# CHEMICAL CLEAVAGE OF DNA :

# THE Cu(II):THIOL SYSTEM

# FOR DNA CLEAVAGE

A thesis submitted to the University of Manchester

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## CONTENTS

## ABSTRACT

The DNA cleaving ability of the Cu(II):thiol system and related Cu(II)-mediated systems was investigated. The reaction of Cu(II) ions with thiols in aerated solution was shown to produce single-strand cleavages and base damage in both supercoiled plasmid DNA and linear plasmid DNA restriction fragments. The reaction caused DNA cleavage to occur with unequal frequencies at base sites in three different DNA fragments. This apparent sequence preference did not reveal a consensus sequence for preferred cleavage sites. Base-labile sites generated by Cu(II):thiol were located predominantly at thymines on all three DNA fragments examined. The apparent cleavage preference was independent of thiol structure, charge distribution and stereochemistry. Replacement of thiol by ascorbate (another reductant) yielded the same preferred cleavage sites. The patterns of preferred DNA cleavage sites on both strands of a given stretch of duplex DNA were at non-identical nucleotide positions. Cleavage of denatured DNA occurred with greater specificity than native DNA. Replacement of reductant by $H_2O_2$ gave a different pattern of preferred cleavage sites although damaged base sites were largely similar to those of the Cu(II):reductant system. Products of the Cu(II):thiol DNA cleavage reaction included DNA with 5'-phosphoryl, 3'-phosphoryl and 3'-phosphatase-inert termini. Cleavage of DNA fragments containing specific inserts of repeating dinucleotide sequences showed preference for one nucleotide of the repeating pair.

Attempts to direct the cleavage reaction to DNA *via* DNA-binding thiol molecules, an intercalator-thiol and an oligonucleotide-thiol, were unsuccessful in obtaining enhanced or specific DNA cleavages, respectively. Attempts to footprint the binding sites of a minor groove binding ligand (Hoechst 33258) on DNA using Cu(II):thiol showed no evidence of footprints under conditions in which Fe(II):EDTA gave clear footprints. Attempts to footprint a protein, Hind III, also showed no evidence of footprints.

The results indicated that the reaction between Cu(II) and reductants (and $H_2O_2$) most likely occurs at the DNA surface *via* predominantly non-specifically bound Cu(II) ions which ultimately cause the degradation of deoxyribose *via* production of hydroxyl radicals. A full comparison of the results to previous literature reports and comparison to known chemical DNA cleavage systems was made and the potential uses of the Cu(II):reductant system as a footprinting reagent and/or as a probe of DNA structure discussed.

## DECLARATION

No portion of the work referred to in the thesis has been submitted in support of an application for another degree or qualification of this or any other University or other institute of learning.

## RESEARCH EXPERIENCE OF CANDIDATE

Since obtaining my first degree in Biological Chemistry at Essex University (1987), I have been studying at Manchester University for a Ph.D. under the supervision of Prof. K. T. Douglas in the Department of Pharmacy.

Acknowledgements

I would like to thank all the people who have who have helped me along my way as a Ph.D. student, especially the following:

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I am grateful to the students and staff (both past and present) of the group that I have worked in for the past few years, their constant good humour (sic.) has made my time here very enjoyable indeed.

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I would also like to thank my family, especially my parents, for their support over the past three years without whom none of this would have been possible.

Finally, I'd like to thank Ms Johan MacKinnon whose contribution has proved invaluable over these last few years.

ABBREVIATIONS

| | |
|---|---|
| A | adenine |
| A-T | adenine-thymine base pair |
| AdoMet | S-adenosyl methionine |
| ATP | adenosine triphosphate |
| BLM | bleomycin |
| Boc | t-butoxycarbonyl |
| C | cytosine |
| CAP | catabolite gene activator protein |
| CT-DNA | calf thymus DNA |
| Cys | cysteine |
| D | dihydrouracil |
| dA | deoxyadenylate |
| dATP | deoxyadenosine triphosphate |
| dC | deoxycytidylate |
| DEPC | diethyl pyrocarbonate |
| dG | deoxyguanylate |
| DiP | 4,7-diphenyl-1,10-phenanthroline |
| DMS | dimethyl sulphate |
| DNA | deoxyribonucleic acid |
| DNAse | deoxyribonuclease |
| ds | double-stranded |
| dT | deoxythymidylate |
| DTNB | 5,5'-dithiobis(2-nitrobenzoic acid) |
| DTT | dithiothreitol |
| dTTP | deoxythymidine triphosphate |

| | |
|---|---|
| EDTA | ethylenediamine tetraacetic acid |
| ENU | N-ethyl-N-nitrosourea |
| eqn. | equation |
| ESR | electron spin resonance |
| EtBr | ethidium bromide |
| Fe(II):EDTA | iron(II) complex of EDTA |
| fig. | figure |
| Fmoc | fluorene-9-methoxycarbonyl |
| G | guanine |
| G-C | guanine-cytosine base pair |
| Glu | glutamate |
| GSH | glutathione |
| His | histidine |
| hr | hour(s) |
| ISH | intercalator-thiol |
| $KMnO_4$ | potassium permanganate |
| min | minutes(s) |
| 3-MPA | 3-mercaptopropionic acid |
| MPE | methidiumpropyl-EDTA |
| MPE:Fe(II) | iron(II) complex of MPE |
| NMR | nuclear magnetic resonance |
| $^1O_2$ | singlet oxygen |
| $O_2^-$ | superoxide ion |
| $^{\bullet}OH$ | hydroxyl radical |
| oligo | oligonucleotide |
| oligo-SH | oligonucleotide linked to a thiol |
| OP | 1,10-phenanthroline |

| | |
|---|---|
| PAGE | polyacrylamide gel electrophoresis |
| phen | 1,10-phenanthroline |
| phi | 9,10-phenenthrenequinone diimine |
| PNK | T4 polynucleotide kinase |
| polyA | polyadenylic acid |
| polyC | polycytidylic acid |
| polyU | polyuridylic acid |
| pu | purine |
| py | pyrimidine |
| RNA | ribonucleic acid |
| RNAse | ribonuclease |
| sc | supercoiled |
| ss | single-stranded |
| T | thymine |
| TMP | 3,4,7,8-tetramethyl-1,10-phenanthroline |
| tRNA | transfer RNA |
| $tRNA^{Phe}$ | phenylalanine-specific tRNA |
| Trp | tryptophan |
| T4MPyP | meso-tetrakis(N-methyl-4-pyridiniumyl)porphine |
| U | uracil |

# CHAPTER 1 : INTRODUCTION

## 1.1 GENERAL INTRODUCTION

The molecular basis of all types of cancer and inheritable genetic diseases, such as muscular dystrophy, cystic fibrosis and the thalassaemias, lies within the genome. All of the above conditions arise from aberrations in the genomic DNA sequence. Such alterations include single base changes as well as deletions and duplications of bases. These changes may lead to expression of proteins differing by one or several amino acids from the "normal" protein or even to the loss of expression of a particular protein or several proteins. The presence of these abnormal proteins or the absence of functional proteins in turn may lead to the phenotypes of uncontrolled cell growth (i.e. cancers) or a particular physiological malfunction (e.g. sickle cell anaemia, thalassaemias).

During the past two to three decades, advances in molecular biology have enabled the positions of aberrant base residues to be mapped to specific sites on the chromosomes. Realistic goals for therapies designed to combat problems such as genetically inherited disorders therefore include:

- the removal of specific sections, or the whole, of an aberrant gene and replacement with the "normal" gene sequence thereby restoring the normal gene function (and thus normal phenotype)
- the addition of specific genes to living cells deficient in a specific known gene function in order to restore the normal phenotype.

Both of the above are examples of so called "gene-therapies". The latter type of therapy, in which recombinant retroviruses are used to insert specific genes into cells, has been shown to have potential for use in, for example,

cancer treatment (tumour infiltrating lymphocytes may be used to direct genes expressing anti-tumour products specifically to tumour sites; Kasid *et al*, 1990) and treatment of some immunodeficiencies (e.g. adenosine deaminase (ADA) deficiency may be treated by insertion of the ADA gene into haematopoietic stem cells which would then differentiate and express the gene throughout all the multiple lineages of stem cell-derived blood cells; Wilson *et al*, 1990). The former type of therapy requires DNA binding and cleaving functionalities to be developed. Many DNA-binding compounds exist in nature (e.g. proteins, oligonucleotides and antibiotics) and work on synthetic analogues of the natural antiviral, antitumour antibiotics netropsin and distamycin has led to the design of molecules that recognise specific DNA sequences *via* binding in the DNA minor groove (so called "lexitropsins"; Lown, 1988). This has provided an approach to generating molecules that can bind to any specific, defined DNA sequence. DNA cleaving molecules also exist in nature, e.g. restriction enzymes and other less specific nucleases and the bleomycins (BLMs), a family of metal-chelating, DNA-binding and cleaving glycopeptides. BLMs have been used as effective therapeutic agents against several types of cancer e.g. squamous cell carcinoma and Hodgkins lymphoma (Carter, 1978). Another agent in clinical use is ionising radiation, which cleaves DNA in dilute aqueous solution *via* the generation of reactive oxygen radical species (reviewed by Hutchinson, 1985). However, it should be noted that both BLMs and ionising radiation are fairly indiscriminate, cleaving at many sites within the genome and destroying both normal and cancerous tissue. However, synthetic cleavage systems have been produced with the long-term aim of attachment to DNA-binding molecules to effect site-specific DNA cleavage. Examples of such systems have commonly been based on redox chemistry using chelated metal

ions, e.g. Fe(II):EDTA and Cu(I):1,10-phenanthroline which, in the presence of dissolved oxygen and a reducing agent, produce oxygen radical species such as hydroxyl radical which ultimately lead to degradation of the deoxyribose ring and DNA strand breakage (see section 1.3.2). Thus, both molecular components required for the specific excision of gene sequences are currently being investigated.

This thesis contains an evaluation of a new DNA cleaving system and accordingly the majority of the following introduction now centres upon DNA cleaving systems, and in particular metal ion-mediated cleavage systems. It is preceeded by a short section on DNA-binding molecules.

## 1.2 BINDING TO DNA

### 1.2.1 THE LIGAND BINDING SITES OF DNA

The structure of DNA, such as B-DNA (figure 1), offers several types of binding sites for potential ligand:DNA interaction; (i) the negatively charged phosphate backbone provides a region for ligands to bind electrostatically, (ii) the major and (iii) minor groove surfaces contain various base residues ($-NH_2$, -N=, >C=O) capable of participating in hydrogen bonding interactions with ligands and (iv) the stacks of base pairs may accept planar aromatic ring structures that can insert between them (intercalate) to take part in hydrophobic and other interactions with the pi-electron systems of the bases. In addition, alterations in DNA structure such as alteration of DNA conformation (e.g. A, B, D, or Z), the presence of single-stranded regions and the supercoiling or otherwise of DNA, lead to variations in the geometric

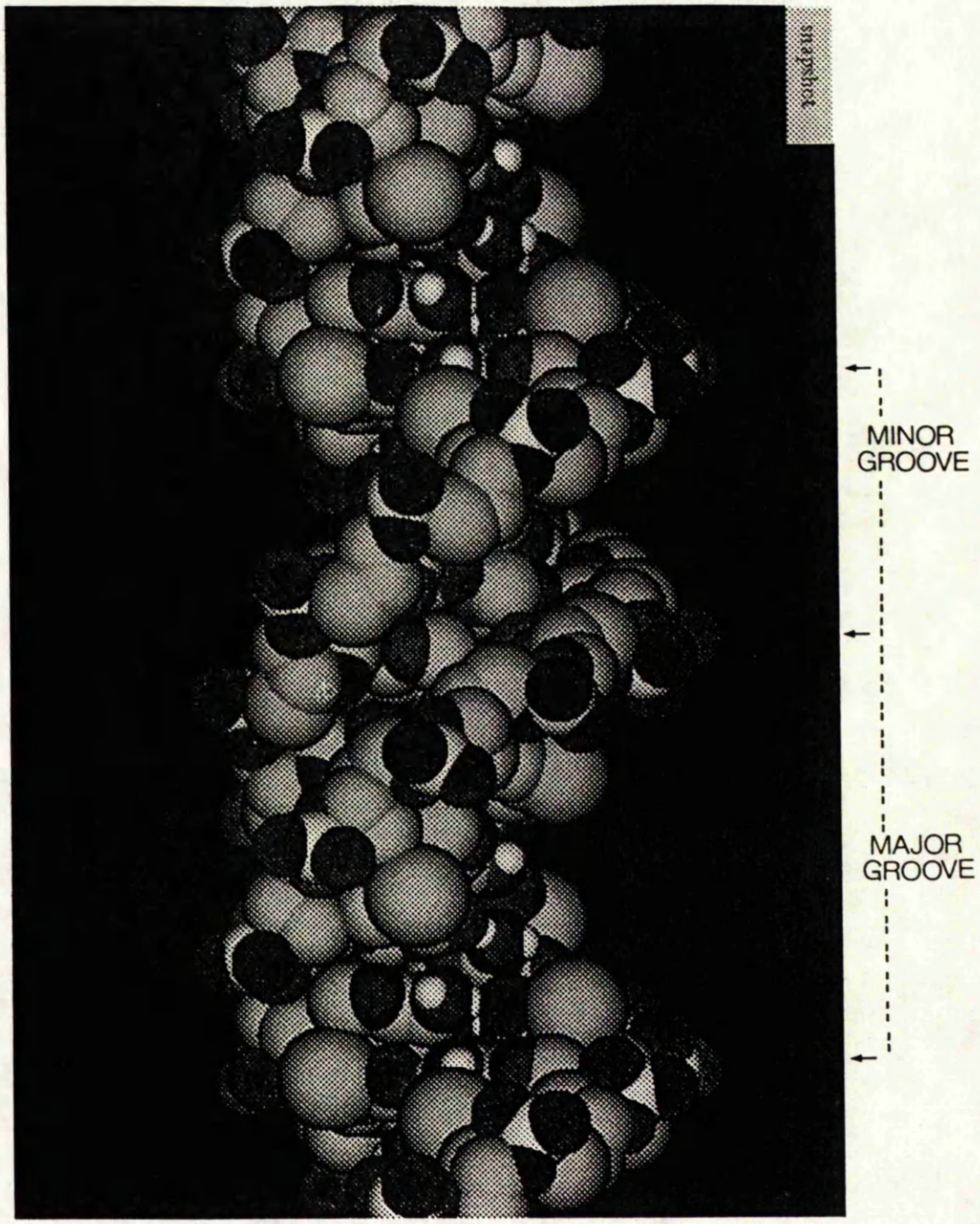



Figure 1. Computer drawn space-filling model of B-DNA helix tilted to show fully the depths of the major and minor grooves.

parameters of the DNA helix (e.g. groove widths, pitch of helix; Table 1) and therefore changes the dimensions and characteristics of the binding sites mentioned above.

Table 1. Some DNA structural types and related parameters (data from Saenger,1984)

| Structure | Number of residues per helix turn | Groove width (Angstroms) | | Groove depth (Angstroms) | |
|---|---|---|---|---|---|
| | | MINOR | MAJOR | MINOR | MAJOR |
| A | 11 | 11.0 | 2.7 | 2.8 | 13.5 |
| B | 10 | 5.7 | 11.7 | 7.5 | 8.5 |
| D | 8 | 1.3 | 8.9 | 6.7 | 5.8 |
| Z | 6 | 8.8 | 2.0 | 3.7 | 13.8 |

### 1.2.2 MAJOR GROOVE BINDING

Proteins, the only class of natural DNA-binding macromolecules (with the possible exception of some types of macromolecular antibiotics e.g. the quinoxalines; Waring and Fox, 1983), generally bind to DNA in a sequence-specific fashion. This is a requirement for their function, for example as controllers of gene transcription, or as bacterial restriction endonucleases (section 1.3.1.1) cleaving foreign DNA sequences. Other DNA binding proteins such as polymerases, nucleases (section 1.3.1.2) and topoisomerases (section 1.3.1.3) show less rigid sequence requirements but respond to sequence-dependent variations in DNA structure. Interactions between the proteins controlling gene expression and their DNA target sites have been elucidated by

means of many techniques including X-ray crystal structure analysis, NMR spectroscopy and DNAse I footprinting (reviewed by Brennan and Matthews, 1989; Struhl, 1989; Steitz, 1990).

At present four major types of structural motif have been identified as the DNA-binding determinants of proteins, namely, the helix-turn-helix, the zinc 'finger', the leucine 'zipper'(figure 2) and the $\alpha$-helices of the dinucleotide (Rossmann) fold. Recognition of DNA by the above motifs occurs in the major groove of B-DNA by specific contacts between amino acid peptide bonds and side chain moieties to the DNA base residues and phosphate groups. Greatest binding is usually seen at specific DNA sequences, greatly reduced binding affinity occurs at sites of similar sequence to the canonical binding site (non-canonical sites). The large loss of binding affinity at a non-canonical site is not just due to the loss of specific favourable protein-DNA base interactions but comes also from the additional loss of intramolecular protein-protein interactions that can occur only on the alignment of protein residues at the correct DNA sequence. Thus, highly specific interactions giving the strongest protein:DNA binding only occur at DNA sites containing, for example, the correct recognition sequence for a given restriction endonuclease. This sequence-specific binding of proteins to the major groove is not entirely unexpected since this groove contains the greater amount of information of DNA sequence (Seeman *et al.*, 1976) *via* the read-out of base pair hydrogen bond donors and acceptors (figure 3). As mutations in base sequence produce a greater effect on the hydrogen bond donor/acceptor groups in the major groove compared to the minor, it is not unreasonable to suggest that the effective, accurate control of gene expression has evolved to be mediated by major groove binding ligands (Wang, 1986).

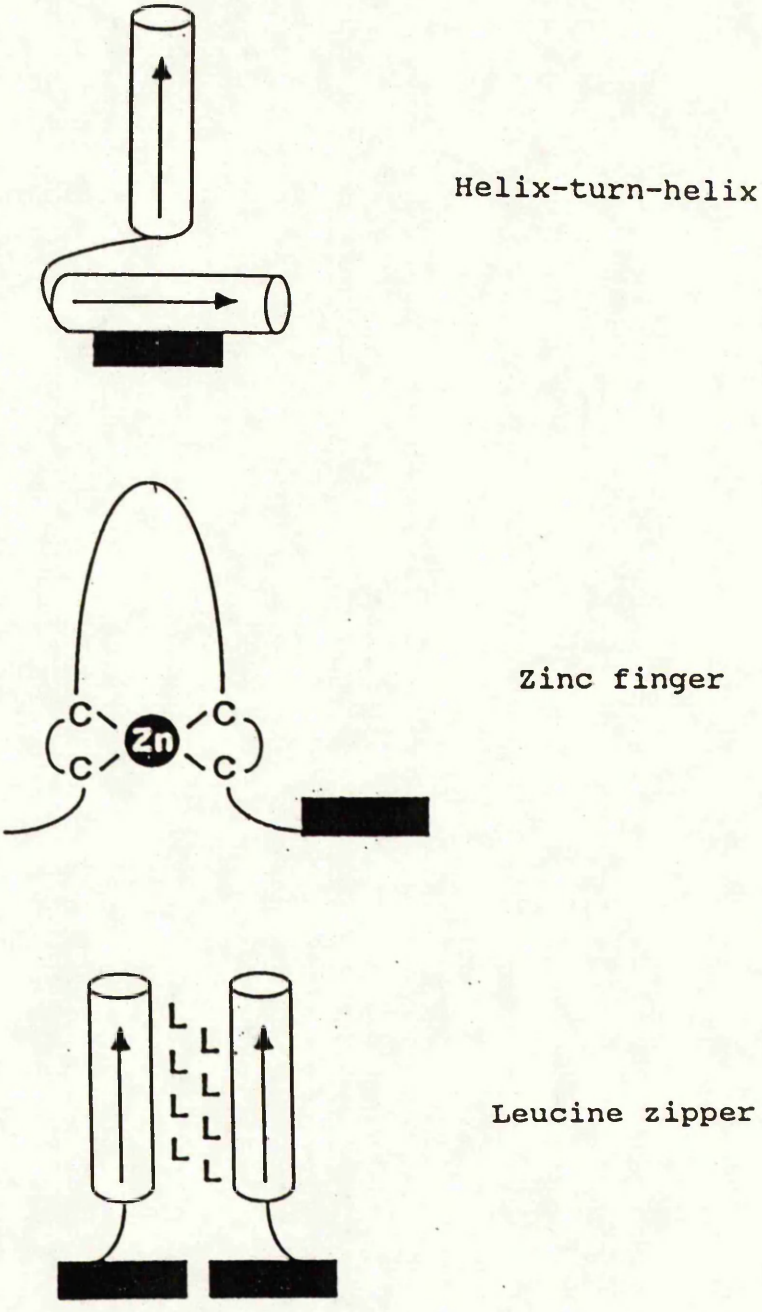



Figure 2. Schematic representation of some DNA-binding motifs. Cylinders represent alpha-helices with arrows indicating directionality. Conserved amino-acid residues are shown in one-letter code (C=cysteine, L=leucine) and zinc ions are shown as black circles. Shaded boxes indicate the regions of the proteins involved in specific contacts to DNA. Taken from Struhl, 1989.

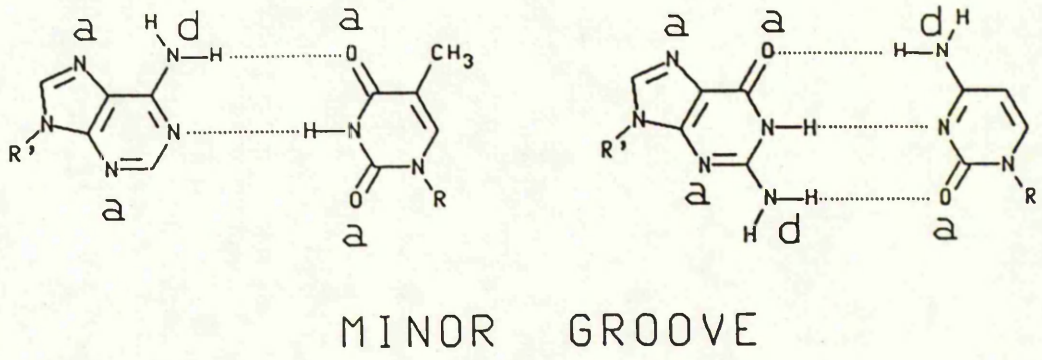



Figure 3. Adenine-thymine (left) and guanine-cytosine (right) base-pairs. R and R' represent connections to C1' of deoxyribose of the component strands of duplex DNA. The dotted lines represent hydrogen bonds and the letters a and d represent hydrogen bond acceptor and donor groups, respectively. The orientation of the base hydrogen bond donor/acceptor groups in relation to the grooves of B-DNA are as shown.

