.

Connors, T. A. "Mechanism of action of 2-chloroethylamine derivatives, sulfur mustards, epoxides and aziridines." In Antineoplastic and Immunosuppressive Agents, Part II, ed. A. C. Sartorelli and D. G. Johns. 18-34. Berlin: Springer-Verlag, 1975.

Connors, T. A., Cox, P. J., Farmer, P. B., Foster, A. B., and Jarman, M. Some studies of the active intermediates formed in the microsomal metabolism of cyclophosphamide and isophosphamide. Biochemical Pharmacology 23 (1974): 115.

D'Andrea, A. D. and Hazeltine, W. A. Modification of DNA by aflatoxin B1 creates alkali labile lesions in DNA at positions of guanine and adenine. Proc. Natl. Acad. Sci. USA 75 (1978): 4120-4124.

Day, R. S., Ziolkowski, C. H. J., Scudiero, D. A., Meyer, S. T., and Mattern, M. Human tumour cell strains defective in repair of alkylation damage. Carcinogenesis 1 (1980): 21-32.

DeNeve, W., Valeriote, F., Tapazoglou, E., Everett, C., Khatana, A., and Corbett, T. Discrepancy between cytotoxicity and DNA interstrand crosslinking of carboplatin and cisplatin in vivo. Invest New Drugs 8 (1 1990): 17-24.

Dervan, PB. Design of sequence-specific DNA-binding molecules. Science 232 (1986): 464-471.

DeVita, V. T., Carbone, P. P., and Owens, A. H. Jr. Clinical trials with 1,3-bis(2-chloroethyl)-1-nitrosourea, NSC-409962. Cancer Research 25 (1965): 1876.

Ding, Z. M. and Hurley, L. H. DNA interstrand cross-linking, DNA sequence specificity, and induced conformational changes produced by a dimeric analog of (+)-CC-1065. Anticancer Drug Des 6 (5 1991): 427-52.

Drew, H. R. and Travers, A. A. DNA structural variations in the E. coli tyr promoter. Cell 37 (1984): 491-502.

Drysdale, R. B., Hopkins, A., and Thompson, R. Y. Some effects of nitrogen and sulphur mustards on the metabolism of nucleic acids in mammalian cells. Br. J. Cancer 12 (1958): 137.

Dzielendziak, A., Butler, J., Hoey, B. M., Lea, J. S., and Ward, T. H. Comparison of the structural and cytotoxic activity of novel 2,5-bis(carboethoxyamino)-3,6-diaziridinyl-1,4-benzoquinone analogues. Cancer Res 50 (7 1990): 2003-8.

Endicott, K. M. US strategy against cancer. New Scientist (1965): 856-858.

Erickson, L. C., Bradley, M. O., Ducore, J. M., Ewig, R. A. G., and Kohn, K. W. DNA cross-linking and cytotoxicity in normal transformed human cells treated with antitumour nitrosoureas. Proc. Natl. Acad. Sci. 77 (1980a): 467-471.

Erickson, L. C., Laurent, G., Sharkey, N. A., and Kohn, K. W. DNA cross-linking and monoadduct repair in nitrosourea treated human tumour cells. Nature 288 (1980b): 727-729.

Ewig, R. A. G. and Kohn, K. W. DNA damage and repair in mouse leukemia L1210 cells treated with nitrogen mustard, 1,3-bis(2-chloroethyl)-1-nitrosourea, and other nitrosoureas. Cancer Res. 37 (1977): 2114.

Ewig, R. A. G. and Kohn, K. W. DNA-protein cross-linking and DNA interstrand cross-linking by haloethylnitrosoureas in L1210 cells. Cancer Research 38 (1978): 3197-3203.

Farrell, N., Qu, Y., Feng, L., and Houten, B. V. Comparison of Chemical Reactivity, Cytotoxicity, Interstrand Cross-Linking and DNA Sequence Specificity of Bis(platinum) Complexes Containing Monodentate or Bidentate Coordination Spheres with Their Monomeric Analogues. Biochemistry 29 (1990): 9522-9531.

Ferguson, L. R., Gourdie, T. A., Valu, K. K., and Denny, W. A. Bacterial mutagenicity studies of DNA-intercalating aniline mustards: an insight into the mode of action of a novel class of anti-tumour drugs. Anticancer Drug Des 4 (3 1989): 209-19.

Filipski, J., Kohn, K. W., and Bonner, W. M. The nature of inactivating lesions produced by platinum(II) complexes in phage λ DNA. Chem. Biol. Interactions 32 (1980): 321-330.

Filipski, J., Kohn, K. W., Prather, R., and Bonner, W. M. Thiourea reverses biological activity in DNA treated with dichlorodiaminoplatinum(II). Science 204 (1979): 181-183.