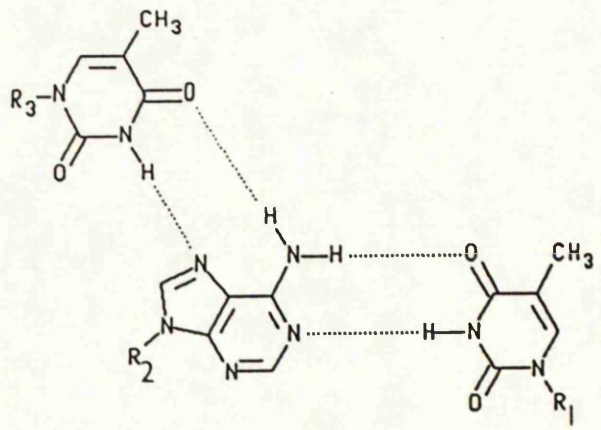
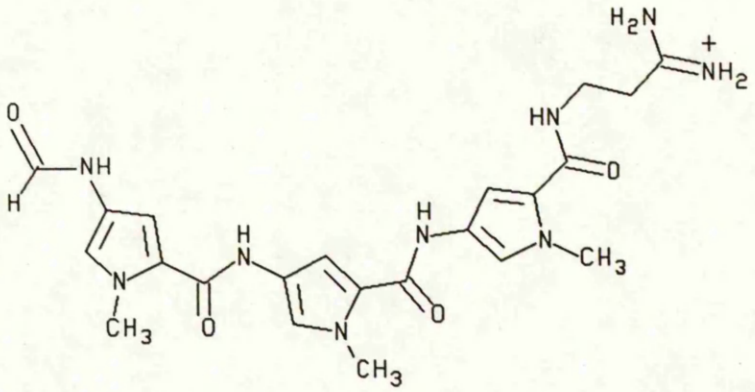


Figure 4. T-A-T base triplet showing Hoogsteen base-pairing of a thymine to an adenine of a Watson-Crick A-T base-pair. R1 and R2 represent connections to deoxyribose of the component strands of a DNA duplex. R3 represents connection to deoxyribose of a single-strand of DNA binding in the major groove of the duplex.

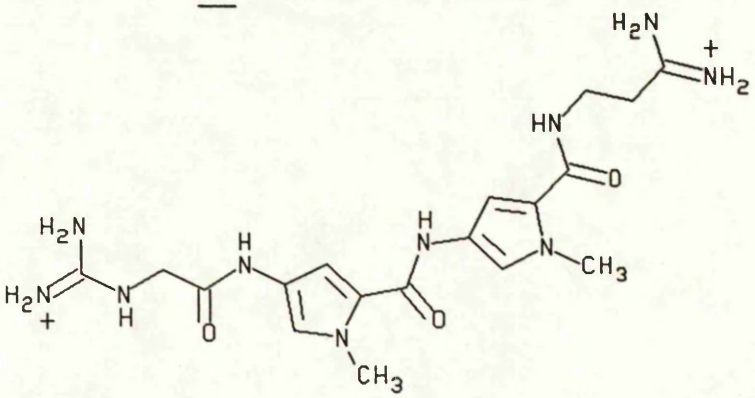
In addition, it is possible for a ssDNA molecule to bind to dsDNA in the major groove, to form a triple helix (Arnott & Selsing, 1974). Oligopyrimidines can bind in the major groove at homopurine.homopyrimidine sequences *via* Hoogsteen base pairings to the homopurine strand to form triplets of hydrogen bonded-bases, i.e. TAT (figure 4) and $C^+GC$ (see also section 1.4.2).

### 1.2.3 MINOR GROOVE BINDING

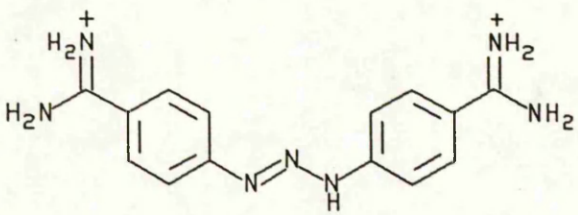
In spite of this cellular utilisation of the major groove, the minor groove is not devoid of sequence information. Natural compounds, such as the antiviral and antitumour antibiotics, distamycin and netropsin (1 and 2, respectively, Dervan *et al.*, 1982; Schultz & Dervan, 1984) and many synthetic compounds such as berenil (3, Laughton *et al.*, 1990) and Hoechst 33258 (4, Harshman & Dervan, 1985), bind within the minor groove usually at A-T rich regions. The molecular characteristics responsible for their specific binding to the minor groove are: (i) molecular dimensions similar to those of the minor groove of B-DNA, (ii) positioning of hydrogen bond donor/acceptor groups complementary to those in A-T rich minor grooves (see figure 5), (iii) either one or both ends of the molecule possessing a positively charged group to provide additional stabilisation *via* electrostatic interactions with the phosphate backbone or (iv) stabilisation of positively charged groups by interaction with the strongly electronegative potential of the minor groove (greatest for A-T rich regions (Pullman and Pullman, 1981)). Apart from the favourable hydrogen bond interactions, the presence of the 2-amino group of guanine projecting out from the minor groove surface sterically hinders the fit of these compounds in G-C sequences. Thus, the A-T preference is due, at least in some part, to an



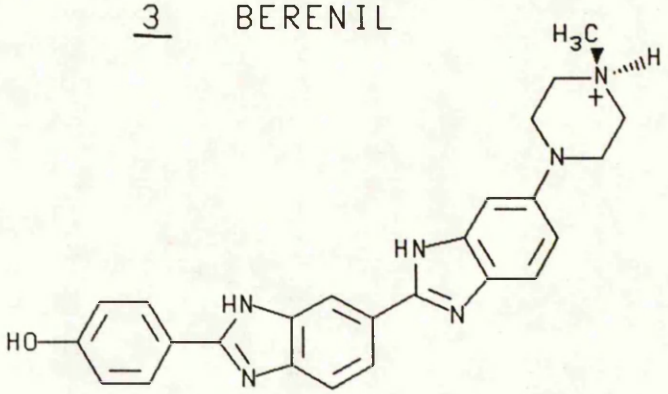
1 DISTAMYCIN



2 NETROPSIN



3 BERENIL



4 HOECHST 33258

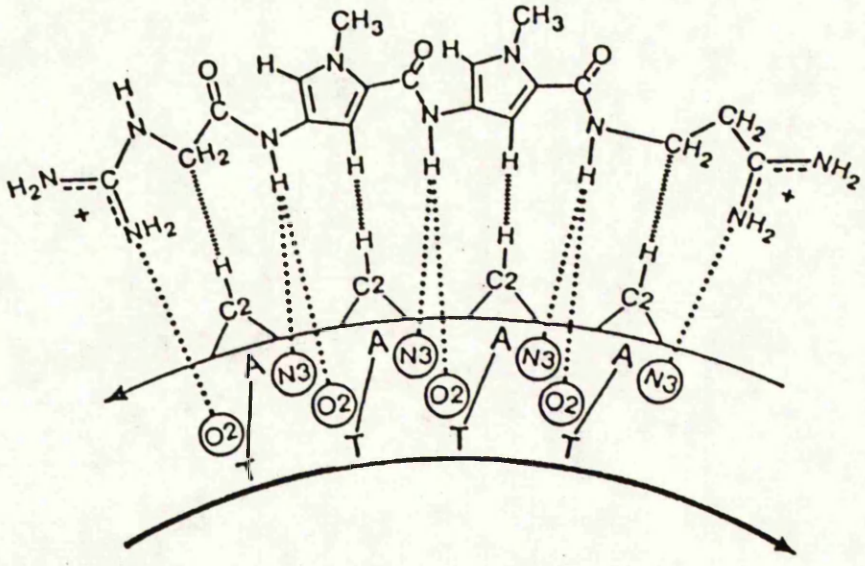
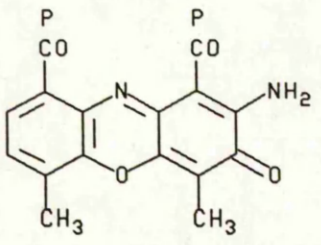



Figure 5. Schematic diagram of molecular recognition between netropsin and duplex DNA of sequence 3'-AAAA-5'. Key: specific hydrogen bonds (..); van der Waals contacts (≡).
(Taken from Lown *et al*, 1986)

exclusion of the ligand from G-C rich sites. Synthetic analogues of netropsin (Lown, 1988), where one or more pyrrole units were replaced by heterocycles capable of accepting a hydrogen bond from the 2-amino group of guanine have been found to exhibit altered sequence preference and can positively bind to G-C rich regions. Thus, it has been possible to generate molecules capable of reading the sequence of DNA *via* minor groove interactions (so called 'lexitropsins').

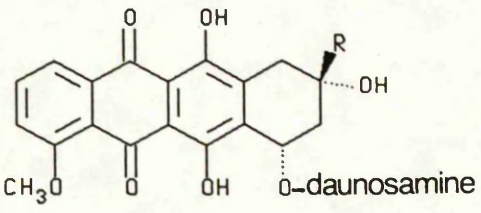
### 1.2.4 INTERCALATIVE BINDING

Intercalative binding of ligands to DNA (reviewed by Berman & Young, 1981 ;Neidle & Abraham, 1984) has inherently lower sequence specificity than the groove binding modes, unless the ligand has both intercalating and potential groove-binding functions. However, intercalation is responsible for the effects of a wide range of drugs which act by impairing the function of DNA as a template, e.g. the antitumour antibiotics actinomycin D, daunomycin and adriamycin (5,6 and 7, Mildner *et al.*, 1978; Chaires *et al.*, 1990; Latt, 1976, respectively), the trypanocidal agent ethidium (8, Hurley & Petinsek, 1979) and various acridine derivatives displaying antitumour/antibacterial properties, e.g. proflavine (9, Pasternack *et al.*, 1983). All of the above molecules possess planar, aromatic ring systems which insert between stacked, adjacent base pairs to participate in hydrophobic stacking interactions. Although the stacking of the bases is not disrupted, the intercalated molecule causes increased separation of the base pairs and a slight unwinding (10-30° per intercalated moiety) of the DNA helix to compensate for the strain resulting from the extension of the backbone. Intercalating molecules generally prefer insertion at



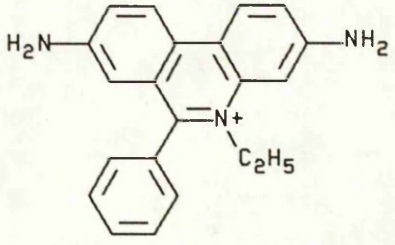

5 ACTINOMYCIN D

( P = -thr-D-val-pro-sar-Meval )

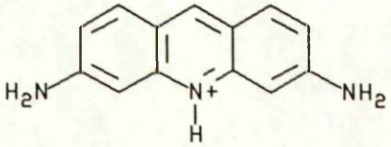



6 DAUNOMYCIN (R=$COCH_3$)

7 ADRIAMYCIN (R=$COCH_2OH$)




8 ETHIDIUM




9 PROFLAVINE

CpG sites, whether 5' to 3' or 3' to 5', e.g. daunomycin binds to both 5'CpG and 5'GpC sites (Chaires *et al.*, 1990).

Linkage of several intercalating moieties together can produce compounds with a great affinity for DNA ($K_{binding}$ is $10^{14}M^{-1}$ for a trimeric acridine derivative (Fiel *et al.*, 1979)) - this is a result of the affinity constant of the polymeric intercalator approximating the product of the three separate monomer affinities.

## 1.3 CLEAVAGE OF DNA

This section describes the properties of the major enzymatic and chemical DNA cleaving systems.

### 1.3.1 ENZYMATIC DNA CLEAVAGE

In the following pages are presented some of the properties of enzymes that interact with DNA to cleave the phosphodiester backbone. At this point it is appropriate to show how DNA cleavage is achieved in natural systems. The three types of enzymes to be discussed are: (1) bacterial restriction endonucleases, (2) nucleases, specifically DNAse I and (3) topoisomerases.

#### 1.3.1.1 Restriction Endonucleases

The restriction endonucleases are enzymes produced by bacteria to cleave foreign DNA that has entered their cells. In addition to restriction endonucleases, bacteria produce specific methylases that methylate their own

DNA at sequences corresponding to specific restriction endonuclease cleavage sites. Since methylated DNA sequences are not cleaved by the endonucleases, the host DNA is protected and the foreign DNA is susceptible to degradation. Three types of restriction endonuclease are known (types I, II and III). Type II enzymes are the least complicated (and most used) system and are described after a brief outline of types I and III.

The type I enzymes are complex, multifunctional proteins consisting of many subunits, which are responsible for the DNA cleavage, DNA methylase, DNA sequence-specificity and ATPase activities of the type I holoenzymes (Adams *et al.*, 1986). These enzymes catalyse either the cleavage of unmethylated target dsDNA (in the presence of AdoMet, ADP and $Mg^{2+}$) or the methylation of hemi-methylated target dsDNA sites. The type III enzymes have only two subunits - a nuclease and a combined methylase and specificity function. They recognise specific, non-symmetrical sites of DNA and cleave the DNA some 20-30 bases away from the 3' ends of those sites (Modrich, 1979).

The type II enzymes consist of two identical subunits and require only $Mg^{2+}$ ions for activity. Most type II enzymes recognise sites of four to six bp in length possessing twofold rotational symmetry, e.g. the EcoRI, PvuI and RsaI sites as shown in figure 6. Cleavage of DNA at the specified site by the dimeric enzyme may produce 3'-recessed or 5'-recessed termini or blunt ends, depending on the cleavage point within the recognition sequence (figure 6). The 'hydrolysis' reaction results in cleaved duplex DNA strands possessing 3'-hydroxyl and 5'-phosphoryl groups on either side of the strand breaks, but information on the protein residues and mechanism of this reaction is not yet fully known. In some cases, however, catalytic steps have been determined e.g. for EcoRI (Rubin and Modrich, 1978), revealing that cleavage of both

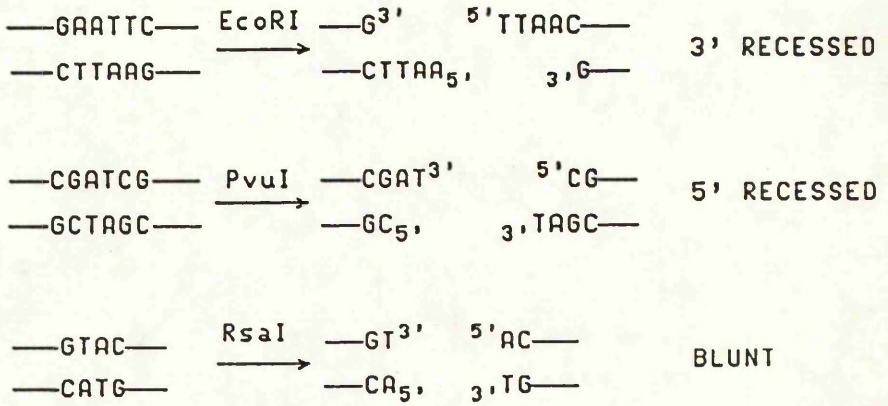



Figure 6. Restriction enzyme cleavage sites and products. Sequences are written 5' to 3' (top strand). Termini generated at the cleavage sites are in all cases 3'-hydroxyl and 5'-phosphoryl.

strands of the target duplex is not necessarily a one-step process and, in fact, may require two separate binding and cleavage events on alternate strands. Crystallographic studies of EcoRI have been carried out (McClarin *et al.*, 1986) and have provided information on the binding of the enzyme to the DNA.

1.3.1.2 DNAse I and other nucleases

DNAses and other nucleases cleave DNA by hydrolysis of phosphodiester bonds to yield free 5'-phosphoryl mononucleotides and oligonucleotides in a reaction requiring divalent metal ions. Examples of such enzymes and their metal ion requirements are: bovine-pancreatic DNAse I, $Ca^{2+}$ and $Mg^{2+}/Mn^{2+}$ (Melgar and Goldthwait, 1968); BAL31 Nuclease, $Ca^{2+}$ (Wei *et al.*, 1983); Nuclease S1, $Zn^{2+}$ (Kroeker and Kowalski, 1978), Exonuclease III, $Mg^{2+}$ (Weiss, 1976). BAL31 and Nuclease S1 are also RNAses while DNAse I and Exonuclease III are DNA specific. Of the above, only DNAse I is capable of degrading DNA endonucleolytically, i.e. at sequences within intact duplex DNA, and its reaction with DNA has been determined in detail. Recent investigations, notably crystallographic studies (Suck and Oefner, 1986; Suck *et al.*, 1988), have added to previous studies (reviewed by Laskowski, 1971) and led to a proposed chemical mechanism for the hydrolysis of DNA by DNAse I. The reaction pathway involves: (i) the binding of a loop of protein to the minor groove of the DNA substrate, (ii) a $Ca^{2+}$ ion binding to position the phosphodiester bond of the DNA correctly within the enzyme active site and (iii) a Glu-His-water proton-donor-acceptor chain (similar to the Asp-His-Ser catalytic triad found in serine proteases (Stryer, 1981)). The Glu-His-water system provides a water hydroxyl group to attack the phosphorus of the P-O-$C_{3'}$

substrate bond nucleophilically (figure 7). This attack is facilitated by the presence of the positively charged $Ca^{2+}$ ion.

DNAse I has very low sequence-specificity, which is required for it to function as an effective general DNAse, although it does exhibit a preference for cleaving sites adjacent to pyrimidine residues. It has been used extensively to "footprint" molecules (proteins or drugs) which are bound to DNA as cleavage of DNA by the enzyme is interrupted (or inhibited) at sites where ligands are bound to DNA leading to gaps in the DNA digestion patterns corresponding to ligand binding sites (figure 8; Galas & Schmitz, 1978; Fox *et al.*, 1986). The other nucleases mentioned above also have found applications making use of their particular DNA cleaving properties, e.g. probing of helical distortions in dsDNA using BAL31 (Wei *et al.*, 1983), cleavage of dsDNA at nicks or gaps in studies of chemical DNA cleavers using S1 nuclease (Kirschenbaum *et al.*, 1988), and footprinting studies to determine the edges of protein binding sites using Exonuclease III (Straney & Crothers, 1987).

### 1.3.1.3 Topoisomerases

Topoisomerases (nicking-closing enzymes) catalyse the interconversion of different topological isomers of DNA, e.g. to reduce or increase the degree of DNA supercoiling. The mechanism involves transient breakage of one (or both) strands of the substrate DNA duplex accompanied by rotation of DNA strands relative to the transient break-point. Two types of topoisomerase exist and are defined by their ability to cut either one (topoisomerase I; topo I) or both strands (topoisomerase II, topo II) of duplex DNA (Adams *et al.*, 1986).

The reaction catalysed by topo I (eukaryotic) produces a 5'-OH terminus

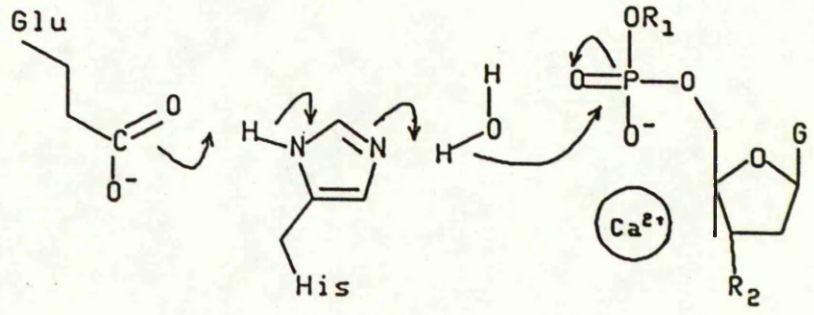



Figure 7. Mechanism of DNA cleavage by DNAse I via charge-relay type system. R1 and R2 represent connections to the polynucleotide chain (after Suck & Oefner, 1986).

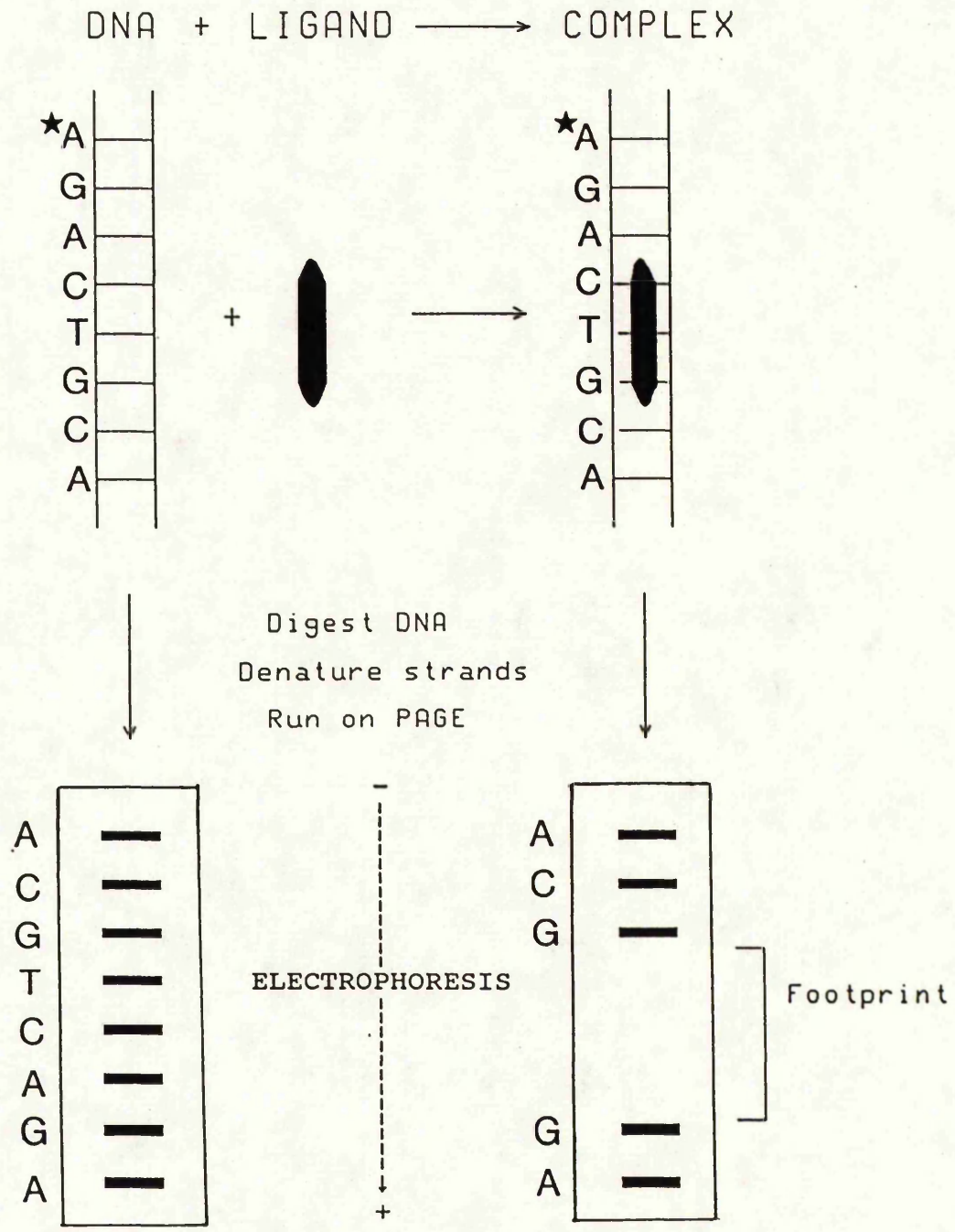



Figure 8. Schematic diagram of footprinting strategy for determining the binding sites of sequence-specific DNA-binding molecules. Inhibition of DNA degradation by DNAse or chemical nuclease, produces a gap (or "footprint") in the ladder of DNA bands which is observed on electrophoresis of the reaction products. The gap corresponds to the sequence of the binding site of the DNA-binding ligand. The asterisk (★), represents the position of radiolabelled nucleotide in DNA.

and a covalently-linked 3'-phospho-enzyme intermediate at the site of transient strand breakage (Champoux, 1981; Wang, 1985). The phosphate group of the DNA is linked *via* a tyrosine-OH ($O^4$)to the enzyme. Topo I (prokaryotic) produces the alternative DNA-enzyme link, i.e. a 5'-phospho-enzyme link together with a 3'-OH at the site of transient strand breakage (Wang, 1985).

Topo II cuts both DNA strands with a four base-pair stagger and becomes covalently linked transiently to the protruding 5'-phosphoryl ends of each broken strand. The enzyme-DNA covalent intermediate is once again an $O^4$-phosphotyrosine bond, a feature common to most known topoisomerases, e.g. *E. Coli* topo I(Tse *et al.*, 1980), rat liver topo I (Champoux, 1981), bacterial gyrase (Tse *et al.*, 1980) and T4 DNA topoisomerase (Rowe *et al.*, 1984).

Both type I and II topoisomerases show sequence preferences for the site of transient DNA breakage (Morrison and Cozzarelli, 1981; Edwards *et al.*, 1982; Been *et al.*, 1984), but the determinants of sequence specificity are not clear.

### 1.3.2 NON-ENZYMATIC DNA (AND RNA) CLEAVAGE

The following sections deal with the cleavage of nucleic acids by free (solvated) metal ions or by metal complexes that do not bind to DNA. Discussion of nucleic acid cleavage by metal ion complexes that bind to DNA, or are directed to bind to DNA by linkage to a DNA-binding ligand, appears in sections 1.3.2.4 and 1.4, respectively.

### 1.3.2.1 Metal ion cleavage of RNA

Metal ion mediated RNA cleavage is different from metal ion mediated DNA cleavage in one major respect, *viz.*, the metal ion alone is required - addition of oxidants or reductants is generally not required. Early work, however, by Eichorn and his colleagues, involved extended incubations of polyribonucleotides or RNA at relatively high temperatures (for up to 20 hours at 64°C) in unbuffered solutions in order to observe scission (Butzow & Eichorn, 1965; Eichorn & Butzow, 1965; Eichorn and Tarien, 1967; Eichorn *et al.*, 1967). Of the metals tested, $Zn^{2+}$, $La^{3+}$, $Ce^{3+}$, $Lu^{3+}$, $Mn^{2+}$, $Co^{2+}$, $Ni^{2+}$, and $Cu^{2+}$ were all effective at producing scission of RNA, although the last four ions were less active than the first four and $Ag^{+}$ was ineffective (Butzow & Eichorn, 1965). The studies indicated that polyribonucleotides (e.g. RNA, polyA, polyC, polyU) were degraded at 64°C in the presence of various metal ions by cleavage of the phosphodiester linkage involving the 2'OH group of the ribose of the substrate to yield 3' phosphomononucleotides as the ultimate degradation product. No cleavage was seen with ds or ssDNA, which indicated that the 2'OH of ribose was essential for this cleavage system. During the above work, however, the presence of a cyclic 2',3'-phosphomononucleotide was not tested for and, in the absence of further work, the reaction mechanism remains undetermined.

A well characterised metal ion-mediated nucleic acid cleavage system is that of yeast $tRNA^{Phe}$ by $Pb^{2+}$. It has been found that $Pb^{2+}$ ions cleave tRNA at specific sites both in solution (Werner *et al.*, 1976) and in the crystalline state (Brown *et al.*, 1983) under mild conditions, including buffered solutions and low temperatures (*cf.* $Zn^{2+}$, $La^{3+}$, $Ce^{3+}$ and $Lu^{3+}$ cleavage above). The

reaction was shown to proceed *via* highly nucleophilic metal ion bound hydroxyl groups (figure 9) present at or near neutral pH. These species facilitate polynucleotide chain scission by abstraction of a proton from ribose 2'OH groups located in their vicinity (Brown *et al.*, 1983). In addition, $Mg^{2+}$ and $Eu^{3+}$ ions can also cleave tRNA site-specifically (Ciesiolka *et al.*, 1989) although they have a slightly different cleavage pattern to that of $Pb^{2+}$. RNA cleavage by all three metal ions is highly sequence-specific occurring at 15 residues out of 76 ($Pb^{2+}$, yeast $tRNA^{met}$) or as few as 7 out of 76 ($Mg^{2+}$, yeast $tRNA^{met}$). This "sequence specificity" is not, however, due to recognition of the cleavage site but to the presence of an appropriate metal ion binding site proximal to, and correctly juxtaposed to, the observed cleavage site.

RNA can also be cleaved using the Fe(II):EDTA system (Wang and Padgett, 1989); the mechanism of RNA cleavage by this reagent is similar to that producing DNA cleavage and is discussed in section 1.3.2.3.

In conclusion, cleavage of RNA and polyribonucleotide by bound metal ions is made possible by the presence of the ribose 2'OH group. These cleaving methods are, therefore, not applicable to DNA whether ds or ss, due to the lack of 2'OH groups. Consequently, we move now to look at chemical methods of DNA cleavage.

### 1.3.2.2 Metal ion cleavage of DNA

Metal ions in association with oxidising and/or reducing agents are capable of DNA degradation *via* mechanisms that generally produce oxygen free radical species, which damage DNA by chemically altering the bases and/or the deoxyribose moieties of DNA (a general redox scheme is shown in

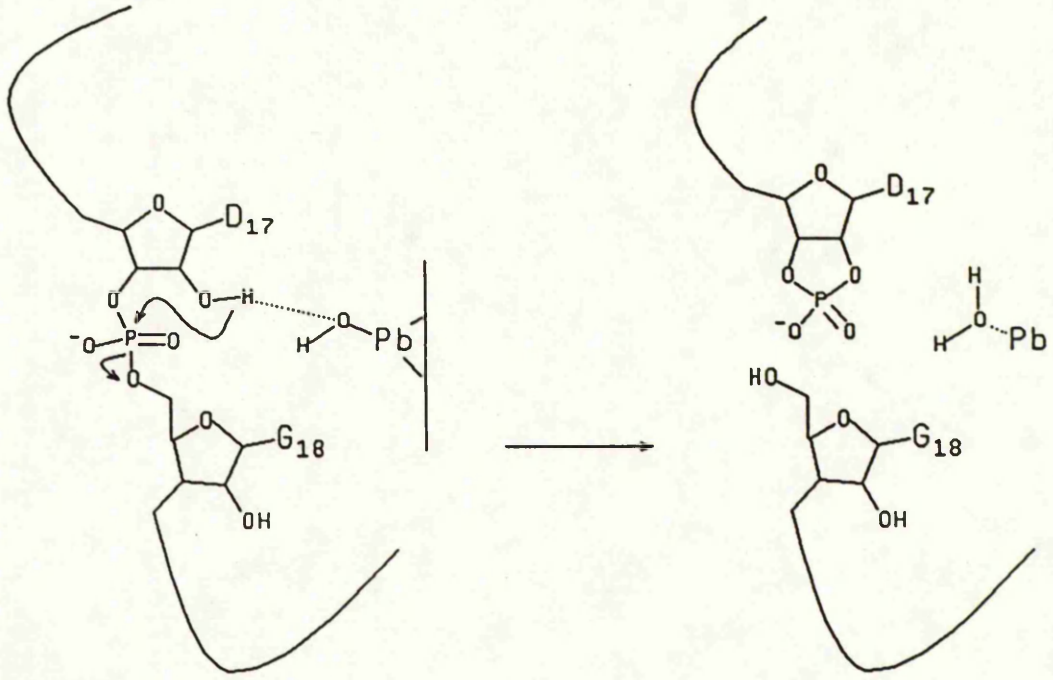



Figure 9. Schematic diagram of $Pb^{2+}$ catalysed cleavage of yeast $tRNA^{Phe}$ (after Brown *et al*,1983). G and D represent guanine and dihydrouracil, respectively.

figure 10). The following metal ions have all been shown to cleave DNA in the presence of various added reagents: chromium, cobalt, copper, iron, manganese, and nickel. Discussions of these cleavage systems are grouped according to the nature of the added reagent required to activate the metal ion. It is noted that of the above metals Fe(II) alone can generate DNA cleaving radicals in the absence of "added" reagents (equations 1-3; Moorhouse *et al.*, 1985).

$$Fe^{2+} + O_2 \rightleftharpoons Fe^{2+}-O_2 \rightleftharpoons Fe^{3+} + O_2^{-} \quad \text{(eqn. 1)}$$

$$2O_2^{-} + 2H^{+} \longrightarrow H_2O_2 + O_2 \quad \text{(eqn. 2)}$$

$$Fe^{2+} + H_2O_2 \longrightarrow Fe^{3+} + {}^{\bullet}OH + {}^{-}OH \quad \text{(eqn. 3)}$$

1.3.2.2.1 Metal ions plus sulfite ions

Metal ion cleavage of DNA in the presence of sulfite ion ($SO_3^{2-}$) has been shown to occur for $Co^{2+}$ and to a lesser extent for $Cu^{2+}$, $Fe^{2+}$ and $Mn^{2+}$ (Kawanishi *et al.*, 1989a) - other metals tested and shown to be inactive were $Ni^{2+}$, $Zn^{2+}$ and $Cd^{2+}$. The reactions for the most active metals $Co^{2+}$, $Cu^{2+}$ and $Mn^{2+}$ were shown to proceed *via* $SO_4^{-\bullet}$, $SO_3^{-\bullet}$ (which in the presence of $O_2$ gives $SO_5^{-\bullet}$)and oxygen radicals, respectively. Examined at the DNA sequence level, damage of DNA by either $Co^{2+}$ or $Cu^{2+}$ in the presence of sulfite ions produced non-identical cleavage patterns both before and after piperidine treatment (to determine base-labile sites), in accordance with the non-identical nature of the radical species formed in the respective reactions. Without piperidine treatment, DNA cleavage by $Co^{2+}$/sulfite occurred almost equally at every base position but on piperidine treatment cleavage at positions of guanine, and guanine 5′ to guanine increased predominantly indicating that

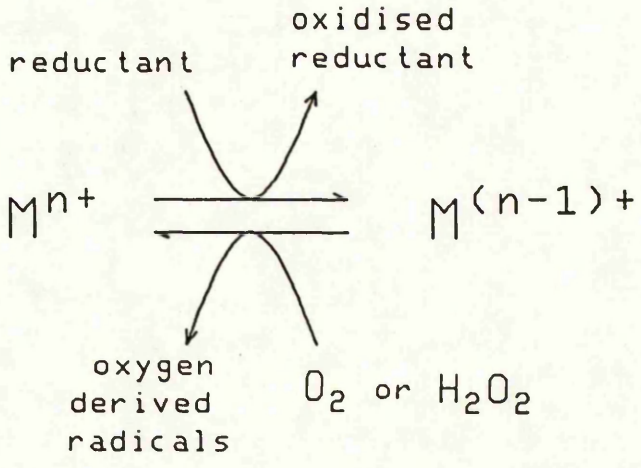



Figure 10. Simplified redox scheme for metal ions.

guanine bases were damaged preferentially.

1.3.2.2.2 Metal ions plus hydrogen peroxide

DNA cleaving activity by $Co^{2+}$, Cr(VI), $Cu^{2+}$, $Fe^{2+}$ and $Ni^{2+}$ ions in the presence of hydrogen peroxide has been demonstrated (Yamamoto *et al.*, 1989 and Moorhouse *et al.*, 1985; Kawanishi *et al.*, 1986; Chiou, 1983 and Chiou *et al.*, 1985; Sagripanti and Kraemer, 1989; Kadiiska *et al.*, 1989 and Lu *et al.*, 1990; Kawanishi *et al.*, 1989b, respectively). In general, hydroxyl radical (or "crypto-hydroxyl" radical, described later in this section) is implicated as the DNA cleaving agent. The reactions involved, however are generally more complex than the simple Fenton-type hydrogen peroxide dismutation, as shown below in equation 4, which occurs for $Fe^{2+}$ and possibly $Co^{2+}$.

$$M^{n+} + H_2O_2 \longrightarrow M^{(n+1)+} + HO^- + {}^{\bullet}OH \qquad \text{(eqn 4)}$$

The above metal ions might bind to DNA *via* electrostatic interactions at the phosphate backbone or even to the bases. Accordingly, detection of reactive intermediates in the DNA cleaving reactions (and therefore mechanistic inferences) can prove difficult due to the increased probability that radicals will react with DNA as opposed to quenching by added scavenger molecules free in solution. Identification of radical species is usually carried out by scavenging techniques where molecules known to react with postulated radicals generated in DNA cleavage reactions are added to the cleavage reaction to determine any inhibitory effect (typical scavengers and target species include DMSO (for

•OH), superoxide dismutase (for $O_2^{\bullet-}$)). ESR experiments (to detect radicals in the absence of DNA) and DNA cleavage experiments with $M^{n+}/H_2O_2$ often show reduced scavenging effects for the latter system (e.g. $Co^{2+}$ ; Moorhouse *et al.*, 1985) and this has been interpreted as evidence for 'hydroxyl radical type' radicals generated from DNA-bound metal ions, so-called "crypto-hydroxyl" radicals (Youngman, 1984; Moorhouse *et al.*, 1985; Kawanishi *et al.*, 1989b; Yamamoto *et al.*, 1989). In addition, singlet oxygen ($^1O_2$) seems to be generated by $Co^{2+}$, Cr(VI) and $Cu^{2+}$ in the presence of $H_2O_2$ (Kawanashi *et al.*, 1986; Yamamoto *et al.*, 1989; Chiou, 1983; Sagripanti and Kraemer, 1989) and sequence analysis of DNA degraded by the above systems shows a relatively even cleavage of DNA (roughly equal at all nucleotide positions) except for $Cu^{2+}$:$H_2O_2$ which has a slight sequence preference according to Chiou (Chiou *et al.*, 1985). Following piperidine treatment (to detect base damage) the most damaged positions correspond to guanine residues. These results are in accord with the fact that $^1O_2$ is known to react readily with deoxyguanylate but scarcely at all with other mononucleotides (Kawanishi *et al.*, 1986). Thus, the pattern of cleavage on a particular fragment of DNA may be the sum of the effects of both hydroxyl radical and $^1O_2$ damage for the $Co^{2+}$, Cr(VI) and $Cu^{2+}$:$H_2O_2$ systems. Both $Fe^{2+}$ and $Co^{2+}$ in the presence of $H_2O_2$ are more active DNA cleavers when bound to EDTA due to modulation of the metal redox potential (see section 1.3.2.3).

1.3.2.2.3 Metal ions plus ascorbate, thiols or reducing sugars

Reactions of metal ions such as $Cu^{2+}$, or $Fe^{3+}$ with reducing agents in aerated solution can lead to the production of oxygen-derived free radical species such as hydroxyl radical. These reactions can be described by the general, simplified redox cycle (figure 10, above). Both $Cu^{2+}$ and $Fe^{2+}$ ions are capable of oxidising ascorbic acid and when this reaction occurs in the presence of aromatic organic compounds (e.g. phenol) hydroxylation of the latter occurs (Udenfriend *et al.*, 1954; Brodie *et al.*, 1954, respectively). The reactive, hydroxylating species (probably hydroxyl radical) generated during the oxidation of ascorbic acid by $Cu^{2+}$ can also damage DNA and protein (Chiou, 1983 and Chiou *et al.*, 1985) and the following reactions were proposed:

$$\text{ascorbate} + 2M^{n+} \longrightarrow \text{dehydroascorbate} + 2M^{(n-1)+} + 2H^{+} \qquad \text{(eqn 5)}$$

$$\text{ascorbate} + O_2 \xrightarrow{M^{n+}} \text{dehydroascorbate} + H_2O_2 \qquad \text{(eqn 6)}$$

$$H_2O_2 + M^{(n-1)+} \longrightarrow M^{n+} + OH^{-} + {}^{\bullet}OH \qquad \text{(eqn 7)}$$

Superficially then, the above system is similar to the $M^{n+}$:$H_2O_2$ systems, discussed in the previous section (section 1.3.2.2.2). It also gives rise to incomplete quenching by hydroxyl radical scavengers leading to the conclusion that radicals may be generated by the metal ion while bound to DNA.

Limited studies on the sequence dependence of $Cu^{2+}$:ascorbate DNA cleavage (Chiou *et al.*, 1985) showed slight preferences for cleavage at sites 3′ to dG and sites 3′ or 5′ to dA and the least susceptible positions were found at pyrimidine clusters (e.g. TTCTC). In addition, the 3′ end-labelled DNA

products were found to bear 5'-phosphoryl termini. $Cu^{2+}$:ascorbate cleavage of DNA fragments is also examined in this thesis (section 3.3).

Scission of plasmid DNA by D-glucosamine and related reducing sugars has been shown to be stimulated by $Cu^{2+}$ (Watanabe *et al.*, 1986) although extended incubations (3hr, 37°C) and relatively high concentrations of co-reactants were required (D-glucosamine-6-phosphate, 0.1M; $Cu^{2+}$, 1mM). Detailed studies using DNA fragments of known sequence showed that base-labile sites generated by $Cu^{2+}$:D-glucosamine-6-phosphate treatment occurred primarily at C and T residues. The reaction was postulated to occur *via* a $Cu^{2+}$-DNA complex that on reduction by D-glucosamine (or other reducing sugars) produced hydroxyl radical as the DNA-damaging species (Kashige *et al.*, 1990).

Thiols auto-oxidise in aerated solutions, a reaction that is catalysed by $Cu^{2+}$ or $Fe^{3+}$ (Ehrenberg *et al.*, 1989 and Munday, 1989) and results in the formation of hydroxyl radicals. Although studies by Chiou (Chiou, 1983) showed very little DNA damage by $Cu^{2+}$ and thiols, this reaction was recently shown to cleave DNA effectively (Reed and Douglas, 1989) and further work on this DNA cleavage system is the main subject of this thesis.

### 1.3.2.3 Non-DNA-Binding Metallocomplexes

The ions $Fe^{2+}$ and $Co^{2+}$ form complexes with EDTA that cleave DNA or hydroxylate organic molecules such as phenol on activation with $H_2O_2$. The cleavage reaction for the chelated metal is enhanced and is accompanied by an increase in the yield of hydroxylated products compared to that of the free ion and $H_2O_2$ (Moorhouse *et al.*, 1985 and Kadiiska *et al.*, 1989). In addition Co(II):EDTA incubated with $H_2O_2$ plus ascorbate increases the total yield of

hydroxylated products by 10-fold (Moorhouse *et al.*, 1985) by increasing the cycling of the system (see figure 10). Similarly the Fe(II):EDTA system (Fe(II):EDTA plus $H_2O_2$ plus ascorbate) is a very efficient hydroxylating system (producing $^{\bullet}OH$ radical, equation 3) and its reaction with DNA has been widely used and studied (Tullius and Dombroski, 1985; Burkoff and Tullius, 1987; Tullius, 1989). Due to its apparent lack of sequence-specificity in DNA cleavage it is ideal for "footprinting" (figure 8) of proteins or small DNA-binding ligands to determine their binding sites on DNA (Galas & Schmitz,1978; Harshman & Dervan, 1985; Tullius & Dombroski, 1986). The Fe(II):EDTA system generates hydroxyl radicals without binding to its DNA target and so cleavage of the DNA occurs in a sequence-neutral manner in regions that are accessible to diffusible hydroxyl radicals generated near to the DNA (Lu *et al.*, 1990) - the binding of the protein/ligand to DNA is therefore unperturbed by the source of DNA cleaving activity. The Fe(II):EDTA system also cleaves ss- and dsDNA at similar rates (Jezewska *et al.*, 1990) and RNA (Wang and Padgett, 1989).

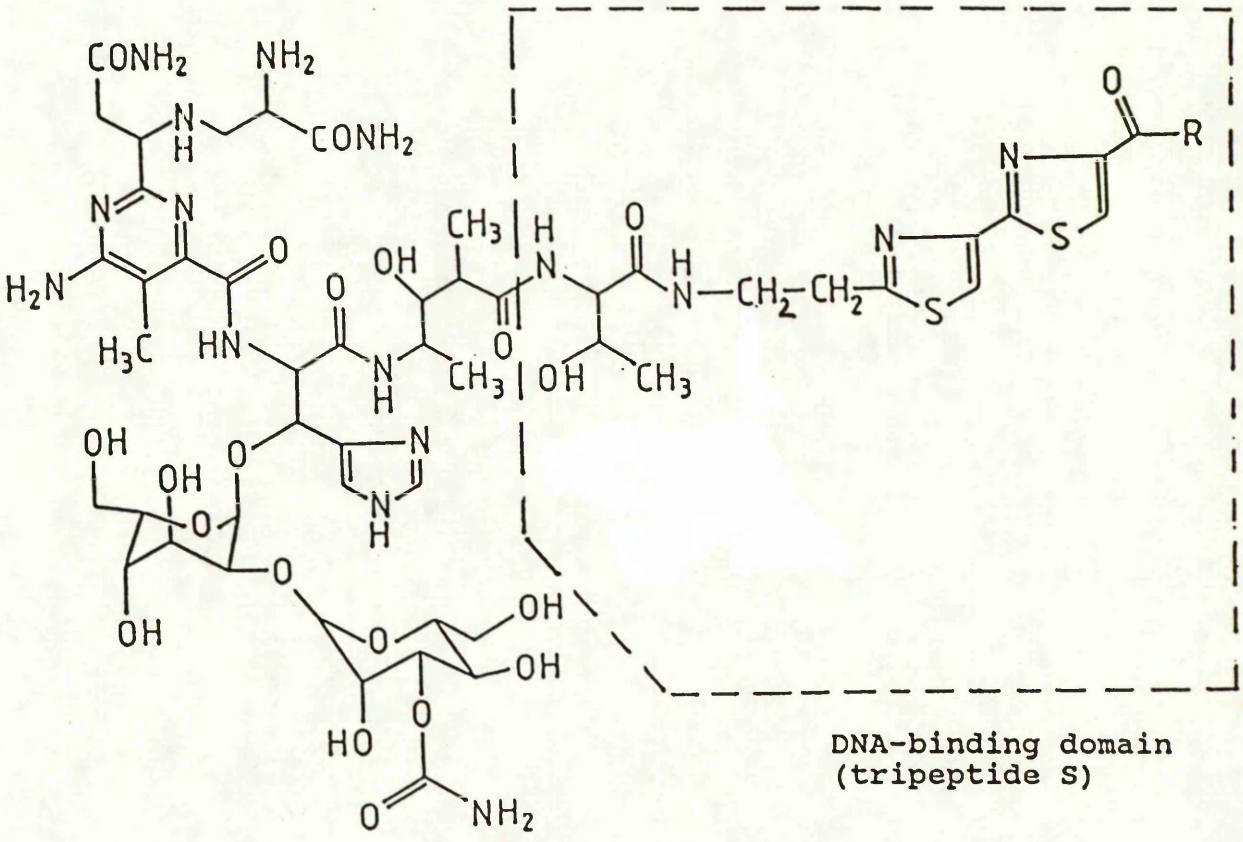
One other system of a metallocomplex which does not bind to DNA yet has DNA cleaving activity is a Cu(II):tripeptide, Cu(II):diglycyl-L-histidine (Chiou, 1983). On addition of $H_2O_2$ or ascorbate to a mixture of DNA and the Cu(II):tripeptide, DNA cleavage is effected, probably *via* hydroxyl radicals. Recently, this system has been attached to a DNA-binding protein (Mack *et al.*, 1988) and this combination has been shown to catalyse site-specific DNA damage (discussed in section 1.4.3).