Fracasso, P. M., Keyes, S. R., and Rockwell, S. Biotransformation of mitomycin C by NADPH-cytochrome P-450 reductase and DT-diaphorase in cultured cells. Proc. Am. Assoc. Cancer Res. 24 (1983): 249.

Fujimoto, B. S. and Schurr, J. M. Dependence of the torsional rigidity of DNA on base composition. Nature 344 (1990): 175-178.

Fujiwara, Y. "Interstrand Cross-Links Produced by Mitomycin C." In DNA Repair. A Laboratory Manual of Research Procedures., ed. E. C. Friedberg and P. C. Hanawalt. 143. 2. New York: Dekker, 1983.

Fujiwara, Y. and Tatsumi, M. Cross-link repair in human cells and its possible defect in Fanconi's anemia cells. J. Mol. Biol. 113 (1977): 635-649.

Futscher, B. W. and Erickson, L. C. Changes in c-myc and c-fos expression in a human tumor cell line following exposure to bifunctional alkylating agents. Cancer Res 50 (1 1990): 62-6.

Futscher, B. W., Pieper, R. O., Dalton, W. S., and Erickson, L. C. Gene-specific DNA interstrand cross-links produced by nitrogen mustard in the human tumor cell line Colo320HSR. Cell Greowth & Differentiation (1992):

Galas, D. J. and Schmitz, A. DNAase footprinting: a simple method for the detection of protein-DNA binding specificity. Nucleic Acids Research 5 (1978): 3157.

Geiduschek, E. P. Reversible DNA. Proc. Natl. Acad. Sci. USA 47 (1961): 950-955.

Gibson, N. W., Hartley, J. A., Butler, J., Siegal, D., and Ross, D. Relationship between DT-Diaphorase-mediated metabolism of a series of aziridinylbenzoquinones and DNA damage and cytotoxicity. Molecular Pharmacology 42 (1992): 531-536.

Gilman, A. and Phillips, F. S. The biological actions and therapeutic applications of B-chloroethylamines and sulfides. Science 103 (1946): 409.

Goldacre, R. J., Loveless, A., and Ross, W. C. J. Mode of production of chromosome abnormalities by the nitrogen mustards, the possible role of cross-linking. Nature (London) 163 (1949): 667-669.

Gomori, G. Histochemical demonstration of sites of phosphoramidase activity. Proc. Soc. Exp. Biol. Med. 69 (1948): 407-409.

Green, C. L., Loechler, E. L., Fowler, K. W., and Essigman, J. M. Construction and characterization of extrachromosomal probes for mutagenesis and carcinogenesis: site specific incorporation of O6-methylguanine into viral and plasmid genomes. Proc. Natl. Acad. Sci. USA 81 (1984): 13-17.

Grueneberg, D. A., Ojwang, J. O., Benasutti, M., Hartman, S., and Loechler, E. L. Construction of a human shuttle vector containing a single nitrogen mustard interstrand, DNA-DNA cross-link at a unique plasmid location. Cancer Res 51 (9 1991): 2268-72.

Gutierrez, P. L. Mechanism(s) of bioreductive activation. The example of diaziquone (AZQ). Free Radic Biol Med 6 (4 1989): 405-45.

Hansson, J., Lewensohn, R., Ringborg, U., and Nilsson, B. Formation and removal of DNA cross-links induced by melphalan and nitrogen mustard in relation to drug-induced cytotoxicity in human melanoma cells. Cancer Res 47 (10 1987): 2631-7.

Hartley, J. A., Berardini, M. , Ponti, M., Gibson, N. W., Thompson, A. S., Thurston, D. E., Hoey, B. M., and Butler, J. DNA cross-linking and sequence selectivity of aziridinylbenzoquinones: a unique reaction at 5'-GC-3' sequences with 2,5-diaziridinyl-1,4-benzoquinone upon reduction. Biochemistry 30 (50 1991a): 11719-24.

Hartley, J.A., Berardini, M.D., and Souhami, R.L. An agarose gel method for the determination of DNA interstrand crosslinking applicable to the measurement of the rate of total and "second-arm" crosslink reactions. Anal. Biochem. 193 (1991b): 131-134.

Hartley, J. A., Bingham, J. P., and Souhami, R. L. DNA sequence selectivity of guanine-N7 alkylation by nitrogen mustards is preserved in intact cells. Nucleic Acids Research 20 (12 1991c): 3175-3178.

Hartley, J. A., Gibson, N. W., Kohn, K. W., and Mattes, W. B. DNA sequence selectivity of guanine-N7 alkylation by antitumour chloroethylating agents. Cancer Research 46 (1986): 1943-1947.

Hartley, J. A., Lown, J. W., Mattes, W. B., and Kohn, K. W. DNA sequence specificity of antitumor agents. Oncogenes as possible targets for cancer therapy. Acta Oncol 27 (5 1988a): 503-10.