### 1.3.2.4 DNA-binding metallocomplexes

Numerous DNA-binding metal complexes can effect DNA scission; Examples include iron or copper BLM (10, Hecht, 1979; Ehrenfield *et al.*, 1984); copper-1,10-phenanthroline and derivatives (11, for a review see Sigman and Chen, 1989); copper complexes of quercetin and related flavonoids (12, Chrisey *et al.*, 1988; Rahman *et al.*, 1989); copper-camptothecin (13, Kuwahara *et al.*, 1986); copper-amsa (14, Wong *et al.*, 1984); cobalt, ruthenium or rhodium complexes of some phenanthroline derivatives (15-17, Barton, 1986; Kirschenbaum *et al.*, 1988); uranyl ion, $UO_2^{2+}$ (Jeppesen and Nielsen, 1989); metalloporphyrins (18, Fiel *et al.*, 1982; Ward *et al.*, 1986). Most of the above systems require addition of co-reactants, e.g. $H_2O_2$, RSH, ascorbate or light in order to cleave DNA. Five of the most studied and useful of these systems are described below:

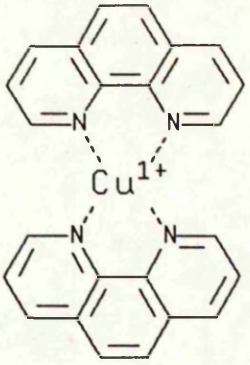
#### 1.3.2.4.1 Bleomycins

The bleomycins (BLMs) are a group of antitumour, glycopeptide-derived antibiotics elaborated by *Streptomyces* (Hecht, 1979) and have been used in the treatment of malignancies such as squamous cell carcinomas and lymphomas (Carter, 1978). Clinical BLM (Blenoxane) is primarily a mixture of two BLMs ($A_2$ and $B_2$, 10a and 10b, respectively) which differ only in the nature of their carboxyl-terminal substituents. The therapeutic effects of BLM are believed to involve DNA strand scission and *in vitro* studies have shown that the Fe(II), Cu(I), Mn(II) and VO(IV) BLM complexes cleave DNA in aerobic solution (Sausville *et al.*, 1978; Ehrenfield *et al.*, 1984; Ehrenfield *et al.*, 1985;

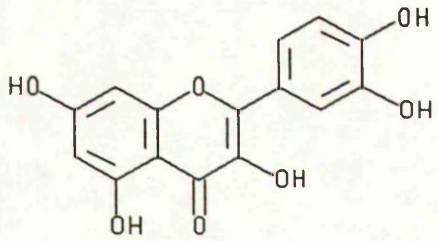



<u>10a</u> Bleomycin $A_2$ : R = $NH(CH_2)_3\overset{+}{S}(CH_3)CH_3$

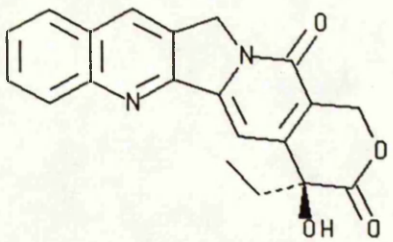
<u>10b</u> Bleomycin $B_2$ : R = $NH(CH_2)_4NHC(=NH)NH_2$



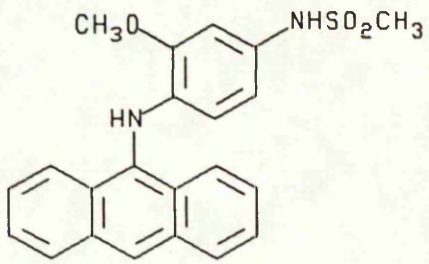

11 Cu(1,10-phenanthroline)$_2$



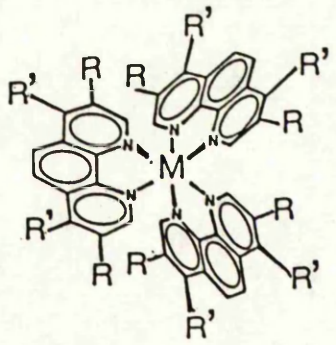

12 QUERCETIN



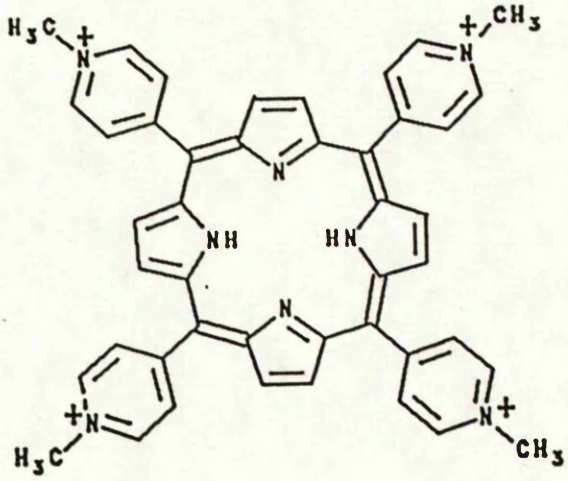

13 CAMPTOTHECIN




14 AMSACRINE (amsa)



15 Λ-Co(DiP)$_3^{2+}$ : M=Co(III), R=H, R'=$C_6H_5$
16 Λ-Ru(TMP)$_3^{2+}$ : M=Ru(II), R= R'=$CH_3$
17 Λ-Rh(DiP)$_3^{3+}$ : M=Rh(III), R=H, R'=$C_6H_5$
(Δ-Rh(DiP)$_3^{3+}$ is mirror image of Λ-isomer)



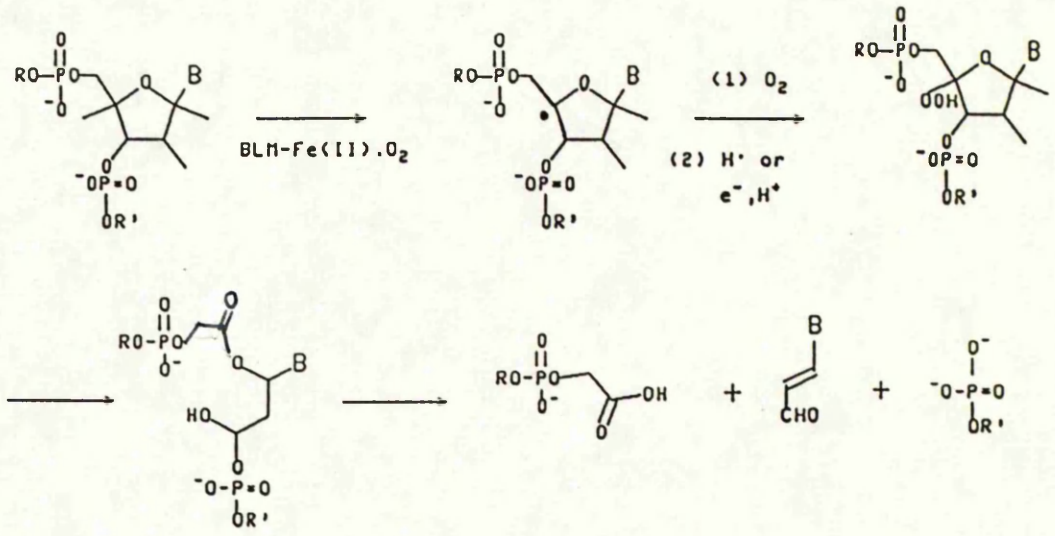
18 Meso-tetrakis(4-N-methylpyridyl)porphine (T4MPyP)

Kuwahara *et al.*, 1985, respectively).

The reaction of Fe(II):BLM with DNA is the best studied of all the BLM complexes (e.g. Hecht, 1986a,b; Stubbe and Kozarich, 1987). Postulated intermediates formed along the DNA degradation pathway are (i) a C-4' hydroperoxide arising *via* a transient C-4' radical (Scheme 1) and (ii) a C-4' hydroxyl derivative (Scheme 2) - see figure 11. Schemes 1 and 2 account for all the products found on degradation of DNA by BLM, i.e. a 5' oligonucleotide containing a 3'-phosphoglycolate terminus, a 3' oligonucleotide containing a 5'-phosphate and base propenals (all Scheme 1), free bases and alkali-labile lesions (Scheme 2).

BLM cleaves dsDNA but not RNA (Hori, 1979) and ssDNA is a poor substrate (Kross *et al.*, 1982). DNA cleavage occurs most frequently at pyGCpu (Mirabelli *et al.*, 1982), the preferred binding site of bleomycin. BLM can be cleaved chemically to remove the iron-chelating (DNA cleaving) moiety from the DNA binding grouping (tripeptide S) and it has been shown that tripeptide S has almost the same affinity for DNA as BLM itself (Chien *et al*, 1977). Hence BLM is composed of two functionally distinct moieties combined to produce a highly specific drug. BLM is therefore a natural compound which has provided the model for the design of sequence-specific DNA-cleaving compounds, i.e. by attachment of cleaving functions to DNA binding functions.

SCHEME 1




SCHEME 2

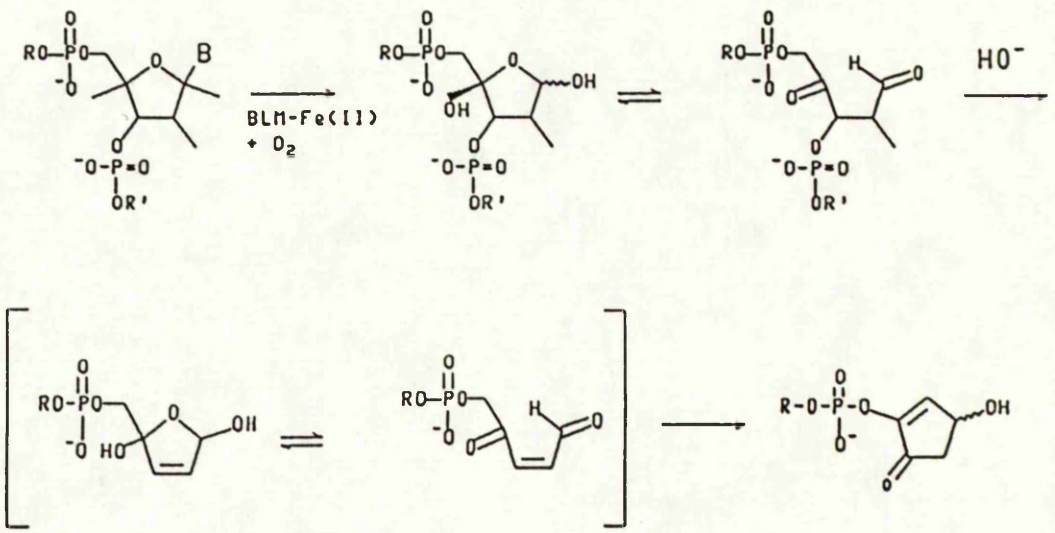



Figure 11. Schemes 1 and 2 for BLM:Fe(II) catalysed DNA cleavage (after Stubbe and Kozarich, 1987).

1.3.2.4.2 Copper-phenanthroline

The copper-phenanthroline chemical nuclease was first described in 1979 (Sigman *et al.*, 1979) but was simultaneously investigated by another group (Downey *et al.*, 1980 and Que *et al.*, 1980). The system described by the former group was copper:1,10 phenanthroline plus a thiol and $H_2O_2$ while that of the latter was copper:1,10-phenanthroline plus a thiol. However, the two systems are equivalent in aerobic solution as $H_2O_2$ may be generated by the latter system from the reaction of a thiol and dissolved oxygen with the cuprous 1,10-phenanthroline complex (equation 11). The information gained over the last 10 years has been reviewed recently (Sigman, 1990) and is summarised below.

The 2:1 complex of 1,10-phenanthroline and $Cu^{2+}$ ($(OP)_2Cu^{2+}$) together with its coreactant $H_2O_2$ are essential for DNA cleavage. The phenanthroline moiety has two roles, (i) moderation of the redox potential for $Cu^{+}/Cu^{2+}$ and (ii) direction of the reaction to DNA by binding to the minor groove. Detailed kinetic studies (Thederahn *et al.*, 1989) showed that the following obligatory ordered mechanism occurred:

$$(OP)_2Cu^{2+} \longrightarrow (OP)_2Cu^{1+} \qquad \text{(eqn 8)}$$

$$(OP)_2Cu^{1+} + DNA \rightleftharpoons [(OP)_2Cu^{1+}\ DNA] \qquad \text{(eqn 9)}$$

$$[(OP)_2Cu^{1+}\ DNA] + H_2O_2 \longrightarrow \text{nicked DNA products} \qquad \text{(eqn 10)}$$

Equation 8 proceeds by the addition of thiol or superoxide (Sigman 1986). The binding of the tetrahedral cuprous complex to DNA (equation 9) is then required before reaction of the complex with $H_2O_2$ (equation 10) to form the

oxidative species which cleaves the DNA. The $H_2O_2$ can be added or generated by the following reactions,

$$2(OP)_2Cu^{1+} + O_2 + 2H^+ \longrightarrow 2(OP)_2Cu^{2+} + H_2O_2 \qquad \text{(eqn 11)}$$

$$2H^+ + 2O_2^- \longrightarrow H_2O_2 + O_2 \qquad \text{(eqn 12)}$$

The former reaction occurs when thiol is added and the latter reaction occurs when superoxide is added. Analysis of the reaction products shows that 3'- and 5'-phosphomonoester and 3'-phosphoglycolate termini are generated together with free bases and 5'-methylene-2-furanone (figure 12). These products are produced by preferential attack of copper-oxo species (not diffusable hydroxyl radical) at C1' of deoxyribose. Attack at C4' occurs to a lesser extent.

The $(OP)_2Cu$ complex exhibits a preference for the DNA sequence at which it cuts (Veal & Rill, 1988; Sigman & Chen, 1989; Yoon *et al.*, 1990), a feature which is due to the stability and reactivity of the complex between $(OP)_2Cu$ and the DNA at the preferred sequences (*cf.* the Fe(II):EDTA system which gives sequence-neutral cleavage, section 1.3.2.3). Besides the primary DNA sequence preference, $(OP)_2Cu$ also exhibits secondary structure specificity, e.g. A-DNA is cut more slowly than B-DNA while Z-DNA and ssDNA are not measurably cleaved (Pope and Sigman 1984). However, ssDNA as part of an RNA polymerase-lac promoter open complex is cleaved (Spassky & Sigman, 1985). Thus, the ability of $(OP)_2Cu$ to cleave nucleic acids is strongly dependent on the availability of a stable binding site for the complex.

$(OP)_2Cu$ has been used to footprint the binding of *E. coli* RNA polymerase to lac promoters (Sigman, 1986) and for the detection of

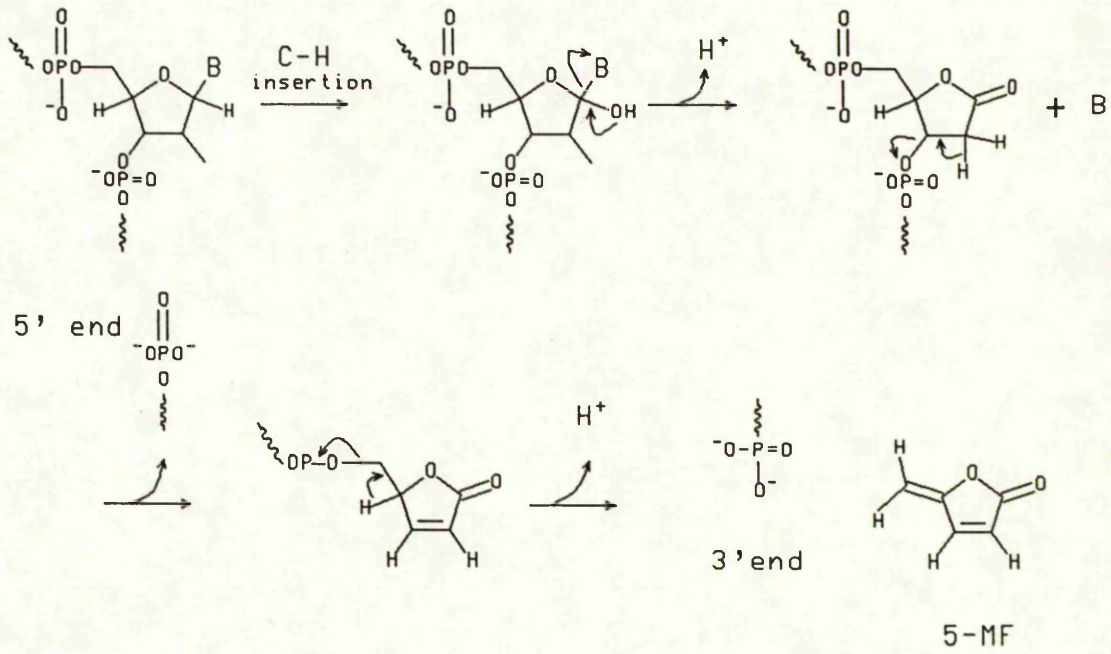



Figure 12. Predominant mechanism of DNA cleavage by $Cu(OP)_2$ (after Sigman & Chen, 1989).

intermediates in transcription initiation by *E. coli* RNA polymerase (Thederahn *et al.*, 1990) but has also been used to cleave DNA when directed to DNA by attachment to oligonucleotides or proteins (discussed in section 1.4.2 and 1.4.3, respectively).

1.3.2.4.3 Tris-phenanthroline derivatives

Studies by Barton and co-workers on a large number of chiral tris-phenanthroline transition metal complexes that bind to and cleave DNA have emphasised the requirement of suitably shaped molecules for binding to particular DNA (and RNA) conformations. Since DNA is chiral (right handed helices for A- and B-forms; left handed helix in the Z-form) the binding of specific enantiomers of chiral metal complexes can be specific for various DNA forms and conformations. In addition, some of the complexes can be activated with light to cleave DNA, enabling their use as conformation-specific DNA-cleaving compounds or probes (Barton, 1986).

The complex Λ-$Co(DIP)_3^{3+}$ (15) binds to and cleaves at Z-DNA sites and sites of alternating purine/pyrimidine residues that may be expected to form Z-DNA-like regions (Barton and Raphael, 1985). The binding only occurs in supercoiled DNA, indicating that the compound does not respond to DNA sequence, but to DNA secondary structure, i.e. the complex fits into the groove of DNA in a Z-like complementary conformation. In addition, it was found that sites of cleavage by the complex occurred mainly at non-coding control regions of the plasmid pBR322 and also of SV40 DNA (Müller *et al.*, 1987). Since these sites of altered conformation are those involved in the biological control of gene expression these results may indicate that DNA

secondary structure (in addition to primary structure) plays an important part in gene regulation.

The complex Λ-Ru(TMP)$_3^{2+}$ (16) binds preferentially to A-form helices (dsRNA or RNA/DNA hybrids) and also cleaves them twice as efficiently as does its Δ-isomer (Mei and Barton, 1986). Rh(DIP)$_3^{3+}$ (17), as both Λ and Δ isomers, cleaves supercoiled DNA (pBR322, pCOLE1 and bacteriophage ϕX174 RF-DNA) at or near sites of cruciforms (Kirshenbaum *et al.*, 1988), the binding of the complex occurring on one side of the double helix close to the base of the cruciform structure. It should be noted that this cleavage is extremely specific, occurring at defined single-base sites (*cf.* Fe(II):EDTA cleavage, section 1.3.2.3). The mechanism of DNA cleavage for the above complexes is believed to be oxidation of the bases by singlet oxygen or *via* electron transfer - depending upon the particular complex. (The degradation of RNA (specifically tRNA$^{Phe}$ from yeast) has been described for the Ru(TMP)$_3^{2+}$, Ru(Phen)$_3^{2+}$, Rh(phen)$_2$phi$^{3+}$ and Rh(DIP)$_3^{3+}$ complexes (Chow and Barton, 1990) although this method requires post-complex treatment with aniline to effect RNA cleavage).

1.3.2.4.4 Uranyl ion

The uranyl (VI) ion, $UO_2^{2+}$, cleaves DNA when irradiated with long wavelength ultraviolet radiation ($300 < \lambda < 420$nm) in an almost sequence-neutral fashion (Nielsen *et al.*, 1988). It is believed that the cleavage is produced by reactive species generated from the excited state of uranyl ion (which is a powerful oxidant) while the ion is bound to DNA. However, whether the cleavage is due to hydroxyl radicals or a direct oxidation of the

deoxyribose unit has not yet been determined. Results of uranyl photofootprinting of *E. coli* RNA polymerase in an open complex with the deo-P1 promoter provide clear regions of protection which are within regions found to be protected by DNAse and hydroxyl radical footprinting (Jeppesen & Nielsen, 1989a) and are closely similar to the results of the latter. Although this method of footprinting is relatively new the early results are very promising and as yet this method is the only one able to probe ligand/DNA-phosphate contacts directly.