Hartley, J. A., Mattes, W. B., Vaughan, K., and Gibson, N. W. DNA sequence specificity of guanine N7-alkylations for a series of structurally related triazenes. Carcinogenesis 9 (4 1988b): 669-74.

Hartley, J. A. and Souhami, R. L. "DNA sequence specificity of anticancer agents." In Cancer Chemotherapy, ed. J. A. Hickman and T. R. Tritton. 251. Oxford: Blackwell, 1993.

Hartley, J. A., Souhami, R. L., and Berardini, M. D. Electrophoretic and chromatographic separation methods used to reveal interstrand cross-linking of nucleic acids. J. Chromatog. Biomed. Appl. 9 (1993): In Press.

Haseltine, W. A., Gordon, L. K., Lindon, C. P., Grafstrom, R. H., Shaper, N. L., and Grossman, L. Cleavage of pyrimidine dimers in specific DNA sequences by a pyrimidine dimer DNA-glycosylase of M. luteus. Nature 285 (1980): 634-641.

Hausheer, F.H., Singh, U.C., Saxe, J.D. , and Colvin, O.M. Identification of local determinants of DNA interstrand crosslink formation by cyclophosphamide metabolites. Anticancer Drug Design, 4 (1989): 281-294.

Hemminki, K. and Ludlum, D. B. Covalent Modification of DNA by Antineoplastic Agents. Journal of the National Cancer Institute 73 (5 1981): 1021-1028.

Hendry, J. A., Homer, R. F., Rose, F. L., and Walpole, A. L. Cytotoxic agents: III, derivatives of ethyleneimine. Br. J. Pharmacology 6 (1951): 357-410.

Hertzberg, R. H., Hecht, S. M., Reynolds, V. L., Molineux, I. J., and Hurley, L. H. DNA sequence specificity of the pyrrolo[1,4]benzodiazepine antitumour antibiotics. Methidiumpropyl-EDTA-iron(II) footprinting analysis of DNA binding sites for anthramycin and related drugs. Biochemistry 25 (1986): 1249-1258.

Hertzberg, R. P. and Dervan, P. B. Cleavage of DNA with methidiumpropyl-EDTA-Iron (II): reaction conditions and product analysis. Biochemistry 23 (1984): 3934-3945.

Hopkins, P.B., Millard, J.T., Woo, J., and Weidner, M.F. Sequence Preferences of DNA Interstrand Cross-Linking Agents: Importance of Minimal DNA Structural Reorganization in the Cross-Linking Reactions of Mechlorethamine, Cisplatin, and Mitomycin C. Tetrahedron 47 (14/15 1991): 2475-2489.

Hurley, L. H., Reck, T., Thurston, D. E., and Langley, D.R. Pyrrolo[1,4]benzodiazepine Antitumor Antibiotics: Relationship of DNA Alkylation and Sequence Specificity to the Biological Activity of Natural and Synthetic Compounds. Chem. Res. Toxicol. 1 (5 1988): 258-268.

Hurley, L. H., Reynolds, V. L., Swenson, D. H., Petzold, G. L., and Scahill, T. A. Reaction of the antitumor antibiotic CC-1065 with DNA. Structure of an adduct with DNA sequence specificity. Science 226 (1984): 843-844.

Islas, A. L., Vos, J. M., and Hanawalt, P. C. Differential introduction and repair of psoralen photoadducts to DNA in specific human genes. Cancer Res 51 (11 1991): 2867-73.

Iyer, V. N. and Szybalski, W. Mitomycin and porfiromycin: chemical mechanism of activation and cross-linking of DNA. Science 145 (1964): 55-58.

Johnson, J. M. and Ruddon, R. W. Interaction of nitrogen mustard with polynucleotides, ribosomes, and enzymes involved in protein synthesis in a cell-free system. Mol. Pharmacol. (3 1967): 195.

Jolley, G. M. and Ormerod, M. G. An improved method for measuring cross-links in the DNA of mammalian cells: the effect of nitrogen mustard. Biochimica et Biophysica Acta 308 (1973): 242-251.

Kalyanaraman, B., Perez-Reyes, E., and Mason, R. P. Spin trapping and direct electron spin resonance investigations of the redox metabolism of quinone anticancer drugs. Biochim. Biophys. Acta. 630 (1980): 119-130.

Karlin, S. Significant potential secondary structure in the Epstein-Barr virus genome. Proceeding of the National Acadamy of Sciences USA 83 (1986): 6915-6919.

Knox, R. J., Friedlos, F., Lydall, D. A., and Roberts, J. J. Mechanism of cytotoxicity of anticancer platinum drugs: evidence that cis-diamminedichloroplatinum(II) and cis-diammine-(1,1-cyclobutanedicarboxylato)platinum(II) differ only in the kinetics of their interaction with DNA. Cancer Res 46 (1986):

Kohn, K. W. Interstrand cross-linking of DNA by 1,3-bis(2-chloroethyl)-1-nitrosourea and other 1-(2-haloethyl)-1-nitrosoureas. Cancer Res. 37 (1977): 1450-1454.