1.3.2.4.5 Porphyrins

Porphyrins, in particular metal derivatives of cationic porphyrins such as meso-tetrakis(N-methyl-4-pyridiniumyl)porphine (T4MPyP, 18)), cleave DNA in the presence of added reducing reagents (Fiel *et al.*, 1982; Praseuth *et al.*, 1986). Zinc(II) and non-metal derivatives of T4MPyP also produce DNA scission when irradiated with visible light at their Soret band maxima, possibly *via* singlet oxygen generation from the interaction of oxygen with the excited triplet state of the porphyrin (Kelly *et al.*, 1985). The Co(III), Fe(III) and Mn(III) derivatives of T4MPyP cleave DNA in the presence of ascorbate (reducing agent), iodosobenzene and superoxide (oxidising agents) by mechanisms involving high-valent metalloporphyrin species generated by reaction of the ascorbate-reduced metalloporphyrins with molecular oxygen or by direct oxidation of the porphyrins effected by superoxide or iodosobenzene. More recently, Mn(III)-T4MPyP, activated by potassium monopersulphate ($KHSO_5$), has been shown to cleave DNA efficiently (Fouquet *et al.*, 1987). The reaction causing DNA cleavage occurs at $C_{1'}$ or $C_{4'}$ of the deoxyribose to produce 5'-

phosphoryl termini (Tapper & Clayton, 1981) and in the case of $KHSO_5$-activated Mn(III)-T4MPyP, 5-methylene-2-furanone, 5'and 3'-phosphoryl termini were detected, indicating preferential attack at $C_1$. of deoxyribose (Bernadou *et al.*, 1989, van Atta *et al.*, 1990). Conflicting results of the sequence dependence of Mn(III)-T4MPyP-mediated cleavage of DNA show A-T preference (when activated by $KO_2$; Ward *et al.*, 1986) or a G preference within TpG or ApG sequences (when activated by $KHSO_5$; van Atta *et al.*, 1990). However, despite these anomalies, Mn(III)-T4MPyP activated by $KHSO_5$ has been used in quantitative footprinting analysis of netropsin (a minor groove binding compound) bound to DNA (Dabrowiak *et al.*, 1989a).

### 1.3.3 METAL ION INDEPENDENT CHEMICAL DNA CLEAVAGE

A wide variety of chemical agents can produce alkylation and/or cleavage of DNA *via* reactions independent of metal ions. Some examples of such chemicals are:

(a) S-(2-haloethyl)mercapturic acid analogues (19, Vadi *et al.*, 1985),

(b) dimethylsulphate (20, section 1.3.4 and references therein),

(c) diethylpyrocarbonate (21, section 1.3.4 and references therein),

(d) diazo-acridine derivatives (22, Jeppesen and Nielsen, 1989b),

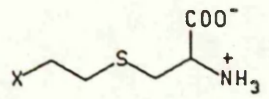
(e) phthalimide hydroperoxides (23, Saito *et al.*, 1990),

(f) tetrachlorohydroquinone (24, Carstens *et al.*, 1990),

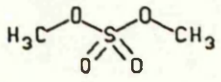
(g) nitrated oligo-N-methylpyrrole carboxamide derivatives (25, Nishiwaki *et al.*, 1990),

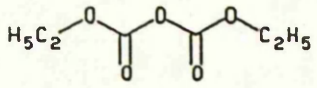
(h) adriamycin (7, Lown *et al.*, 1977),

(i) ionising radiation (reviewed by Hutchinson, 1985),



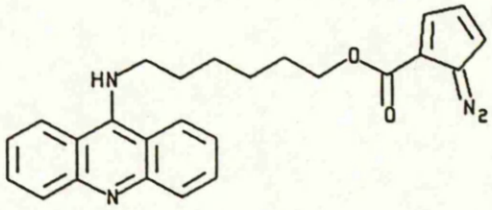
19 S-(2-haloethyl)-L-cysteine
( X = Br or Cl )



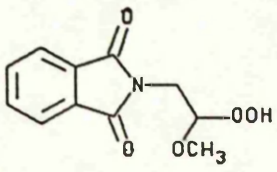


20 Dimethylsulphate

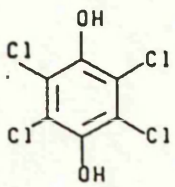
21 Diethylpyrocarbonate



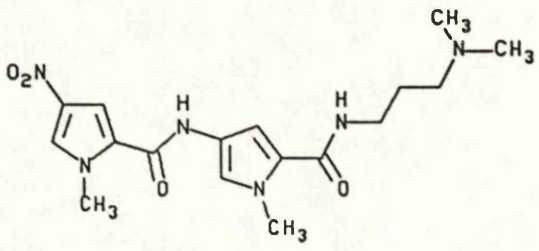
22 9-[6-(2-diazocyclopentadienylcarbonyloxy) hexylamino] acridine



23 N-(2-hydroperoxide-2-methoxyethyl)phthalimide



24 Tetrachlorohydroquinone



25 N-methylpyrrole carboxamide derivative

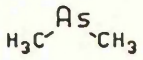
(j) dimethylarsine (26, Yamanaka *et al.*, 1990),

(k) ellipticine derivatives (27, Perroualt *et al.*, 1990),

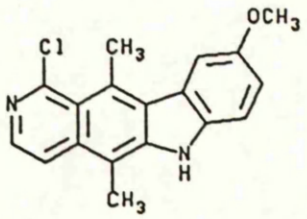
(l) neocarzinostatin (28, Hatayana *et al.*, 1978; Charnes and Goldberg, 1984).

The above reagents provide examples of various important mechanisms of producing DNA cleavage/modification. Although detailed discussions of the individual mechanisms are not given here, brief outlines of the general mechanisms involved are given below for illustration:

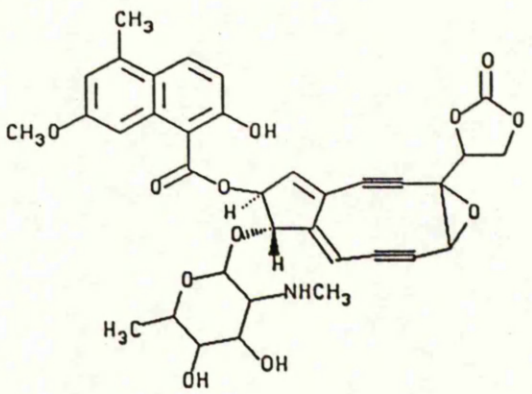
Reagents such as (a)-(d) all produce covalent adducts (usually alkylation of DNA bases) on reaction with DNA, although DNA strand cleavage is seen for (b)-(d) only after further chemical treatments designed to remove the modified base. Reagent (a) can produce strand scissions without further treatment, possibly by reaction with the phosphodiester backbone. Reagents (b) and (c) are extensively used as probes of DNA structure and their reaction with DNA is discussed in detail in section 1.3.4. Reagents (e)-(i) are all postulated to cleave DNA *via* production of hydroxyl radicals (the ultimate DNA cleaving species generated by many metal ions or metal ion complexes (section 1.3.2)) although the mechanisms of hydroxyl radical production vary greatly. For example, reagent (e) degrades to produce hydroxyl radical in a photo-induced intramolecular reaction; reagent (h), a representative of the anthracyclines (a major class of anticancer drugs ; reviewed by Brown, 1983), generates hydroxyl radical in a reaction requiring added reducing reagents and dissolved oxygen; reagent (i) produces hydroxyl radicals *via* reaction of the radiation with water (reviewed by Hutchinson, 1985). Reagent (j) is postulated to react with DNA *via* a peroxyl radical. Reagents (d), (e), (g) and (k) are all photoactive,




26 Dimethylarsine




27 1-chloro-5,11-dimethyl-9-methoxyellipticine




28 Neocarzinostatin chromophore

producing DNA cleavage either directly upon irradiation (reagents (e), (g) and (k)) or after alkali-treatment of the DNA-reagent photoadducts (reagent (d)). Reagent (l), the non-protein, DNA-cleaving component of neocarzinostatin, a polypeptide antitumour antibiotic, causes single-strand scissions in an oxygen-dependent reaction involving $O_2^{-}$, $H_2O_2$ and $^{\cdot}OH$ species.

### 1.3.4 CHEMICAL PROBES OF DNA STRUCTURE

Although the following compounds do not cleave DNA directly, their reactions with the bases or the phosphate backbone of DNA have been used to yield information on the various conformations of DNA and the features of DNA-protein or DNA-ligand interfaces. The information produced by the use of these so-called "probes" and the information gained from the use of DNA-cleaving reagents (see sections 1.3.1 and 1.3.2) is complementary and has led to the more complete characterisation of the molecular contacts occurring in a wide range of DNA-complexes (see Nielsen, 1990 for a review). The probes have been used generally in protection experiments (where modification of DNA by probe is blocked by protein or small ligand bound to DNA), interference experiments (where binding of protein or small ligand is inhibited by the presence of probe-modified DNA at specific binding site residues) or experiments using DNA of different conformations. The majority of DNA probes modify the bases of DNA and such compounds are discussed below along with an example of a probe that reacts with the phosphodiester backbone.

The following reagents modify DNA bases in a manner which labilises the sugar-base glycosidic bond, to gives cleavage on subsequent treatment with piperidine. This ultimately leads to DNA strand breakage.

Dimethylsulphate (DMS, 20) is an alkylating agent which selectively methylates N7 of guanine (figure 13) and N3 of adenine in B-DNA (dsDNA). In contrast, DMS can also methylate N3 of cytosine and N1 of adenine in ssDNA as these sites are no longer involved in base pair hydrogen bonds in ssDNA. Many protein/DNA and ligand/DNA complexes have been analysed by using DMS to probe for the accessibility of guanine N7, e.g. RNA polymerase-DNA interactions (Carpousis and Gralla, 1985; Straney and Crothers, 1987) and antibiotic-DNA interactions (Fox and Howarth, 1985; McLean & Waring, 1988). Guanine N7 methylation by DMS has also been used in interference experiments, e.g. RNA polymerase-DNA contacts (Siebenlist & Gilbert, 1980).

Diethylpyrocarbonate (DEPC, 21) ethoxycarbonylates N7 of purines (figure 13) in Z-DNA and ssDNA efficiently, but those of B-DNA with less efficiency due to the greater relative exposure of the purine N7 moiety in the former DNA conformations.

Osmium tetroxide ($OsO_4$) and potassium permanganate ($KMnO_4$) both oxidise the thymine (and, to a lesser extent, cytosine) alkene double bond to 5,6-cis-diols (figure 13). Oxidation takes place from the face of the aromatic ring, and therefore the thymines in B-DNA are relatively resistant to such oxidation due to the stacking of the bases. Thymines present in DNA conformations where exposure of the thymine 5,6 double bond is increased are consequently more susceptible to oxidation. (Potassium permanganate and $OsO_4$ probe identical features of DNA and therefore for safety reasons $KMnO_4$ is the reagent of choice)

Bromo- and chloro-acetaldehyde (29) both react with adenines and cytosines at the N1-N6 and N3-N4 moieties, respectively, (if these positions are not involved in hydrogen bonding) to form cyclic adducts.

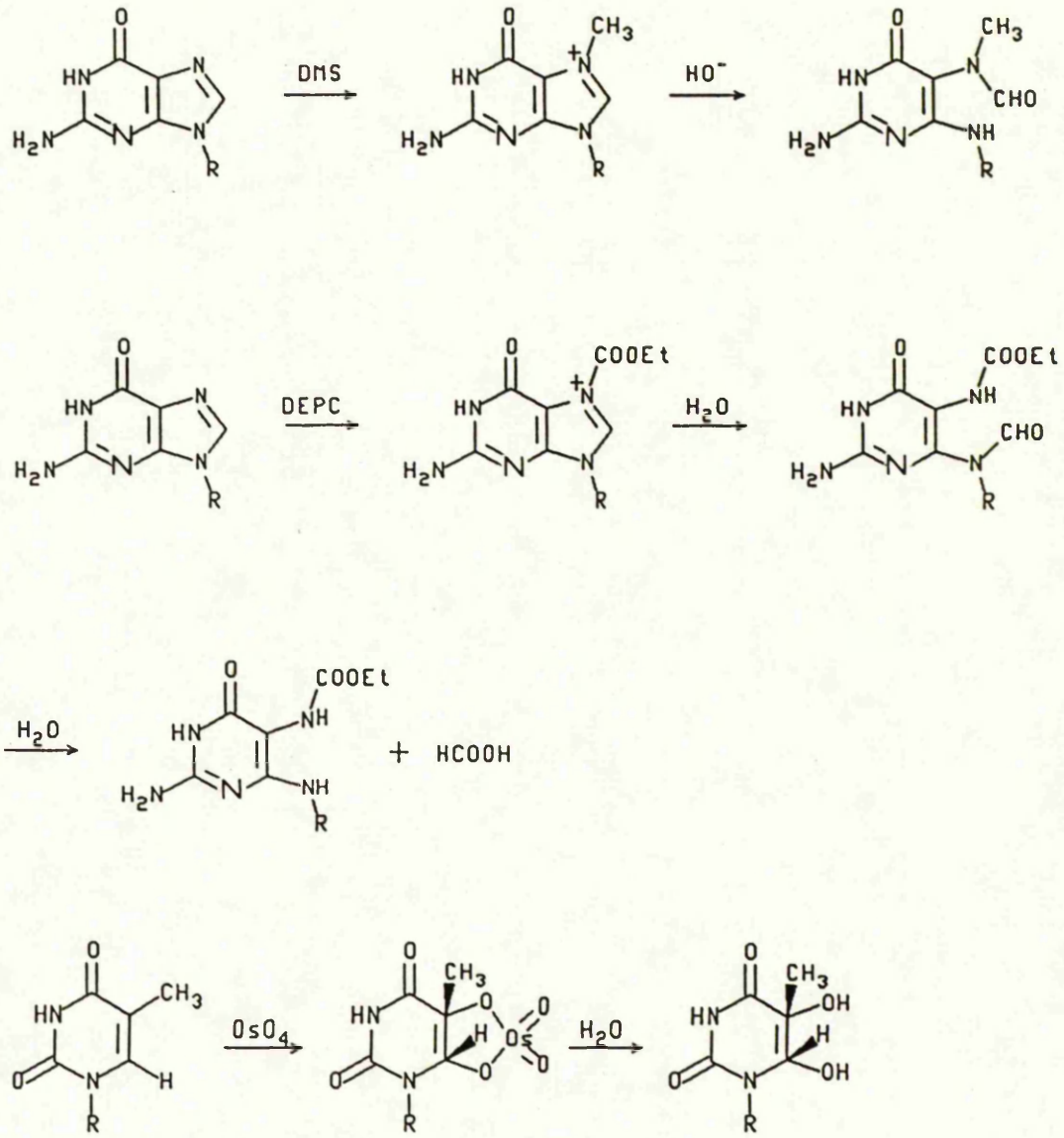
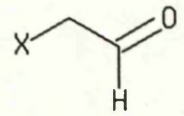
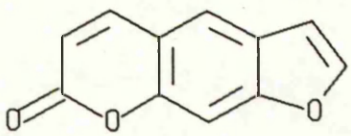


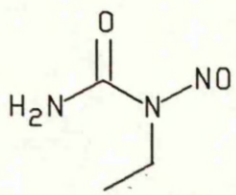

Figure 13 Reactions of DNA bases with various probes. In each case the modified base species is detected by treatment with piperidine which displaces the base and ultimately leads to strand scission proximal to the damaged base site. R represents connection to C1' of deoxyribose.




29 Haloacetaldehyde (X=Br or Cl)




30 Psoralen




31 N-ethyl-N-nitrosourea

As each of the above probes react markedly differently with the bases in B-DNA and non-B-DNA structures, they have all found use as probes of structural irregularities or transitions in B-DNA. Probing of such structures is usually carried out using more than one probe in order to characterise the system fully. The following are examples of systems probed by such chemicals:

- Single-stranded DNA in hairpin loops (Furlong & Lilley, 1986; Furlong *et al*, 1989)

- B-DNA/Z-DNA junctions/transitions (Johnston & Rich, 1985; Kohwi-Shigematsu *et al*, 1987)

- Intercalation (especially bis-intercalation) - induced DNA structure changes (McLean & Waring, 1988)

- The role of DNA sequence in the formation of Z-DNA versus cruciforms (McLean & Wells, 1988)

Another probe that reacts with the DNA bases is psoralen (30), and its derivatives, which photo-reacts with thymine 5,6 double bonds in a cyclo-addition reaction using either of the two reactive double bonds of the psoralen structure. Since the photobinding of psoralens is prevented by sequence-specific binding of proteins to DNA, psoralens have been used for photofootprinting studies (Zhen *et al.*, 1988). Interstrand cross-links may be formed by reaction of one psoralen molecule with two thymines at sites

containing adjacent thymines on opposite strands (e.g. TA sites). A probe that reacts with DNA phosphates is N-ethyl-N-nitrosourea (ENU, 31) which forms two stereoisomeric phosphotriesters, with the ethyl groups in either the minor or the major grooves. Direct footprinting (protection) experiments are not possible, however, due to the conditions required for DNA modification (50°C, 50% ethanol). However, this ethylation has been used to detect phosphates essential for protein binding by the interference method, e.g. RNA polymerase/promoter contacts (Siebenlist & Gilbert, 1980). The steric hindrance by the ethyl group, the removal of the negative charge of the original phosphate group and changes in DNA conformation induced by phosphate ethylation are all factors contributing to the interference of protein binding.

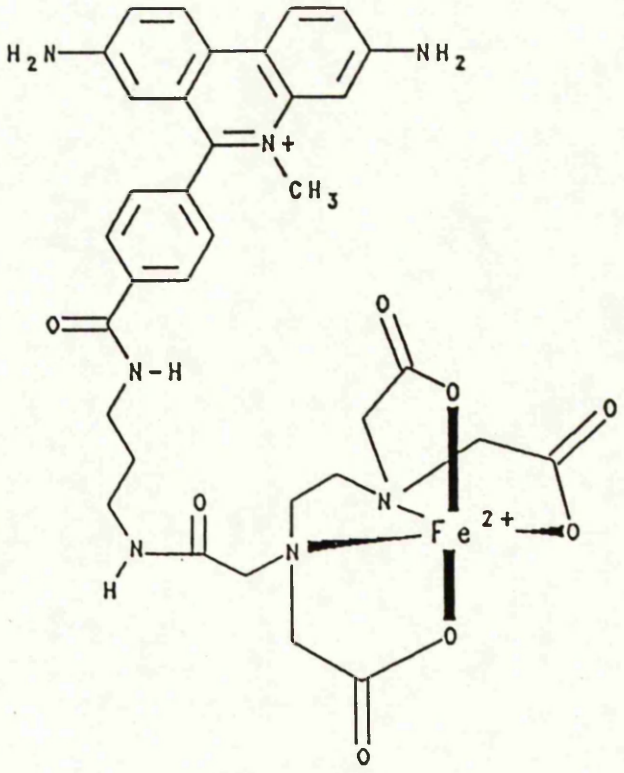
## 1.4 SYNTHETIC COMBINATIONS OF DNA BINDING AND CLEAVING MOIETIES

BLM (section 1.3.2.4.1) is the naturally occurring prototype of a DNA binding/cleaving reagent. It has, however, limited specificity and does not recognise long sequences of DNA. The advent of novel chemical DNA-cleaving systems, e.g. $(OP)_2Cu$ and EDTA:Fe(II) (sections 1.3.2.4.2 and 1.3.2.3, respectively), spurred the design and synthesis of sequence-specific DNA-binding and cleaving compounds by chemically linking existing DNA-binding molecules to DNA-cleaving molecules thus producing hybrid molecules capable of cleaving at any length (in principle) of specific sequence. Furthermore, the attachment of chemical cleaving systems to relatively sequence-nonspecific DNA-binding moieties (such as intercalators), to produce "sequence-neutral"

DNA-cleaving molecules, has led to the assignment of more accurate "footprints" of DNA-binding ligands on DNA compared to the large stretches of protections observed with DNAseI. Some combinations of DNA-binders with chemical cleavers are now described.

### 1.4.1 CLEAVAGE SYSTEMS LINKED TO INTERCALATOR AND MINOR GROOVE BINDING DRUGS

In one of the earliest studies in the field, the Fe(II):EDTA DNA-cleaving system (section 1.3.2.3) was linked to an intercalating molecule, methidium, to form the hybrid molecule MPE:Fe(II) (32). This was shown to cause DNA cleavage in the presence of reducing agents at concentrations some two orders of magnitude lower than required for Fe(II):EDTA alone (Hertzberg and Dervan, 1984). Thus, the reaction of Fe(II):EDTA with DNA was made more efficient by intimately directing the cleavage chemistry to the DNA. It was further shown that MPE:Fe(II), produced roughly "sequence-neutral" cleavage of dsDNA (e.g. Van Dyke *et al.*, 1982 and Van Dyke and Dervan, 1982) and, therefore, was an ideal reagent for the footprinting of small molecules on DNA. The minimum binding sites required for several compounds, including minor groove-binding molecules, were defined by this method, e.g. distamycin, netropsin and actinomycin (Van Dyke *et al.*, 1982 and Van Dyke and Dervan, 1982), chromomycin, mithramycin and olivomycin (Van Dyke and Dervan, 1983a), Hoechst 33258 (Harshman and Dervan, 1985) and a bis-N-methylimidazole lexitropsin (Dabrowiak *et al.*, 1989b). Comparisons of DNAse I and MPE:Fe(II) footprinting results showed that smaller, more accurate footprints were reported by the latter (Van Dyke and Dervan, 1983b). The resolution of footprinting had




32 Methidium-propyl-EDTA:Fe(II) complex
( MPE:Fe(II) )

thus been improved considerably relative to that of DNAse I.

The traditional footprinting approach was then reversed by Dervan and co-workers, who attached the EDTA:Fe(II) moiety to minor groove-binding ligands such as those footprinted by MPE:Fe(II) above. The result was minor groove-directed molecules that reported their own binding sites on DNA (so-called affinity cleaving). Cleavage of DNA occurred at positions proximal to the end of the molecule carrying the EDTA:Fe(II) moiety, e.g. distamycin and penta-N-methylcarboxamide derivatives (Schultz *et al.*, 1982; Schultz and Dervan, 1984). By linkage to longer DNA binding moieties (e.g. some which could adopt a helical trajectory similar to that of a groove on DNA) cleavage adjacent to DNA sites of up to 16 base pairs was achieved (Youngquist & Dervan, 1985 and 1987). Attachment of Fe(II):EDTA at both ends of a DNA binder was reported to effect cleavage at either end of the binding site (Youngquist & Dervan, 1985) but only brief, preliminary detail was given. It should be noted that hybrid minor groove-binding DNA-cleaving molecules, such as the above, have not been described for any other DNA-cleaving system to date. It is also worth mentioning studies of a minor groove-binding compound, N-bromoacetyl-distamycin, which alkylates DNA proximal to the binding site of distamycin (Baker & Dervan, 1985). Cleavage of DNA at this site was achieved by piperidine treatment and revealed that alkylation occurred at a single adenine. Thus, by combining a DNA-binding molecule and an alkylating reagent sequence-specific DNA modification at a single base was achieved.