Kohn, K. W. "DNA as a target in cancer chemotherapy: measurement of macromolecular DNA damage produced in mamalian cells by anti-cancer agents and carcinogens." In Methods in cancer research, ed. V. T. Devita and H. Busch. 291. 16. New York: Academic Press, 1979.

Kohn, K. W. In Molecular actions and targets for cancer chemotherapeutic agents, ed. A. C. Sartorelli. p. 3. New York: Academic Press, 1981.

Kohn, K. W., Erickson, L. C., and Ewig, R. A. G. Fractionation of DNA from mammalian cells by alkaline elution. Biochemistry 15 (1976): 4629.

Kohn, K. W., Ewig, R. A. G., Erickson, L. C., and Zwelling, L. A. In DNA repair a laboratory manual of research procedures, ed. E. C. Friedberg and P. C. Hanawalt. p. 379. 1. New York: Marcell Decker, 1981.

Kohn, K. W., Hartley, J. A., and Mattes, W. B. Mechanisms of DNA sequence selective alkylation of guanine-N7 positions by nitrogen mustards. Nucleic Acids Research 15 (1987): 10531-10549.

Krumbhaar, E. B. and Krumbhaar, H. D. The blood and bone marrow in yellow cross gas (mustard gas) poisoning: Changes produced in the bone marrow of fatal cases. J. Med. Res. 40 (1919): 497.

Lane, M. J., Dabroviak, J. C., and Vournakis, J. N. Sequence specificity of actinomycin D and netropsin binding to pBR322 DNA analyzed by protection from DNAse I. Proc Natl. Acad. Sci. USA 80 (1983): 3260-3264.

Lawley, P. D. Reaction of N-methyl-N-nitrosourea (MNUA) with P-labelled DNA: evidence for formation of phosphotriesters. Chem. Biol. Interact. 7 (1973): 127.

Lawley, P. D., Lethbridge, J. H., Edwards, P. A., and Shooter, K. V. Inactivation of bacteriophage T7 by mono- and difunctional sulfur mustards in relation to cross-linking and depurination of bacteriophage DNA. J. Molec. Biol. 39 (1969): 181-198.

Lawley, P. D., Orr, D. J., and Jarman, M. Isolation and quantification of products from alkylation of nucleic acids: ethyl-isopropyl purines. Biochem. J. 145 (1975): 73-84.

Lee, C. S., Hartley, J. A., Berardini, M. D., Butler, J., Siegel, D., Ross, D., and Gibson, N. W. Alteration in DNA cross-linking and sequence selectivity of a series of aziridinylbenzoquinones after enzymatic reduction by DT-diaphorase. Biochemistry 31 (N11 1992): 3019-3025.

Lewis, M. R. and Crossley, M. L. Retardation of tumor growth in mice by oral administration of ethyleneimine derivatives. Arch. Biochem. 26 (1950): 319-320.

Loveless, A. Possible relevance of O6 alkylating of deoxyguanosine to the mutagenicity and carcinogenicity of nitrosamines and nitrosamides. Nature 233 (1969): 206.

Loveless, A. and Ross, W. C. J. Chromosome alteration and tumour inhibition by nitrogen mustards: the hypothesis of cross-linking alkylation. Nature 166 (1950): 1113.

Loveless, A. and Stock, J. C. (I) The nature of the inactivation of T2 phage in-vitro by certain alkylating agents. Proc. R. Soc. B150 (1959a): 423-445.

Loveless, A. and Stock, J. C. (II) Properties of coliphage T2 treated with di(2-chloroethyl)methylamine. Proc. R. Soc. B150 (1959b): 486-496.

Loveless, A. and Stock, J. C. (V) A comparison of the inactivation of phages T2, T7 and two strains of T4 by alkylating agents. Proc. R. Soc B151 (1959c): 148-155.

Lown, J. W., Begleiter, A., Johnson, D., and Morgan, A. R. Studies related to antitumor antibiotics. Part V. Reactions of Mitomycin C with DNA examined by ethidium fluorescence assay. Can. J. Biochem. 54 (1976): 110-119.

Lown, J. W. and Chen, H. H. Evidence for the generation of free hydroxyl radicals from certain quinone antibiotics upon reductive activation in solution. Can. J. Chem. 59 (1981): 390-395.

Ludlum, D.B. DNA alkylation by the haloethylnitrosoureas: nature of modifications produced and their enzymatic repair or removal. Mutation Res. 233 (1990): 117-126.

Ludlum, D. B., Kramer, B. S., and Wang, J. Reaction of 1,3-bis(2-chloroethyl)-1-nitrosourea with synthetic polynucleotides. Biochemistry 14 (1977): 5480.