### 1.4.2 CLEAVAGE SYSTEMS LINKED TO OLIGONUCLEOTIDES

Sequence-specificity can be conferred on a cleavage reagent by attaching it to an oligonucleotide of defined sequence, and then annealing the derivatised oligonucleotide to DNA bearing the correct complementary sequence. Addition of the co-reactants in the DNA cleavage reaction then activates cleavage of the target strand at positions proximal to the modified end of the bound, derivatised oligonucleotide (method 1, figure 14). Such an approach was used to produce sequence-specific cleavage of ssDNA by oligo-EDTA:Fe(II) (Chu and Orgel, 1985; Dreyer and Dervan, 1985) and by oligo-(OP)Cu systems (Chen & Sigman, 1986). Although experimental details differ slightly, the former system cleaved the target DNA strand at each of up to 8 nucleotides either side of the modified nucleotide residue carrying Fe(II):EDTA. The latter (Cu) system, however, cleaved over approximately 4 or 5 nucleotides range only. The differences in the specificity of the two systems lies in the chemistry causing the cleavage, *viz.*, Fe(II):EDTA produces a diffusable radical whereas, (OP)Cu does not (see section 1.3). The disadvantage of the systems described above is that ssDNA is required for the oligo to bind to the target DNA and thus the system is incapable of cleaving dsDNA.

This problem has been attacked recently and overcome, to some extent at least, for limited classes of base sequence. The ability of poly(purine).poly(pyrimidine) sequences to form triple helices (Arnott & Selsing, 1974; Lee *et al.*, 1984) was exploited to produce dsDNA scission by oligonucleotide-linked DNA-cleaving systems. Oligopyrimidines can bind into the major groove of DNA at homopurine.homopyrimidine sequences where a

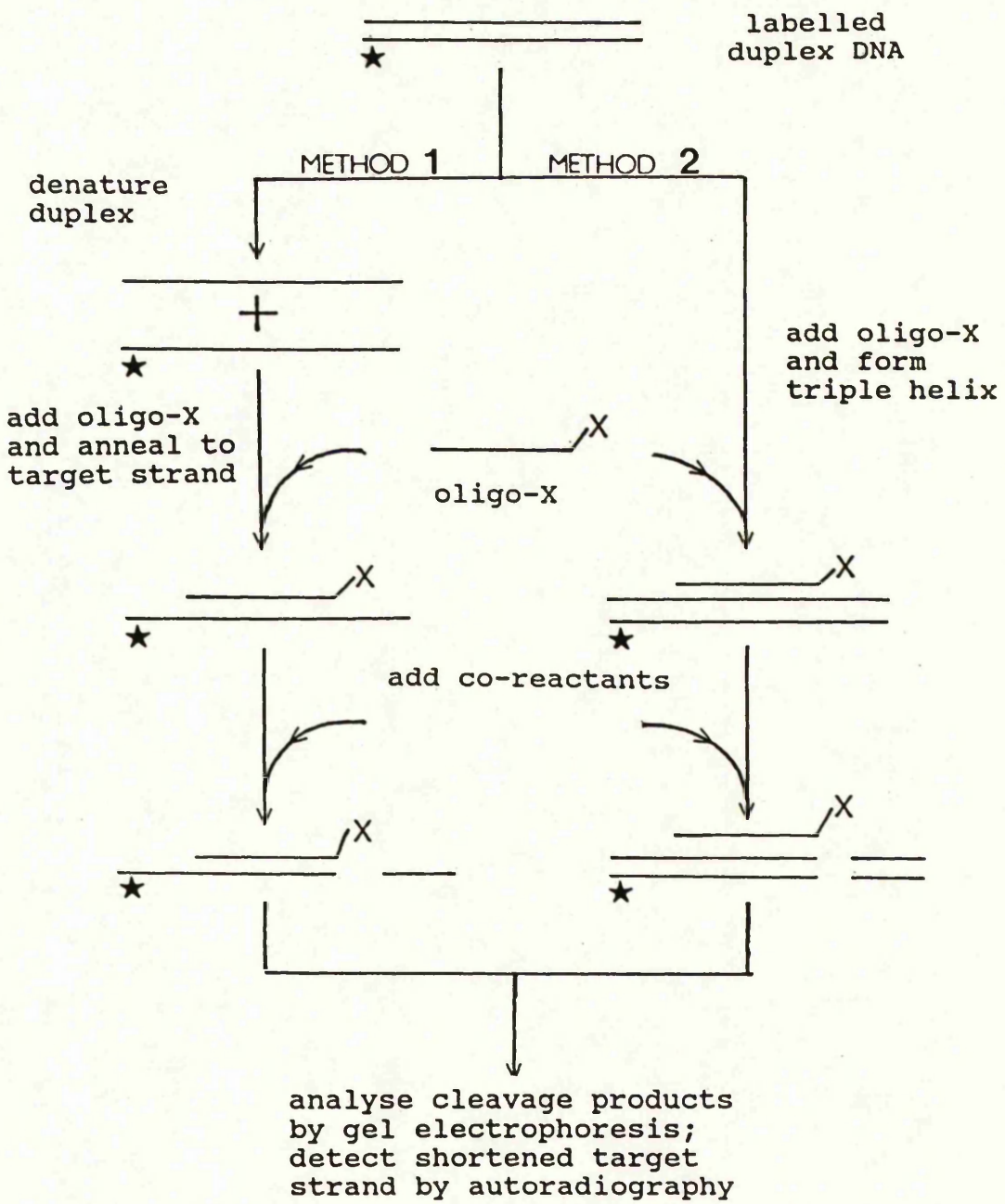



Figure 14. Schematic diagram of methods used in experiments designed to target DNA cleavage to specific DNA sequences via the specificity of oligonucleotide binding. Oligo-X is an oligonucleotide carrying a DNA cleavage system and the asterisk (★) represents the position of radiolabel in the target DNA strand.

thymine of the oligonucleotide forms two Hoogsteen hydrogen bonds with an adenine already engaged in a Watson-Crick A-T base pair (figure 4) and protonated cytosine forms two hydrogen bonds with a guanine involved in a Watson-Crick G-C base pair. Dervan and co-workers have demonstrated site-specific dsDNA cleavage by a 15mer oligo(pyrimidine) modified with an EDTA function at the 5' end (Moser and Dervan, 1987). This bound to target dsDNA of similar sequence *via* Hoogsteen base-pairings to form a triplex. Addition of the co-reactants of the Fe(II):EDTA reaction then caused scission of the duplex DNA at a site proximal to the 5' (modified) end of the oligonucleotide (method 2, figure 14). The cooperative binding of two different oligo(pyrimidine)-EDTA molecules demonstrated cleavage at a DNA sequence of 18 base pairs within the bacteriophage lambda genome (48.5 Kbp; Strobel & Dervan, 1989). Analogously, oligopyrimidine nucleotides modified by attachment of the OP group have also been shown to induce sequence-specific cleavage while part of a triple helix complex (Francois *et al.*, 1988). Cleavage of DNA by irradiation of an 18mer derivatised with the photoreactive reagent ellipticine bound to DNA as part of a triple helix complex has also been observed (Perroualt *et al.*, 1990). Direct cleavage of DNA was observed (not base damage) and was most likely to have occurred *via* direct interaction of the photo-excited state of ellipticine with the DNA.

### 1.4.3 CLEAVAGE SYSTEMS LINKED TO DNA-BINDING PROTEINS

Gene transcription can be modulated *via* sequence-specific DNA-binding proteins which bind to regions of DNA to produce activation or repression of the transcription of specific genes (Adams *et al.*, 1986). These and other

sequence-specific DNA-binding proteins provide opportunities for the construction of artificial sequence-specific DNA-cleaving proteins *via* their modification with existing DNA-cleaving factors. Four such examples have appeared to date and are discussed below.

The Fe(II):EDTA cleavage system was attached to a 52-residue peptide that constitutes the DNA-binding domain of Hin recombinase (Sluka *et al.*, 1987). As a dimer the enzyme binds to a specific 26bp site. The EDTA:Fe(II) derivative of the Hin peptide (amino-terminus modified with EDTA) cleaved DNA specifically at the known Hin binding sites (in the presence of ascorbate and $H_2O_2$) and therefore revealed the location of the amino terminus of the peptide within the DNA-protein complex. The above experiments utilised sequencing gel methods and only offered indirect evidence of ds cuts in the DNA. However, dsDNA scission was also proven directly at lower resolution using agarose gel methods.

Two different DNA-binding proteins/peptides have been modified with the chelator 1,10-phenanthroline, namely, the *E. coli* trp repressor (Chen and Sigman, 1987) and the *E. coli* catabolite gene activator protein (CAP; Ebright *et al.*, 1990). In the first example, four 1,10-phenanthroline moieties were added per subunit of the trp repressor. Although the protein has four lysyl residues (at which modification with 1,10 phenanthroline was to occur) it was possible that the N-terminus of the protein was among those residues modified. On binding this modified trp repressor to its target DNA sequence, however, the addition of cupric ion and thiol (as co-reactants, see section 1.3.2.4.2) effected site-specific cleavage within the recognition sequence. The second example is more sophisticated in that it involved incorporation of 1,10 phenanthroline at a single site within the CAP molecule. The CAP molecule

possesses a sole solvent-accessible cysteine residue, occurring within a helix-turn-helix motif, which is located close to the DNA duplex on CAP-DNA complex formation. Modification of this cysteine residue provided site-specific DNA cleavage within the 22bp, two-fold-symmetric DNA-recognition site of CAP. This was demonstrated by cleavage of a 40bp radiolabelled DNA fragment and a 7164bp substrate (to show ds cleavage directly). From these successful experiments, Ebright *et al.* proposed that a general method for production of site-specific DNA-cleaving proteins is *via* site-directed mutagenesis to introduce similar, unique, solvent-accessible cysteine residues into helix-turn-helix motifs of other sequence-specific DNA-binding proteins and subsequent modification of these residues with 1,10-phenanthroline (Ebright *et al*, 1990) or other cleaving systems.

Attachment of a diglycyl histidine peptide to the DNA-binding domain of Hin recombinase has also been reported to produce a site-specific DNA-cleaving protein (Mack *et al.*, 1988). Incubation of this modified protein with its DNA substrate in the presence of Cu(II), hydrogen peroxide and sodium ascorbate (see section 1.3.2.3 for Cu(II):diglycylhistidine nuclease activity) produced specific cleavage of DNA at four sites identified as Hin binding sites.

In conclusion, it should be noted that, while the above systems all catalyse sequence-specific degradation of DNA, the "accuracy" of targetting (spread of cleaved DNA positions around the major cleavage site) of cleavage at the target site is greatest for the Cu(II):diglycylhistidine system (2bp) compared to CAP(OP)Cu (4bp) and Hin-EDTA:Fe(II) (5-10bp). Thus, targetting the cleavage to a specific DNA sequence *via* the site-specific binding of a DNA-binding protein produces site-specific DNA scission but the accuracy of the

cleavage is still governed by the chemistry of the attached cleaving function. To date this cleavage chemistry has been free radical based.

## 1.5 SUMMARY OF CLEAVAGE SYSTEMS

The methods of cleaving DNA described in the previous sections may be grouped into two broad types, namely, (i) free radical mediated and (ii) non-free radical mediated cleavage. Virtually all of the presently used synthetic DNA-cleaving systems are of the former type while natural (enzymatic) systems are often of the latter type. Both systems, however, have advantages and disadvantages according to their desired use in DNA-cleaving applications.

The most obvious differences between enzymic and non-enzymic systems is that the cost of the enzymes is greater than that of the chemicals and that, in general, enzymes function only under specific and mild buffer conditions (metal ion requirements and pH) whereas chemical systems may function under a variety of buffer and pH conditions (e.g. Fe(II):EDTA; Shafer *et al.*, 1989). The cleavage caused by many chemical systems has obvious advantages over enzymatic DNA cleavage in the technique of footprinting of ligands bound to DNA, for example,

- the use of photoactivated cleavage reagents should be invaluable for footprinting short-lived or low affinity DNA-ligand complexes (e.g. using uranyl; section 1.3.2.4.4),

- the smaller "size" of the chemical nucleases compared to enzymes produces smaller, more accurate footprints than those obtained with DNAseI due to the

penetration of free radicals to the DNA at all but the areas tightly associated with the ligand,

- the indiscriminate (sometimes sequence-neutral) chemical cleavage produces clearer, more easily determined, footprints since cleavage is effected at almost every nucleotide equally whereas enzymic cleavage, for example by DNAseI, may have non-cleaved sequences even in the control samples (with no ligand present),

- the use of "nucleases" that do not bind to DNA causes less disturbance to DNA-ligand complexes during the footprinting reaction and therefore footprints are determined under conditions closer to ideal.

The free radical-mediated and even the 'crypto-radical'-mediated cleaving systems, by virtue of the reactivity of the radicals generated, cause many modifications to the DNA at the site of cleavage, i.e. not just phosphodiester bond cleavage but base damage and sugar damage also. This would present problems if the cleaved DNA were to be used further, e.g. for processing with DNA-modifying enzymes (radiolabelling, ligation, etc.). Thus, enzymatic cleavage, which produces distinct DNA termini at the site of cleavage, has a distinct advantage in this situation.

Both free radical and enzymatic systems produce many cleavages per given cleaving molecule; free radical systems rely on redox cycling to regenerate the reagents required for cleavage (generally oxygen radicals) and enzymes require hydrolysis of enzyme-substrate complex intermediates to regenerate catalytically active enzyme. An important difference in the two systems is that, whereas enzymes cleave only once at a particular site on DNA

before diffusing to another site, the chemical systems which have been directed to a particular site on DNA (for example by means of a protein or a drug (section 1.4)), will carry out many radical mediated DNA-damaging events at or near the same local DNA site. The spread of these damaged sites around a particular target sequence depends upon the particular chemical nuclease used and thus on the type and origin of the free radical generated by the nuclease (section 1.4). Design and examination of new cleavage reagents is therefore required to produce systems yielding more "accurate" cleavage (i.e. cleavage restricted to a single nucleotide position).

## 1.6 AIMS OF THE THESIS

The aim of this study of the Cu(II):thiol DNA cleavage system was examination of DNA cleavage at the nucleotide level. Detailed goals were as follows:

(1) to determine the sequence-specificity, or otherwise, of the cleavage reaction, by cleavage of DNAs of differing sequence.

(2) to determine the effect of varying thiol structure, stereochemistry and charge distribution on the cleavage patterns.

(3) to determine the effect of replacing thiol by another reductant, or by an oxidant, on the cleavage patterns.

(4) to determine the chemical nature of the DNA products generated by cleavage using this system.

(5) to compare these results with characteristics of other DNA-cleaving systems to determine any similarities or differences and to determine the cause of Cu(II):thiol DNA-cleaving ability.

(6) to attempt to direct the cleavage of Cu(II):thiol to DNA via the use of thiols linked to DNA-binding molecules.

(7) to determine the ability of the Cu(II):thiol system to be used as a footprinting reagent.

# CHAPTER 2 : MATERIALS AND METHODS

## 2.1 MATERIALS

### 2.1.1 CHEMICALS

Reagents were of the highest quality available and purchased from the suppliers indicated below:

BDH Ltd., Liverpool: Acrylamide, agarose (agarose "10"), ammonium persulphate, boric acid, butanol, chloroform, dihydrogen sodium phosphate, dimethyldichlorosilane (2% solution in 1,1,1-trichloroethane), disodium hydrogen phosphate, ethylenediaminetetraacetic acid (disodium salt), formamide, glacial acetic acid, glucose, glycerol, magnesium chloride, N,N'-methylenebisacrylamide, N,N,N',N'-tetramethylethylenediamine, phenol, propan-2-ol, sodium acetate, sodium azide, sodium chloride, sodium hydroxide, thiourea, tris(hydroxymethylaminomethane), urea.

Sigma Chemical Company Ltd., Poole: Ampicillin, calf-thymus DNA (type I-highly polymerised), D-cysteine hydrochloride monohydrate, 1,2-dimercaptopropanol, 5,5'-dithio-bis(2-nitrobenzoic acid), dithiothreitol, glutathione (free acid), manganese chloride, 2-mercaptoethanol, 3-mercaptopropionic acid, $\gamma$-methacryloxypropyltrimethoxysilane, 1,3-propanedithiol, sodium cacodylate and xylene cyanol.

Aldrich Chemical Company Ltd., Poole: Copper tetrasulfophthalocyanine, copper sulphate pentahydrate, dimethylsulphate, piperidine.

FSA Laboratory Supplies, Loughborough: Ammonium ferrous sulphate, bromophenol blue, and caesium chloride.

Fluka Chemicals Ltd., Glossop: L-cysteine hydrochloride, cystamine dihydrochloride, ethidium bromide and Hoechst 33258 (bisbenzimidazole

trihydrochloride).

Oxoid, Basingstoke: Bactotryptone and yeast extract.

### 2.1.2 RADIOCHEMICALS

5'[$\alpha$-$^{32}$P] deoxyadenosinetriphosphate (aqueous; specific activity >6000 Ci/mmol) and 5'-[$\gamma$-$^{32}$P] adenosinetriphosphate (50% aqueous ethanol; specific activity >6000 Ci/mmol) were both purchased from Amersham International PLC, Buckinghamshire.

### 2.1.3 RESTRICTION ENDONUCLEASES, DNA AND DNA MODIFYING ENZYMES

Calf-intestinal alkaline phosphatase, deoxyribonuclease I (bovine pancreatic), lambda DNA, RsaI, T4 DNA ligase and T4 polynucleotide kinase were purchased from Boehringer Mannheim UK, Lewes. AvaI, DNA polymerase I large fragment (Klenow fragment, labelling grade) and EcoRI were from GIBCO-BRL, Paisley.

### 2.1.4 BACTERIA/PLASMID STOCKS

The following transformed bacterial stocks were received as gifts from academic sources:

Plasmid pSP64 (Maniatis *et al.*, 1982) in *E. coli* HW87 ((araD139-Leu), lacX74, GalH, galK, hsrK, rpsL, str, recA) from Dr. J. Rosamund, Department of Molecular Biology and Biochemistry, Manchester University, England.

Plasmid pBR322 (Maniatis *et al.*, 1982) in *E. coli* EQ82 (met$^-$, hsdR, SupE, supF) from Dr. J. Andrews, Pharmacy Department, University of Manchester, England.

Plasmid pKMΔ98 (Drew and Travers, 1985) in *E. coli* CB1 (thr$^-$, leuB6, lacY1, tonA21, supE44, recA$^-$, galk$^-$, Tet$^R$) from Dr. K.R. Fox, Department of Physiology and Pharmacology, University of Southampton, England.

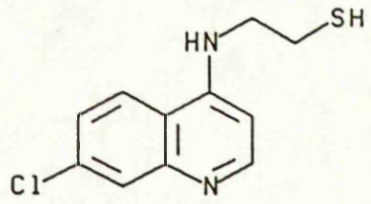
Plasmids pRW1011 and pRW1015 were from Dr. M. McClean, ICI Diagnostics, U.K., and were transformed into *E. coli* TG1 (K12,Δ(lac-pro), supE, thi, proA$^+$B$^+$, hsdD5/F' traD36, laclq, lacZ, ΔM15). These plasmids were derived from plasmid pRW790 (Wells, 1988) which was produced from pBR322 by deletion of base pairs 45-2434 and insertion of a 50bp EcoRI/HindIII multiple cloning site fragment, derived from M13mp9, between its EcoRI and HindIII sites. Plasmids pRW1011 and pRW1015 were produced by ligation of oligonucleotide sequences (inserts) into the BamHI site - note the resulting ligated molecules possess one or two incomplete BamHI sites as a result (McLean and Wells, 1988) - the inserts were $(TG)_{12}$ and $(TG)_6(AC)_6$ for pRW1011 and pRW1015, respectively (for full sequence around these inserts see figures 19 and 20).

### 2.1.5 MISCELLANEOUS

Fuji RX X-ray film and SMIT RAPID UL intensifying screens were from Everything X-ray Ltd, Watford. Sephadex G-50 (medium) was from Pharmacia-LKB, Milton Keynes. Duolite MB6113 mixed bed ion-exchange resin was from BDH.

## 2.1.6 INTERCALATOR-THIOL BIFUNCTIONAL MOLECULE

An intercalator modified with a thiol group, N-(7-chloro-4-quinolinyl)cysteamine (ISH, 33), synthesised and purified (as the hydrochloride) by the procedures of Demonchaux and co-workers (Demonchaux *et al.*, 1989), was a gift from Dr. S. Cameron (AFRC, Harpenden, Herts) . This compound when assayed by method 1 of the thiol assay (section 2.2.8.1) was found to possess 90 ± 5% free thiol calculated by weight.

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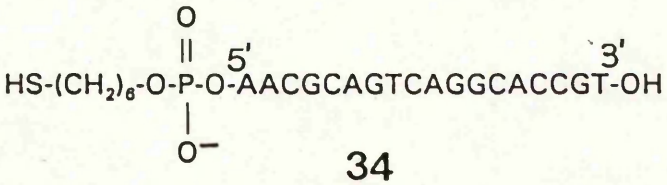
## 2.1.7 5' MODIFIED OLIGONUCLEOTIDE (OLIGONUCLEOTIDE-THIOL)

An 18mer oligonucleotide bearing a thiol moiety attached to the 5' end (oligo-SH, 34) was a kind gift from Dr. D. Tidd (Liverpool University) and was stored lyophilised at -20°C.

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Samples were redissolved (in deaerated water) and assayed for thiol titre by method 2 of the thiol assay (section 2.2.8.1). Reaction with DTNB in the assay was complete only after prolonged incubation (45 min). Thiol titres of 90 ± 8% of expected values calculated on an $A_{260}$ unit basis were obtained.

## 2.1.8 INSTRUMENTATION

### Spectrophotometers

Spectrophotometric determinations were carried out using a Perkin-Elmer Lambda 3B spectrophotometer with a HAAKE GH/D8 circulating thermostat and data output to a Perkin-Elmer R100A chart recorder. Thermostat temperature control was ±0.5°C. DNA melting-point determinations were carried out using a Pye-Unicam PU8800 spectrophotometer fitted with an electrically heated cuvette block, the temperature of which was controlled by a Pye-Unicam temperature controller. Absorbance and temperature readings were recorded simultaneously on a Phillips PM8271 X-Yt plotter.

### pH Meter

Determinations of pH were performed using a Corning pH meter 240 and BDH Colourkey buffer standards as reference solutions. The electrode was stored in saturated KCl when not in use.

### Water Purification

All water used was distilled and purified by ion-exchange and charcoal filtering using a MilliQ system (Millipore Ltd).