Lusthof, K. J., de Mol, N. J., Janssen, L. H. M., Prins, B., Verboom, W., and Reinhoudt, D. N. Interactions between potential anti-tumour 2,5-bis(1-aziridinyl)-1,4-benzoquinone derivatives and gluathione: reductive activation, conjugation, and DNA damage. Anti-Cancer Drug Design 5 (3 1990): 283-290.

Lusthof, K. J., Groothuis-Pielage, I. L., Decuyper, J., de Mol, N. J., Janssen, L. H. M., Verboom, W., and Reinhoudt, D. N. Potential anti-tumour 2,5-bis(1-aziridinyl)-1,4-benzoquinones: relationship between cytotoxicity against DNA repair deficient E. coli, inactivation of bacteriophage M13mp19, and in-vitro and in-vivo antitumour activity. Anti-Cancer Drug Design 3 (3 1988): 147-155.

Madhani, H. D., Bohr, V. A., and Hanawalt, P. C. Differential DNA repair in transcriptionally active and inactive proto-oncogenes: c-abl and c-mos. Cell 45 (3 1986): 417-23.

Maniatis, T., Fritsch, E. F., and Sambrook, J. Molecular cloning. A laboratory manual. Cold Spring Harbor Laboratory, 1982.

Mattes, W. B. Lesion selectivity in blockage of lambda exonuclease by DNA damage. Nucleic Acids Research 18 (13 1990): 3723-3730.

Mattes, W. B., Hartley, J. A., and Kohn, K. W. DNA sequence selectivity of guanine-N7 alkylation by nitrogen mustards. Nucleic Acids Research 14 (1986a): 2971-2987.

Mattes, W.B., Hartley, J.A., and Kohn, K.W. Mechanism of DNA strand breakage by piperidine at sites of N7-alkylguanines. Biochimica et Biophysica Acta 868 (1986b): 71-76.

Mattes, W.B., Hartley, J.A., Kohn, K.W. , and Matheson, D.W. GC-rich regions in genomes as targets for DNA alkylation. Carcinogenesis, 9 (1988): 2065-2072.

Maxam, A. M. and Gilbert, W. A new method for sequencing DNA. Proc. Natl. Acad. Sci. U.S.A. 74 (1977): 560-564.

Maxam, A.M. and Gilbert, W. Sequencing End-Labeled DNA with Base-Specific Chemical Cleavages. Methods in Enzymology 65 (1980): 499-560.

Mehta, J.R., Przybylski, M. , and Ludlum, D.B. Alkylation of guanesine and deoxyguanosine by phosphoramide mustard. Cancer Res. 40 (1980): 4183-4186.

Millard, J.T., Raucher, S., and Hopkins, P.B. Mechlorethamine Cross-Links Deoxyguanosine Residues at 5'-GNC Sequences in Duplex DNA Fragments. J. Am. Chem. Soc. 112 (1990): 2459-2460.

Millard, J.T., Weidner, M.F., Kirchner, J.J., Ribeiro, S., and Hopkins, P.B. Sequence preferences of DNA interstrand crosslinking agents: quantitation of interstrand crosslink locations in DNA duplex fragments containing multiple crosslinkable sites. Nucleic Acids Research 19 (8 1991): 1885-1891.

Millard, J.T., Weidner, M.F., Raucher, S., and Hopkins, P.B. Determination of the DNA Cross-linking Sequence Specificity of Reductively Activated Mitomycin C at Single-Nucleotide Resolution: Deoxyguanosine Residues at CpG Are Cross-Linked Preferentially. J. Am. Chem. Soc. 112 (1990): 3637-3641.

Mitchell, M. A., Johnson, P. D., Williams, M. D., and Aristoff, P. A. Interstrand DNA cross-linking with dimers of the spirocyclopropyl alkylating moiety of CC-1065. J. Am. Chem. Soc. 111 (1989): 6428-6429.

Montgomery, J. A. "The development of the nitrosoureas: a study in congener synthesis." In Nitrosoureas: Current Status and New Developments, ed. A. W. Prestayko, S. T. Crooke, L. H. Baker, S. K. Carter, and P. S. Schein. 3-8. New York: Academic Press, 1981.

Moore, P. D., Bose, K. K., Rabkin, S. D., and Strauss, B. S. Sites of termination of in-vitro DNA synthesis on ultraviolet- and N-acetylaminofluorene-treated ϕX174 templates by prokaryotic and eukaryotic DNA polymerases. Proc. Natl. Acad. Sci. USA 78 (1 1981): 110-114.

Moore, P. D. and Strauss, B. S. Site of inhibition if in-vitro DNA synthesis in carcinogen- and UV-treated ΦX174 DNA. Nature 278 (1979): 664-666.

Nielsen. Sequence selective DNA recognition by synthetic ligands. Bioconjugate Chemistry 2 (1 1991): 1-12.

Nissen, N. I., Pajak, T, F., and Glidewell, O. A comparative study of a BCNU containing 4-drug program versus MOPP versus 3-drug combination in advanced Hodgkin's disease. Cancer 43 (1979): 31.

Norman, D., Live, D., Sastry, M, Lipman, R., Hingerty, B. E., Tomasz, M., Broyde, S., and Patel, D. J. NMR and computational characterization of mitomycin cross-linked to adjacent deoxyguanosines in the minor groove of the d(TACGTA)-d(TACGTA) duplex. Biochemistry 29 (1990): 2861-2875.

O'Connor, P. M. and Kohn, K. W. Comparative pharmacokinetics of DNA lesion formation and removal following treatment of L1210 cells with nitrogen mustards. Cancer Commun. 2 (1990): 387-394.

Ojwang, J. O., Grueneberg, D. A., and Loechler, E. L. Synthesis of a duplex oligonucleotide containing a nitrogen mustard interstrand DNA-DNA cross-link. Cancer Res 49 (23 1989): 6529-37.

Pacheco, D., Siegel, D., Butler, J., Hartley, J. A., Gibson, N. W., and Ross, D. The relationship between DT-diaphorase mediated metabolism of AZQ analogs and cytotoxicity in HT-29 human colon carcinoma cells. Proc. Am Assoc. Cancer Res. 32 (1991): 321.

Pan, S. S., Andrews, P. A., and Glover, C. J. Reductive activation of MMC and MMC metabolites catalyzed by NADPH cytochrome P-450 reductase and xanthine oxidase. J. Biol. Chem. 259 (1984): 959-966.

Pera, M., Rawlings, C. J., Shackleton, J., and Roberts, J. J. Quantitative aspects of the formation and loss of DNA interstrand cross-links in chinese hampster cells following treatment with cis-diamminedichloroplatinum(II) (cisplatin). Biochemica et Biophysica Acta 655 (1981): 152-166.

Ponti, M, Forrow, S. M., Souhami, R. L., D'Incalci, M., and Hartley, J. A. Measurement of the sequence specificity of covalent DNA modificationby antineoplastic agents using taq DNA polymerase. Nucleic Acids Research 19 (1991a): 2929-2933.

Ponti, M., Souhami, R. L., Fox, B. W., and Hartley, J. A. DNA interstrand crosslinking and sequence selectivity of dimethanesulphonates. Br J Cancer 63 (5 1991b): 743-7.

Powis, G. Free radical formation by antitumor quinones. Free Radic Biol Med 6 (1 1989): 63-101.

Price, C. C. "Chemistry of Alkylation." In Antineoplastic and Immunosuppressive Agents, Part II, ed. A. C. Sartorelli and D. G. Johns. 1-5. Berlin: Springer-Verlag, 1975.

Price, C. C., Gaucher, G. M., and Koneru, P. Relative reactivities for monofunctional nitrogen mustard alkylation of nucleic acid components. Biochim. Biophys. Acta 166 (1968): 327.

Pullman, A. and Pullman, B. Molecular electrostatic potential of the nucleic acids. Q. Rev. Biophys. 14 (1981): 289-380.

Reynolds, V. L., Molineux, I. J., Kaplan, D. J., Swenson, D. H., and Hurley, L. H. Reaction of the Antitumor Antibiotic CC-1065 with DNA. Location of the site of Thermally Induced Strand Breakage and Analysis of DNA Sequence Specificity. Biochemistry 24 (1985): 6228-6237.

Richardson, F. C., Bouchern, J. A., Skopek, T. A., and Swenberg, J. A. Formation of O6-methyldeoxyguanosine at specific sites in a synthetic oligonucleotide designed to resemble a mutgenic hotspot. J. Biol. Chem. 264 (1989): 838-841.

Roberts, J. J., Brent, T. P., and Crathorn, A. R. Evidence for the inactivation and repair of the mammalian DNA template after alkylation by mustard gas and half mustard gas. Eur. J. Cancer 7 (1971): 515.

Ross, D., Siegel, D., Gibson, N. W., Pacheco, D., Thomas, D. J., Reasor, M., and Wierda, D. Activation and deactivation of quinones catalyzed by DT-

diaphorase. Evidence for bioreductive activation of diaziquone (AZQ) in human tumor cells and detoxification of benzene metabolites in bone marrow stroma. Free Radic Res Commun 8 (4-6 1990): 373-81.

Ross, W. E., Ewig, R. A. G., and Kohn, K. W. Diferences between melphalan and nitrogen mustard in the formation and removal of DNA cross-links. (1978):

Ruddon, R. W. and Johnson, J. M. The effect of nitrogen mustard on DNA template activity in purified DNA and RNA polymerase systems. Mol. Pharmacol 4 (1968): 258.