## 2.2 METHODS

### 2.2.1 EXPERIMENTAL PRECAUTIONS

Many of the chemicals used in the following methods are either toxic, harmful or carcinogenic (or all of these). Gloves (latex type) should be worn at all times when handling these materials in addition to laboratory coat and safety spectacles. **The ethidium bromide and dimethylsulphate used are carcinogens.** Care must be exercised in handling ethidium bromide during the plasmid purification method (section 2.2.3) and during the staining and visualisation of nucleic acids in agarose gels (section 2.2.4.1). Waste ethidium bromide must be disposed of after degradation of the carcinogen (see Maniatis *et al.*, 1982) and should therefore be collected and decontaminated in bulk before discarding down sinks. Solid waste should be incinerated. DMS is used in lower quantities for G-tracking (section 2.2.6.4) and must only be used in a fume hood: pipette tips, tubes or solutions containing it must be immersed (for at least 1hr) in 5M NaOH to degrade the carcinogen. The handling of *E. coli* strains was carried out under conditions of good microbiological practice with containment category zero, decontamination of broths with "chloros" bleach (sodium hypochlorite) and of lab-ware by autoclaving. Manipulation of radiochemicals was carried out according to radiological protection regulations at a designated $^{32}P$ bench space close to a designated sink, with samples stored in a designated fridge/freezer. Any necessary use of radio-labelled DNA outside this area was accompanied by clear notice of the fact and checks for contamination both before and after the work.

## 2.2.2. BACTERIAL STOCKS AND CULTURES

Solutions:

Ampicillin - 25mg/ml aqueous; filter sterilised and stored frozen in 1ml aliquots.

Luria broth - equal volumes of potassium phosphate buffer (0.1M, pH 7.0) and a solution containing NaCl (2%), yeast extract (1%), bactotryptone (2%) and glucose (0.2%). This solution was then autoclaved (section 2.2.8.9).

Glycerol - 20% aqueous; autoclaved in bijou bottles in 4ml aliquots.

Stocks of *E.coli* containing plasmids were maintained as frozen (-80°C) stocks in glycerol solution. Overnight cultures of *E.coli* were set up by adding a loopful of the desired glycerol stock to 4ml of Luria broth containing ampicillin (40$\mu$g/ml), and the sample incubated with shaking at 37°C. For large-scale cultures (section 2.2.3.1) the overnight culture was added to 400 or 800ml of pre-warmed Luria broth containing ampicillin (40$\mu$g/ml) and the cultures incubated overnight (approximately 15hr) before harvesting. Glycerol stocks of bacteria will keep for long periods but after each overnight culture or large-scale culture, fresh stocks were made by adding 1ml of overnight culture to 4mls of glycerol solution and storing the 'glycerol stock' at -80°C.

## 2.2.3 ISOLATION, PURIFICATION AND STORAGE OF PLASMID DNA

### 2.2.3.1 Plasmid DNA Maxi-Prep Method

Solutions:

(a) :Tris-HCl (25mM), EDTA (10mM), glucose (50mM), pH 8.0

(b) :NaOH (0.2M), SDS (1% w/v) prepared immediately before use.

(c) :NaOAc (3M, pH 4.8)

TE Buffer :Tris-HCl (10mM), EDTA (0.1mM), pH 8.0.

An overnight culture (section 2.2.2) of an *E.coli* strain containing the appropriate plasmid with a gene for ampicillin resistance was used to inoculate Luria broth (500mls) containing ampicillin (40$\mu$g/ml). The broth was incubated with shaking at 37°C overnight and the cells harvested by centrifugation (3,000 xg, 10min). The cells were resuspended in solution (a) (18mls), lysozyme (2mls of 10mg/ml in solution (a)) was added and the suspension incubated for 10min at room temperature. The cellular DNA and protein were then denatured by adding solution (b) (40mls) and incubating for 15min on ice. The suspension was neutralised by addition of precooled solution (c) (20ml) and further incubation for 15min on ice. Sterile distilled water (5ml) was added and the sample centrifuged at 4°C (10,000 xg, 10 min). The supernatant (containing the plasmid DNA) was filtered off from the solution using a plastic tea-strainer to remove floating debris and the precipitate (containing chromosomal DNA, RNA and protein) was discarded. Plasmid DNA was precipitated from the supernatant by the addition of isopropanol (0.6 volumes), and centrifugation at 4°C (10,000 xg, 10 min). The supernatant was discarded

and the DNA pellet desiccated under vacuum for 15 min. The pellet was resuspended in TE buffer (11.3mls) to which was added caesium chloride (12.0g) and ethidium bromide (0.7ml, 10mg/ml). This solution was clarified by centrifugation (10,000 xg, 10min), pipetted into ultracentrifuge tubes and ultracentrifuged (54,000 rpm, > 20 hours). The supercoiled plasmid DNA-containing band (lower band) was removed from the caesium chloride gradient using a 1ml sterile disposable syringe fitted with a wide-gauge sterile needle (19 gauge) - (Maniatis *et al.*, 1982)

The plasmid DNA solution was placed in sterile 1.5ml Eppendorf tubes and TE-saturated butanol added (0.5-1.0 volumes). The samples were thoroughly mixed by manual inversion and spun in a bench centrifuge (13,400 xg, 1 min). The upper (butanol) layer, containing ethidium bromide, was removed and the procedure repeated until the pink colour was removed from both lower (aqueous) and upper (butanol) phases. The aqueous plasmid sample was then placed in dialysis tubing (section 2.2.8.6) and extensively dialysed against TE buffer (4 litres) at 4°C with at least two buffer changes over 2-3 days. The dialysed sample was then examined spectrophotometrically to determine the concentration and purity of the DNA (section 2.2.3.2).

### 2.2.3.2 Determination of DNA purity and concentration

Solutions of DNA (i.e. plasmid DNA and calf thymus DNA) were assessed spectrophotometrically to determine both the concentration and the purity of the DNA. Absorbance readings were taken at 260 and 280nm of the DNA solution in quartz cuvettes using the buffer alone as a blank.

**Determination of DNA concentration**

The $A_{260}$ value was used to calculate the concentration of the DNA in base pairs (bp) using the extinction coefficient, $\in_{260}$ (DNA bp) = 13,100 $M^{-1}cm^{-1}$ (Pasternack *et al.*, 1983). The amount of DNA was converted into $\mu$g by using the mean value for the molecular weight of one DNA base pair as 650, e.g. 1ml of an $A_{260}$ = 1.0 DNA solution in a 1cm pathlength cuvette. Calculations are shown below:

Concentration of DNA (bp) = 1 / 13,100

= $76.34 \times 10^{-6}$M

Amount of DNA in 1ml = $76.34 \times 10^{-6} \times 1/1000 \times 650$g

= $50 \times 10^{-6}$g

= 50$\mu$g

**DNA purity assessment**

DNA solutions of an $A_{260}/A_{280}$ ratio of 1.8-1.9 were considered suitable for use (Berger, 1987). Abnormally high ratio values (due to RNA contamination) were not found. Abnormally low $A_{260}/A_{280}$ values (protein contamination) were also not found. The general absence of abnormal ratios indicated that no significant amount of protein or RNA was present in the DNA preparations. By the above criteria the DNA was considered to be free of both RNA and protein.

The identity of the plasmid DNA was also checked by digestion with restriction enzymes (section 2.2.5.1) and subsequent agarose gel

electrophoresis (section 2.2.4.1) to check the size and number of fragments obtained with reference to an authentic plasmid sample.

### 2.2.3.3 Phenol Extraction of DNA

Protein was removed from DNA solutions (e.g. after a restriction digest or other enzymatic procedure) by the following method:

A mixture of buffer-saturated phenol (see section 2.2.8.5), chloroform and butan-2-ol (25:24:1) was prepared and clarified by bench centrifugation (13,400 xg, 2min) - the lower phenolic phase was used. To a given volume of DNA solution was added an equal volume of phenol/chloroform/butanol. The phases were mixed by inversion and then separated by centrifugation (13,400 xg, 2 min). Denatured protein appeared at the interface of the two phases and 90% of the upper, aqueous phase was removed to a clean tube without disturbing the interface. The extraction was repeated on the retrieved aqueous phase. The sample was then mixed with an equal volume of chloroform and centrifuged as above, the resulting aqueous layer removed to a clean tube and the DNA ethanol precipitated (section 2.2.3.6) before further use.

### 2.2.3.4 Removal of RNA from DNA solution

When necessary, RNA contaminating a DNA solution (e.g. from a plasmid preparation) was removed by enzymatic digestion using bovine pancreatic RNAse A. RNAse A (5mg/ml, in 10mM tris-HCl, pH 7.5, 15mM NaCl) was rendered DNAse-free by boiling at 100°C for 15 min. After cooling slowly to room temperature the solution was separated into small aliquots and

stored at -20°C. The contaminated DNA solution was incubated with 1/50$^{th}$ its volume of RNAse A solution for 1 hr at room temperature. The enzyme was then removed by phenol extraction and the aqueous phase ethanol precipitated (section 2.2.3.6) prior to use.

2.2.3.5 Spun Column Chromatography

The following procedure was used to separate DNA from lower molecular weight substances (e.g. radiolabelled nucleotides) by chromatography through a column of gel filtration resin - the higher molecular weight DNA is excluded in the void volume of the column and smaller molecular weight nucleotides are retained.

A 1ml disposable syringe was plugged with a small amount of sterile glass wool (section 2.2.8.8) and the syringe filled to the top with a slurry of Sephadex G-50 (section 2.2.8.7) equilibrated in STE buffer (100mM NaCl, 10mM tris-HCl, pH8.0, 0.1mM EDTA). The syringe was then supported in a 10ml glass test tube and centrifuged at 1600xg for 4 min at room temperature in a swinging bucket rotor in a bench centrifuge. More resin slurry was then added and the centrifugation repeated until the packed column volume was about 0.9ml. Three separate column washes with 0.1ml of STE buffer and centrifugation were carried out. The DNA sample(made up to a volume of 0.1ml using STE buffer) was applied to the column, which was attached to a decapped microcentrifuge tube (to collect the effluent), and the whole assembly supported in a 10ml test-tube. The sample was then spun (as above) and the eluted DNA recovered and transferred to a sterile capped tube. The syringe was then checked for radioactivity and discarded appropriately. The

DNA recovered in this way was either ethanol precipitated or lyophilised before further use.

### 2.2.3.6 Ethanol Precipitation of DNA

This procedure was used to concentrate an aqueous DNA sample, stop reactions and/or to remove low molecular weight contaminants (e.g. nucleotides, buffer salts).

To a given volume of DNA solution (in a microcentrifuge tube) was added 0.1 volumes of sodium acetate (3M, adjusted to pH 5.5 with glacial acetic acid) and 2 volumes of absolute ethanol. The sample was inverted several times to mix and either frozen in a dry ice-isopropanol (-50°C) bath for 15min or incubated in a freezer (-20°C) for at least 1 hour before centrifugation in a bench centrifuge (13,400 xg, 20min). After removal of the supernatant, the DNA pellet was washed by addition of ice-cold ethanol (250$\mu$l, 70% aqueous). The pellet was dislodged by flicking the tube and the sample centrifuged (13,400 xg, 5min). The supernatant was removed carefully and the sample vacuum desiccated.

### 2.2.3.7 Storage of DNA

Plasmid DNA was either stored frozen or lyophilised at -20°C. Some degradation of a supercoiled DNA to nicked DNA occurred on prolonged storage. Calf-thymus DNA was stored at 4°C for periods up to one week for use in thermal denaturation (Tm) experiments. Sonicated calf-thymus DNA (see section 2.2.8.3) was stored in small aliquots at -20°C and thawed before use.

## 2.2.4 GEL ELECTROPHORESIS OF DNA AND AUTORADIOGRAPHY

### 2.2.4.1 Agarose Gel Electrophoresis

Agarose gels were cast and run in horizontal trays and tanks built in a similar design to that in most standard texts (Maniatis *et al.*, 1982; Sealey and Southern, 1982). Gels of size 10 x 7cm and 10 x 20cm (length x width) and of depth 0.5cm were cast and were run in the appropriate horizontal gel tanks with the gel surface immersed under buffer. DNA size-resolving properties of the gels were varied by dissolving agarose powder in 1 X TBE buffer (89mM tris-HCl, 89mM boric acid, 2.5mM EDTA, pH 8.3) so that the percentage of agarose was between 0.5 and 1.0 (w/v). The dissolution of agarose was achieved by heating the suspension in a microwave oven. DNA samples (aqueous) were prepared for electrophoresis by the addition of 1/5th volume of agarose gel loading buffer (0.02% bromophenol blue, 1 x TBE, 30% glycerol). The amount of DNA in a sample was 0.1-1.0$\mu$g and was generally loaded in a volume of 20$\mu$l (or less) per gel slot. In practice, most gels (0.8%) were run for 1.5hr (10 x 7cm) or 3-4hr (10 x 20cm) at 100 volts (constant voltage). On completion of electrophoresis, agarose gels were immersed in a solution of EtBr (0.5$\mu$g/ml) for 0.5-1.0hr. The DNA present in the gel was then visualised by the fluorescence of DNA-bound EtBr emitted upon irradiation of the gel with UV light from below, using a mid-range ($\lambda_{max}$ = 302nm) UV transilluminator (TM-20, UV Products Ltd.). In general, a sample of 100-200ng (as a single band) is easily visible, although restriction digests yielding multiple bands may require up to 1$\mu$g/lane. Background fluorescence (caused by non DNA-bound EtBr) was decreased by destaining the gel in running tap water for

0.5-1hr and contrast between sample and gel was greatly increased. Gels were photographed while being irradiated from below with UV light (see above). A Kodak CU-5 Land camera, equipped with a fixed focal length lens and hood unit, a Wratten gelatin no.22 filter, and Polaroid instant film was used. Polaroid type 667 or type 665 film (for positive or positive and negative reproduction, respectively) were used according to the manufacturer's instructions. Camera settings used for the 2 types of film were: Type 667; f11, 0.5 sec and Type 665; f4.5, 5-10 sec.

### 2.2.4.2 Polyacrylamide Gel Electrophoresis

Solutions

(a) 40% acrylamide stock:

A solution of acrylamide (38g) plus N,N'-methylenebisacrylamide (2g) was made up to 100ml with water and stirred for 30 min with 5g of Duolite MB6113 (mixed-bed ion-exchange resin). After filtering twice to remove the resin, the solution was stored in the dark at 4°C. On some occasions this reagent was purchased as a ready-made solution (Acrylogel 5, BDH) and gave the same results as that prepared as above.

(b) 10 x TBE buffer stock:

This was 0.89M tris-HCl, 0.89M boric acid and 0.025M EDTA, adjusted (if necessary) to pH 8.3 using hydrochloric acid.

(c) Formamide loading buffer:

Xylene cyanol (2mg), bromophenol blue (2mg), EDTA (1ml of 0.2M) and formamide (9ml), deionised using the above procedure for acrylamide.

## GLASS PLATE PREPARATION

Gels were cast between a pair of glass plates (40 x 20 x 0.2cm and 37.5 x 20 x 0.2cm, respectively). The plates were first cleansed thoroughly with detergent, rinsed well with tap water, wiped with ethanol and dried with tissues. Separation of these plates after gel electrophoresis was facilitated by pre-siliconising the smaller of the two plates. A small volume (0.5-1.0ml) of a solution of dimethyldichlorosilane (2% in 1,1,1-trichloroethane) was wiped over the surface of the plate and the plate left to air-dry for 5 min. The plate was then rinsed with water before wiping with ethanol and leaving to air-dry.

In order to dry denaturing gels onto the large plate prior to autoradiography, the large plate was coated to prevent the gel from curling up, shrinking or cracking whilst being dried. The plate was treated with gamma-methacryloxypropyltrimethoxysilane before use. To do this, a solution of the silane (30$\mu$l) in ethanol (10ml) was prepared to which was added 10% acetic acid (300$\mu$l). This solution was wiped evenly across the larger plate, which was then left to dry for 5 min and then wiped with ethanol and dried with tissues.

The two plates were assembled together separated by two 400 x 15 x 0.2mm "plastikard" spacers and the sides and bottom of the plates sealed with waterproof electrical tape to prevent leakage of gel prior to polymerisation.

DENATURING PAA GELS

The gel solution was made to a volume of 50ml and the volume of 40% acrylamide stock (solution (a), above) was varied according to the percentage acrylamide required in the gel. The amount of solution (a) required was determined from the following relationship:

$$x = (vy)/z$$

where, v = final volume of gel solution (50ml)

x = ml of solution (a) required

y = percentage of gel required

z = percentage of stock solution (a) (40%)

The gel was therefore prepared from 21g urea, x ml acrylamide stock solution (a), 5.0ml 10 x TBE (solution (b)) and water to give a 50ml total volume. Gels were 8% polyacrylamide unless otherwise stated. Gels containing formamide in addition to urea were used where rigorous denaturing conditions were required to resolve band compressions in conventional gels (Shafer *et al.*, 1989). These gels were prepared by including 30% (v/v) deionised formamide in the gel solution as detailed above. The solution was stirred for 20-30 min to dissolve the urea. Before pouring the gel, the radical polymerisation catalysts ammonium persulphate (3% aqueous, 0.9ml) and TEMED (50$\mu$l) were added and the solution quickly mixed. The gel solution was then poured from a small, lipped beaker into the space between the assembled plates and the sample-well comb inserted. The cast gel was left to set for at least 45 min at an angle of 5-20° to the horizontal. The gel could be stored at 4°C overnight by placing a wet tissue over the top of the gel and sealing the open end of the gel with

cling film to prevent dehydration. Before electrophoresis the bottom edge of the gel was exposed by cutting away the sealing tape and the sample-well comb was removed whilst the gel was supported vertically in its gel tank with both reservoirs full of running buffer (1 x TBE). Excess urea and acrylamide were removed from the wells by gently squirting buffer through the wells with a Pasteur pipette, before pre-running the gel for 0.5-1hr at 1500V constant voltage (20-25mA, 30W). Samples for electrophoresis were resuspended in formamide loading buffer (solution (c) above), heated at 90-100°C for 3 min, and snap-cooled on ice-water prior to loading. Before loading samples, the wells were cleaned again as above and the samples loaded in a volume generally less than 15$\mu$l. 8% gels were run for 1.5-2 hrs (or 3-4 hrs gels containing 30% formamide), 20% gels were run for 6-8 hours.

Following electrophoresis, and gel-mould disassembly, the front (smaller) plate was removed and the gel (stuck to the back, large, plate) fixed (to stop diffusion of DNA bands) by soaking in 10% acetic acid for 10 min. The plate was then washed for 30 min in running water to remove excess urea and the gel placed in an oven (80°C) to dry (approx. 1hr). The dehydrated gel was then ready to be autoradiographed (section 2.2.4.3).

NON-DENATURING POLYACRYLAMIDE GELS

Non-denaturing gels were not dried down onto the large plate and so before casting this type of gel only the front plate was treated. Non-denaturing gels were cast using the same glass-plate system, solutions, and apparatus as for denaturing gels. Non-denaturing gel solution (50ml) was prepared by mixing the following, xml acrylamide stock solution (x is defined as previously for

denaturing gels), 5ml 10 x TBE buffer solution (b), and 45-xml water.

After addition of the polymerisation catalysts, i.e. ammonium persulphate (3% aqueous, 0.9ml) and TEMED (50$\mu$l) the solution was mixed and poured in the same way as for denaturing gels. Non-denaturing gels were electrophoresed at or below a constant voltage of 20 V/cm - voltages and electrophoresis times were as stated in results. Following electrophoresis the gel (attached to the larger (back) plate) was covered with Saran wrap in preparation for autoradiography (section 2.2.4.3).

### 2.2.4.3 Autoradiography of Polyacrylamide Gels

The DNA samples which were electrophoresed through polyacrylamide gels were restriction fragments labelled at the 3' or 5' ends with $^{32}P$. The radioisotope $^{32}P$ emits high-energy $\beta$-particles which can be detected photographically on X-ray film. For highly radioactive bands (> 200 CPS), or in situations where quantitation of the intensity of a film image was not required, "direct" autoradiography at room temperature was employed. When the distribution of radioactivity through a gel was large so that each band had a low radioactive count and/or if quantitation of the film image was required then autoradiography using intensifying screens and low temperatures was employed. Both methods are elaborated below.

#### Direct Autoradiography

Direct exposure of a gel to an X-ray film, with which it is in close contact, was used for detecting highly radioactive samples of $^{32}P$-labelled DNA

restriction fragments on preparative gels. Gels, not dried down, were wrapped in Saran-wrap to prevent moisture reaching the film (and thus blackening it). Film was placed over a wrapped gel and both gel and film pressed together in an autoradiography cassette. In this situation there is a linear relationship between the intensity of the film image and the radioactivity of the samples.

Autoradiography with intensifying screens

One limitation of direct autoradiography is that highly energetic $\beta$-particles (e.g. $^{32}P$, $^{125}I$) can pass through and beyond the film so that only a small proportion of their energy is absorbed and recorded as an image on the film. By using an inorganic scintillator (e.g. a screen of calcium tungstate) just beyond the film, emissions passing through the film are absorbed efficiently by the scintillator which then emits UV light. This light superimposes a photographic image onto the autoradiographic image and increases the sensitivity of detection of a radioactive sample (by 10.5 fold for $^{32}P$; Laskey, 1984; Laskey, 1980) and also enables quicker detection of low activity samples. The increase in detection sensitivity is offset by two problems, (i) the consequent decrease in resolution resulting from the fact that both the primary emissions and the secondary scintillations disperse, and (ii) the fact that once radioactive emissions have been converted to light the response of the film is no longer linear. The decrease in resolution is generally outweighed by the gain in sensitivity but the second problem has to be overcome to prevent serious misinterpretations of data by quantitation of the film images.

Photographic emulsions are disproportionately insensitive to very low light intensities. To obtain any image at all it is necessary to overcome the non-

linear response of film to low light intensities ("low intensity reciprocity failure"). This can be done by a combination of: (1) hypersensitization of the film by pre-exposure to a short flash of light ("preflashing") and (2) cooling the film to approximately -70°C for exposure. The theories behind the above two procedures are well documented and are explained in detail in an Amersham booklet (Laskey, 1984).

In this thesis preflashing was carried out by flashing the film (in complete darkness) with a commercially-available flashgun (flash duration < 1msec). The intensity of the flash was adjusted by means of a Wratten no. 22 filter plus several layers of Whatman filter paper and by varying the distance between the flashgun and the film. These variables were altered so that, when flashed, the absorbance at 540nm of the developed film was raised by 0.1-0.2 absorbance units relative to unflashed film. Film preflashed to a level within these limits would be suitable for accurate quantitation of film images after exposure to a gel. Preflashed film was placed in contact with a dehydrated gel and sealed in an autoradiography cassette containing one intensifying screen in its lid - the order of contents was intensifying screen-film-gel. The cassette was then placed in a -80°C freezer for the required exposure time (from 10 hr to several days). It was essential that the exposed preflashed film was developed in complete darkness (not under red safety light) in order to avoid any further increase in the background absorbance of the film. Exposed films were processed in Kodak chemicals prepared according to the manufacturers instructions -films were immersed in developer solution (4 min), wash solution (1 min) and fixer solution (3 min) before finally rinsing under running water (5 min) and leaving to drip and air-dry.