Sanger, F., Nicklen, S., and Coulson, A. R. DNA sequencing with chain terminating inhibitors. Proc. Natl. Acad. Sci. USA 74 (12 1977): 5463-5467.

Schultz, P. G. and Dervan, P. B. Sequence-specific double-strand cleavage of DNA by penta-N-methylpyrrolecarboxamide-EDTA Fe(II). Proc. Natl. Acad. Sci. USA 80 (1983): 6834-6837.

Scudiero, D. A., Meyer, S. A., Clatterbuck, B. E., Mattern, M. R., J., Ziolkowski C. H., and Day, R. S. Sensitivity of human cell strains having different abilities to repair O6-methylguanine to inactivation by alkylating agents including chloroethylnitrosoureas. Journal 4 (1984): 2467-2474.

Siegel, D., Gibson, N. W., Preusch, P. C., and Ross, D. Metabolism of diaziquone by NAD(P)H:(quinone acceptor) oxidoreductase (DT-diaphorase): role in diaziquone-induced DNA damage and cytotoxicity in human colon carcinoma cells. Cancer Res 50 (22 1990a): 7293-300.

Siegel, D., Gibson, N. W., Preusch, P. C., and Ross, D. Metabolism of mitomycin C by DT-diaphorase: role in mitomycin C-induced DNA damage and cytotoxicity in human colon carcinoma cells. Cancer Res 50 (23 1990b): 7483-9.

Singer, B. All oxygens in nucleic acids react with carcinogenic ethylating agents. Nature, 264 (1976): 333-339.

Singer, B. and Fraenkel-Courat, H. Human pancreatic enzymes: purification and characterization of a nonelastolytic enzyme, protease E, resembling elastase. Biochemistry 14 (1975): 722.

Skipper, H. E., Bennett, L. L., and Langham, W. H. Overall tracer studies with C14-labeled nitrogen mustard in normal and leukemic mice. Cancer 4 (1951): 1025.

Stacey, K. A., Cobb, M., Cousens, S. F., and Alexander, P. The reactions of the "radiometric" alkylating agents with macromolecules in-vitro. Ann. N. Y. Acad. Sci. 68 (1958): 682-701.

Strauss, B., Ayers, K. N., Bose, K., Moore, P., Sklar, R., and Jatsumi, K. In DNA Repair and Mutagenesis in Eucaryotes, ed. W. M. Generoso and F. J. De Serres. 25-43. New York: Plenum, 1979.

Sunters, A., Springer, C. J., Bagshawe, K. D., Souhami, R. L., and Hartley, J. A. The cytotoxicity, DNA cross-linking ability and DNA sequence selectivity of the aniline mustards melphalan, chlorambucil, and 4-[bis(2-chloroethyl)amino]benzoic acid. Biochemical Pharmacology 44 (1 1992): 59-64.

Tanaka, M. and Yoshida, S. Inhibition of deoxyribonucleic acid polymerases α and β and terminal deoxynucleotidyl transferase from calf thymus by mitomycin C-deoxyribonucleic acid. Biochemical Pharmacology 30 (1981): 299-303.

Teng, S.P., Woodson, S.A., and Crothers, D.M. DNA Sequence Specificity of Mitomycin Cross-Linking. Biochemistry 28 (1989): 3901-3907.

Tomasz, M., Mercado, C. M., Olson, J., and Chatterjie, N. The mode of interaction of mitomycin C with deoxyribonucleic acid and other polynucleotides in-vitro. Biochemistry 13 (24 1974): 4878-4886.

Tomasz, M. A. H2O2 generation during the redox cycle of MMC and DNA-bound MMC. Chem. Biol. Interact. 13 (1976): 89-97.

Tong, W.P., Kohn, K.W. , and Ludlum, D.B. Modification of DNA by different haloethylnitorsoureas. Cancer Res. 42 (1982): 4460-4464.

Tong, W. P. and Ludlum, D. B. Crosslinking of DNA by busulfan. Formation of diguanyl derivatives. Biochimica et Biophysica Acta 608 (1980): 174-181.

Tullius, T. D. and Dombroski, B. A. Iron(II)EDTA used to measure the helical twist along any DNA molecule. Science 230 (1985): 679-681.

Tullius, T. D. and Dombroski, B. A. Hydroxyl radical footprinting: a high resolution method for mapping protein-DNA contacts. Methods Enzymol. 155 (1987): 537-558.

Ultmann, J. E., Hyman, J. A., and Crandall, C. Methylene thio-phosphoramide (thio-TEPA) in the treatment of neoplastic disease. Cancer 10 (1957): 902.

Van Dyke, M. W., Hertzberg, R. P., and Dervan, P. B. Map of distamycin, netropsin and actinomycin binding sites on heterogeneous DNA: DNA cleavage inhibition patterns with methidiumpropyl-EDTA Fe(II). Proc. Natl. Acad. Sci. USA 79 (1982): 5470-5474.

Van, Houten B., Gamper, H., Hearst, J. E., and Sancar, A. Construction of DNA substrates modified with psoralen at a unique site and study of the action mechanism of ABC excinuclease on these uniformly modified substrates. J Biol Chem 261 (30 1986a): 14135-14141.

Van, Houten B., Gamper, H., Holbrook, S. R., Hearst, J. E., and Sancar, A. Action mechanism of ABC excision nuclease on a DNA substrate containing a psoralen crosslink at a defined position. Proc Natl Acad Sci U S A 83 (21 1986b): 8077-81.

Varga, J. M., Wiesehahn, G., Bartholemew, J. C., and Hearst, J. E. Dose related effects of psoralen and ultraviolet light on the cell cycle of murine melanoma cells. Cancer Research 42 (1982): 2223-2226.

Verly, W. G. Monofunctional alkylating agents and apurinic sites in DNA. Biochem. Pharmacol. 23 (1974): 3.

Vos, J. M. and Hanawalt, P. C. Processing of psoralen adducts in an active human gene: repair and replication of DNA containing monoadducts and interstrand cross-links. Cell 50 (5 1987): 789-99.

Wakaki, S., Marumo, H., and Tomioka, K. Isolation of new fractions of antitumor mytomycins. Antibiot. Chemother. 8 (1958): 228-240.

Wassermann, K., Kohn, K. W., and Bohr, V. A. Heterogeneity of nitrogen mustard-induced DNA damage and repair at the level of the gene in Chinese hampster ovary cells. J. Biol. Chem. 265 (23 1990): 13906-13913.

Weidner, M.F., Millard, J.T., and Hopkins, P.B. Determination at Single Nucleotide Resolution of the Sequence Specificity of DNA Interstrand Cross-Linking Agents in DNA Fragments. J. Am. Chem. Soc. 111 (1989): 9270-9272.

Weidner, M. F., Sigurdsson, S. T., and Hopkins, P. B. Sequence preferences of DNA interstrand cross-linking agents: dG-to-dG cross-linking at 5'-CG by structurally simplified analogues of mitomycin C. Biochemistry 29 (39 1990): 9225-33.

Weinstein, I. B., Jeffrey, A. M., and Jennette, K. W. Benzo[a]pyrene diol epoxides as intermediates in nucleic acid binding in vitro and in vivo. Science 195 (1976): 592.

Wheeler, G. P. Studies related to the mechanism of action of cytotoxic alkylating agents: a review. Cancer Research 22 (1962): 651.

Wheeler, G. P. and Alexander, J. A. Effects of nitrogen mustard and cyclophosphamide upon the synthesis of DNA in vivo and in cell-free preparation. Cancer Research 29 (1969): 98.

Wurdeman, R. L. and Gold, B. The effect of DNA sequence, ionic strengh, and cationic DNA affinity binders on the methylation of DNA by n-methyl-n-nitrosourea. Chem. Res. Toxicol 1 (1988): 146-147.

Yarosh, D. B., Barnes, D., and Erickson, L. C. Transfection of DNA from a chloroethylnitrosourea resistant tumor cell line (Mer+) to a sensitive tumor cell line (Mer-) results in a tumor cell line resistant to MNNG and CNU that has increased) O6-methylguanine-DNA-methyltransferase levels

and reduced levels of DNA interstrand cross-linking. Carcinogenesis 7 (1986): 1603-1606.

Zerial, M. , Salinas, J., Filipski, J. , and Bernardini, G. Gene distribution and nucleotide sequence organization in the human genome. Eur. J. Biochem. 160 (1986): 479-485.

Zhen, W., Buchardt, O., Nielson, H., and Nielson, P. E. Site specificity of psoralen-DNA interstrand cross-linking determined by nuclease Bal31 digestion. Biochemistry 25 (1986): 6598-6603.

Zlotogorski, C. and Erickson, L. C. Pretreatment of normal human fibroblasts and human colon carcinoma cells with MNNG allows chloroethylnitrosourea to produce DNA interstrand crosslinks not observed in cells treated with chloroethylnitrosourea alone. Carcinogenesis 4 (1983): 759-763.

Zlotogorski, C. and Erickson, L. C. Pretreatment of human colon tumor cells with DNA methylating agents inhibits their ability to repair chloroethyl monoadducts. Carcinogenesis 5 (1984): 83-87.

Zwelling, L.A., Michaels, S., Schwartz, H., Dobson, P.P. , and Kohn, K.W. DNA crosslinking as an indicator of sensitivity and resistance of mouse L1210 leukemia to cis-diamminedichloroplatinum (II) and L-phenylalanine mustard. Cancer Res. 41 (1981): 640-649.