## 2.2.5 ENZYMATIC MODIFICATION OF DNA

### 2.2.5.1 Restriction Endonuclease Digestion

Plasmid DNA was digested into smaller fragments by using specific restriction endonucleases to cleave the plasmid at particular DNA sequences. The reactions were carried out in buffer solutions supplied by, and strictly according to the instructions issued by, the manufacturer of the particular restriction enzyme, although a general method is given below. The following solution was prepared:

| | |
|---|---|
| x$\mu$l | plasmid DNA in water |
| 5.0$\mu$l | 10X restriction enzyme buffer |
| (45-x-y)$\mu$l | distilled water |
| y$\mu$l | restriction enzyme |

where the amount of restriction enzyme units added was 1.5X the amount of plasmid in $\mu$g. The solution was mixed gently and incubated at 37°C for 1-2 hours. If required the products of the reaction were examined by agarose gel electrophoresis (section 2.2.4.1) to ensure that reaction was complete. Generally, restriction enzyme digestions were terminated by EDTA (1$\mu$l of 0.5M, pH 8.0) and heating (65°C, 10-15 min) to inactivate the enzyme, followed by phenol extraction (section 2.2.3.3) and ethanol precipitation (section 2.2.3.6).

2.2.5.2 Removal of 5'-Phosphoryl Groups

Two different methods using calf-intestinal alkaline phosphatase (CIP) were employed to remove the 5'-phosphoryl groups of DNA fragments according to the requirements of subsequent manipulations.

Method a:

Prior to the kinase procedure double-stranded DNA fragments were dephosphorylated by the following method: restriction-digested DNA (20$\mu$g, e.g. pBR322/EcoR1 ≈ 14 pmoles of 5' ends) dissolved in buffer (50$\mu$l, 50mM tris-HCl, pH 9.0, 10mM $MgCl_2$, 1mM $ZnCl_2$ and 10mM spermidine) was incubated with CIP (1.0$\mu$l, 1.0 Unit) for 1 hr at 37°C. The reaction was terminated by adding water (40$\mu$l), 10X STE buffer (10$\mu$l) and SDS (5$\mu$1, 10% w/v) and heating (68°C, 15 min). The sample was phenol extracted twice, washed with chloroform and applied to a Sephadex G-50 spun-column equilibrated in TE buffer (section 2.2.3.5). The eluate recovered was ethanol precipitated and stored lyophilised.

Method b:

This method includes a denaturation step and was therefore used when the dephosphorylated DNA was not required to be in the double-stranded form for subsequent use, e.g. when it was the procedure immediately prior to denaturing PAGE. Linearised plasmid DNA was redissolved in buffer (45$\mu$l of 50mM tris-HCl, pH 9.0, 10mM $MgCl_2$, 1mM $ZnCl_2$, 10 mM spermidine), heat-denatured (90°C, 2 min) and snap-cooled in ice-water. CIP (5 Units) was added, the sample incubated (37°C, 1hr) and the DNA ethanol precipitated.

### 2.2.5.3 Removal of 3'-Phosphoryl Groups

This procedure used T4 polynucleotide kinase (PNK) as a specific 3'-phosphatase under the conditions given by Hertzberg and Dervan (Hertzberg and Dervan, 1984). Ethanol precipitated DNA was redissolved in water (20$\mu$l), the sample heat-denatured (90°C, 2min) and snap-cooled in ice-water. To the heat-denatured DNA was added buffer (20$\mu$l of 20mM tris-HCl, pH 6.6, 20mM $MgCl_2$, and 10mM $\beta$-mercaptoethanol), followed by PNK (15 units). After incubation (37°C, 1 hour) the DNA was recovered by ethanol precipitation.

## 2.2.6 RADIOLABELLING, PURIFICATION AND SEQUENCING OF DNA

DNA was radiolabelled to produce singly end-labelled linear DNA fragments. The general scheme was as follows:

Step 1: Restriction enzyme digest of supercoiled DNA

Step 2: End-labelling procedure (5' or 3' end)

Step 3: Second restriction enzyme digest

Step 4: Recovery of singly end-labelled fragments

These procedures are shown diagramatically in figure 15 and the nucleotide sequences of the fragments produced are shown in figures 16-20.

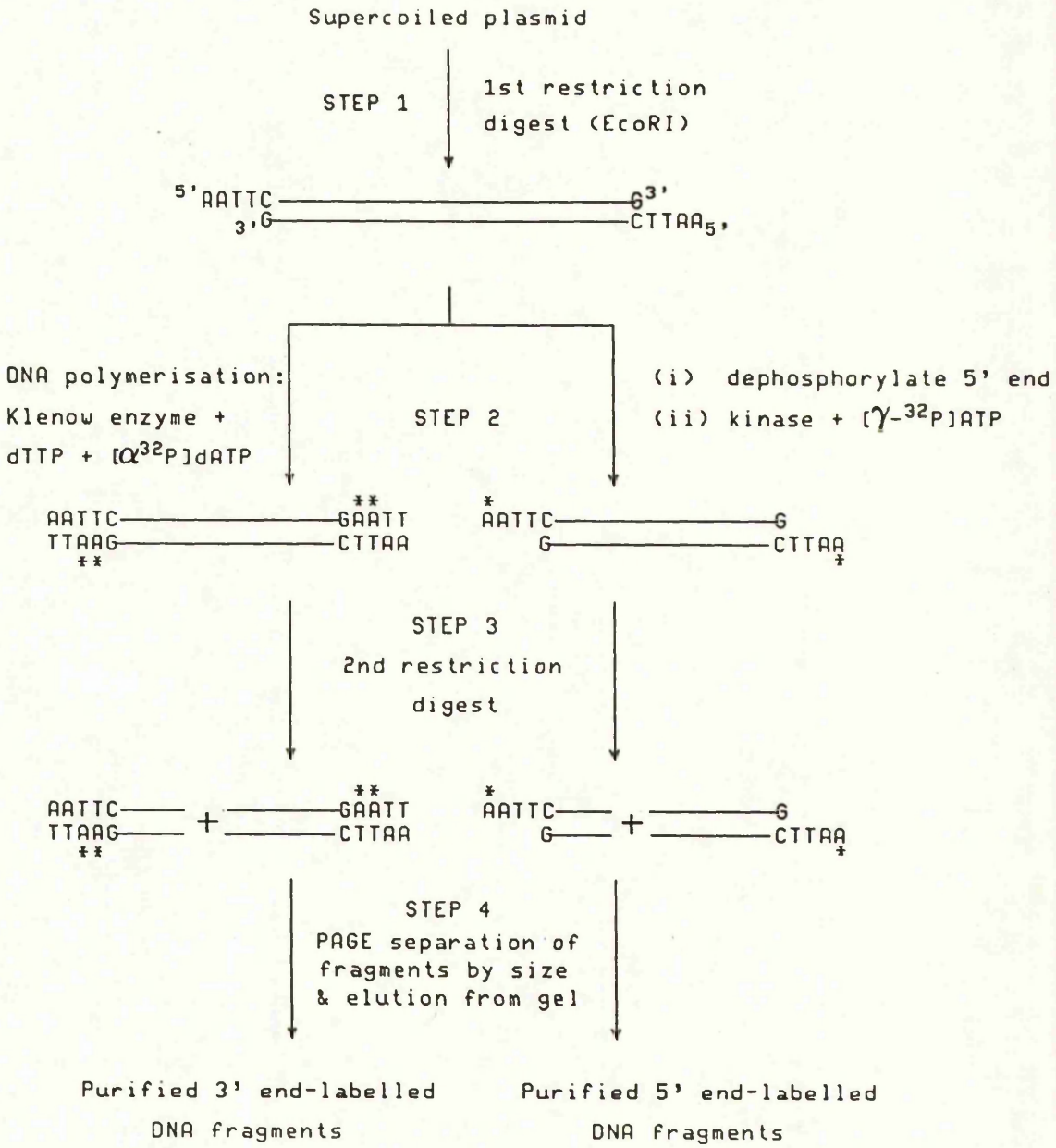



Figure 15. Schematic diagram of preparation of singly end-labelled DNA fragments from supercoiled plasmid DNA. Asterisk indicates $^{32}P$-labelled nucleotide.

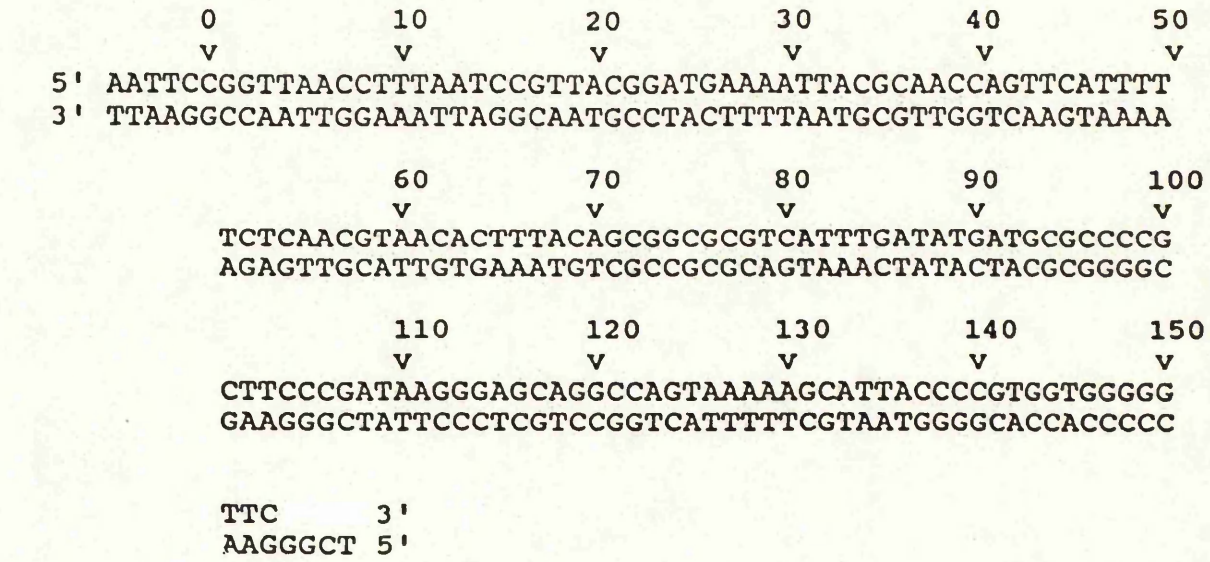



Figure 16. Nucleotide sequence of the 163bp tyrT fragment produced by EcoRI/AvaI digestion of pKMΔ98. 3' end-labelled fragment radiolabelled at nucleotide positions -2 and -3, lower strand. Sequence taken from Fox, 1988 and numbered according to Laughton et al, 1990. Note that this sequence possesses a single extra base at position 6 compared to earlier sequence data, as given by Drew and Travers, 1985.

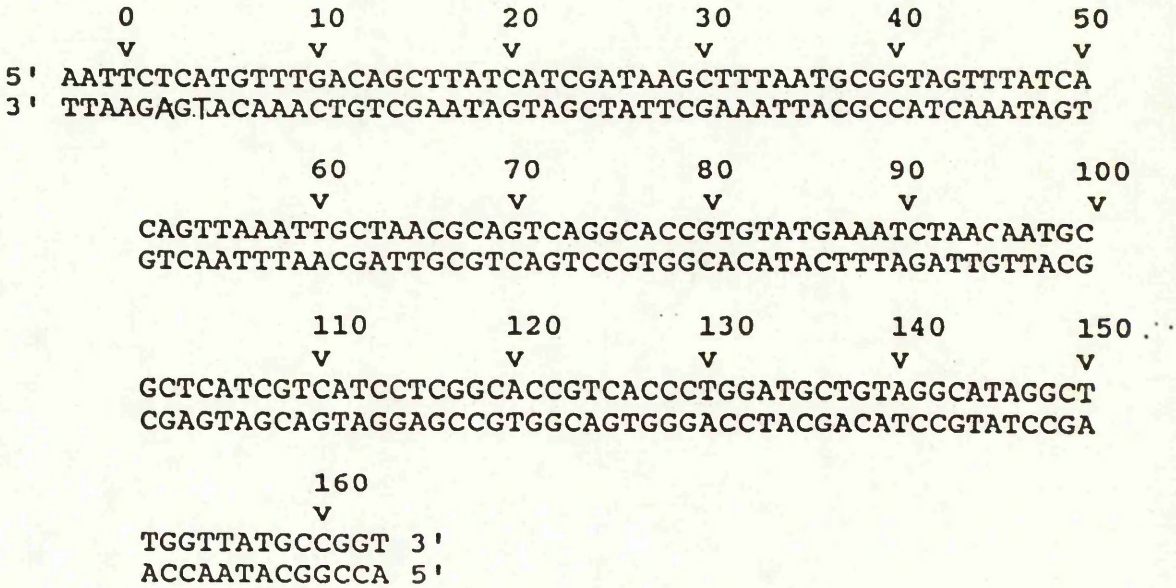



Figure 17. Nucleotide sequence of the 167bp fragment produced by EcoRI/RsaI digestion of pBR322. 3' end-labelled fragment radiolabelled at nucleotide positions 0 and -1, lower strand; 5' end-labelled radiolabelled at position -3, upper strand. Sequence taken from Maniatis *et al*,1982

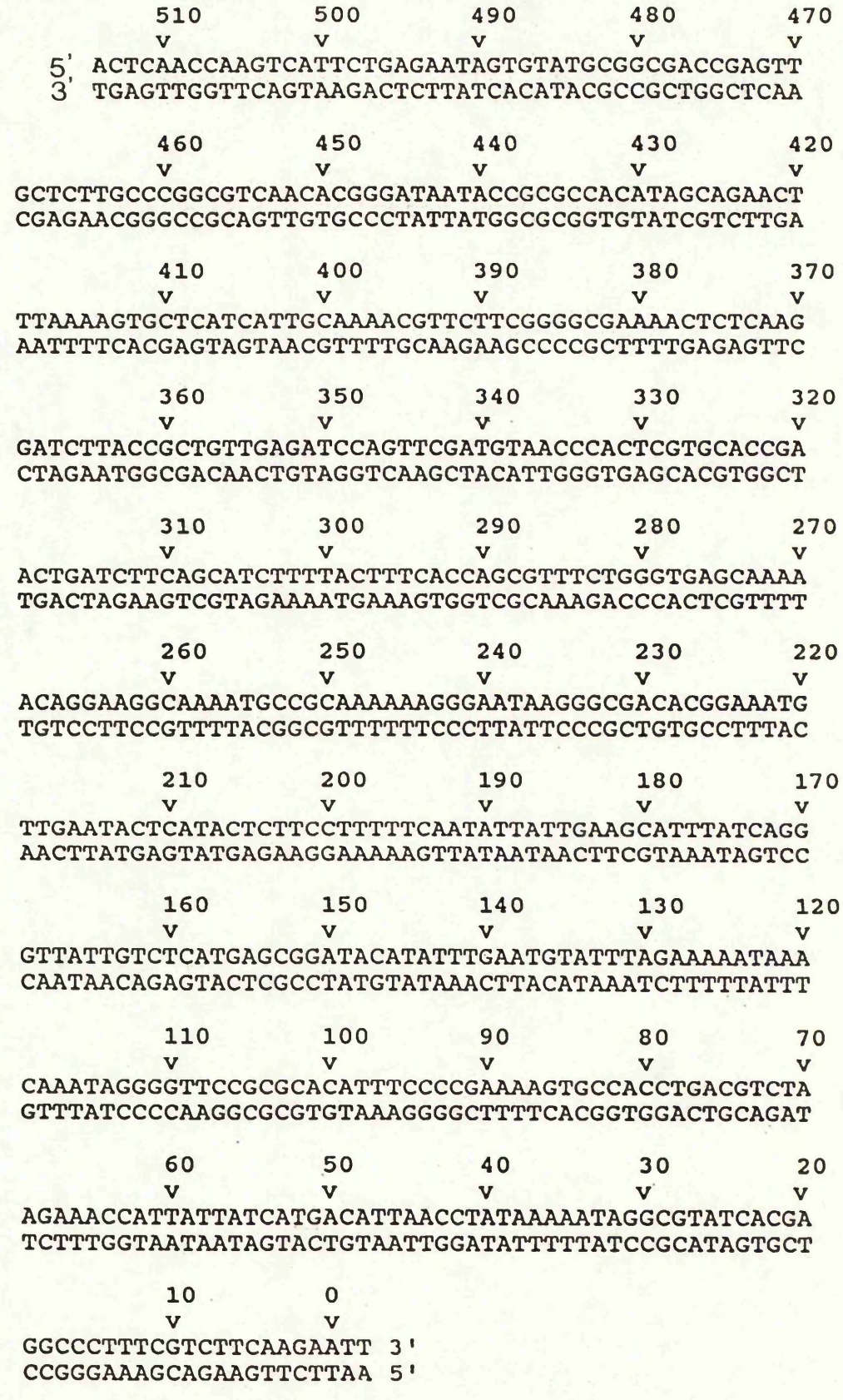



Figure 18. Nucleotide sequence of the 517bp fragment produced by EcoRI/RsaI digestion of pBR322. 3' end-labelled fragment radiolabelled at nucleotide positions 0 and +1, upper strand;5' end-labelled fragment radiolabelled at position -2, lower strand. Sequence taken from Maniatis *et al*,1982.

```
      0         10        20        30        40        50
      v         v         v         v         v         v
5' AATTCGAGCTCGCCCGGGGATCTGTGTGTGTGTGTGTGTGTGTGGATCCTCT
3' TTAAGCTCGAGCGGGCCCCTAGACACACACACACACACACACACCTAGGAGA

                60
                v
       AGAGTCGACCTGCAGCCCA     3'
       TCTCAGCTGGACGTCGGGTTCGA 5'
```

Figure 19. Nucleotide sequence of 70bp fragment produced by EcoRI/HindIII digestion of pRW1011. 3' end-labelled fragment radiolabelled at positions 0 and -1, lower strand. Sequence taken from McLean & Wells,1988.

```
      0         10        20        30        40        50
      v         v         v         v         v         v
5' AATTCGAGCTCGCCCGGGGATCTGTGTGTGTGTGACACACACACACGATCCTCT
3' TTAAGCTCGAGCGGGCCCCTAGACACACACACACTGTGTGTGTGTGCTAGGAGA

                60
                v
       AGAGTCGACCTGCAGCCCA     3'
       TCTCAGCTGGACGTCGGGTTCGA 5'
```

Figure 20. Nucleotide sequence of 70bp fragment produced by EcoRI/HindIII digestion of pRW1015. 3' end-labelled fragment radiolabelled at positions 0 and -1, lower strand. Sequence taken from McLean & Wells,1988.

2.2.6.1 3' end-labelling

3' end-labelling was achieved by filling in the 3' recessed end of a piece of DNA resulting from digestion by EcoR1 restriction enzyme that produces 3'-recessed (5'-overhang) ends. The Klenow fragment of DNA polymerase I uses the 5'-overhang sequence as a template and the 3'-recessed end as a primer for the addition of nucleotides complementary to the 5'-overhang sequence to "fill-in" the EcoRI "sticky end". The following sample was prepared:

40$\mu$l EcoR1 restriction endonuclease-digested DNA
($\approx$ 20$\mu$g, $\approx$ 10-20pmoles 3' ends)

5.0$\mu$l restriction enzyme buffer 10X solution

1.5$\mu$l dTTP (2mM)

3.0$\mu$l [$\alpha$-$^{32}$P]dATP (specific activity > 6000 Ci/mmol; 10$\mu$Ci/$\mu$l)

4.0$\mu$l Klenow fragment (20 units)

Note: to facilitate labelling and the second restriction digestion, without requiring ethanol precipitation before the digest, the labelling reaction was carried out in the second restriction enzyme buffer (Klenow fragment works effectively in most buffers). For example, for pBR322, fragments of 167 and 517bp were produced by a second digest with RsaI, or for pKMΔ98 the 160bp fragment was produced by a second digest with AvaI. The buffers used in the labelling reactions were therefore RsaI 10X buffer (pBR322) and AvaI 10X buffer (pKMΔ98).

The sample was incubated for 40min at 30°C before addition of 'cold' (non-radioactive) dATP and further incubation for 20min. The reaction was

terminated by heating (70°C, 5 min; Maniatis *et al.*, 1982) after which the second restriction enzyme (40 Units) was added and the sample incubated for 1-1.5 hr followed by adjustment of the sample to 20mM EDTA and heating to 70°C for 10 min to terminate the reaction. The sample was then ready for electrophoretic separation and purification of the radiolabelled restriction fragments (section 2.2.6.3).

2.2.6.2 5' end-labelling

Restriction fragments of plasmid DNA were labelled by the forward reaction of bacteriophage T4 polynucleotide kinase (PNK). The DNA was initially dephosphorylated by incubation with alkaline phosphatase (section 2.2.5.2) to create a 5'OH group which is the substrate for the transfer of the $\gamma$-$^{32}$P group of [$\gamma$-$^{32}$P]ATP in the reaction catalysed by PNK. The following solution was prepared:

40$\mu$l restriction enzyme-digested DNA, dephosphorylated ($\approx$ 20ng, $\approx$ 10-20pmoles 5' ends)

5$\mu$l 10 x kinase buffer (0.5M tris-HCl, pH 7.6, 0.1M $MgCl_2$, 50mM DTT, 1mM spermidine-HCl and 1mM EDTA, pH 8.0)

This solution was added to a sample of 100$\mu$Ci dried-down [$\gamma$-$^{32}$P]ATP (specific activity 3000 Ci/mmole; 10$\mu$Ci/$\mu$l). PNK (20 Units) was added and the sample made up to 50$\mu$l with water. The reaction mixture was incubated for 45 min at 37°C, the reaction terminated by the addition of EDTA (2$\mu$l, 0.5M pH 8.0) and the solution was phenol extracted once (section 2.2.3.3). Unincorporated

[$\gamma$-32P]ATP was then separated from the labelled DNA by spun-column chromatography (section 2.2.3.5). The sample was then ethanol precipitated (section 2.2.3.6) and digested with a second restriction enzyme to generate singly end-labelled fragments. The fragments were separated and purified as in section 2.2.6.3.

### 2.2.6.3 Separation and Purification of Radiolabelled DNA

Singly end-labelled DNA fragments were generated by linearisation of plasmid DNA by restriction endonuclease cleavage at a unique site, radioactive labelling of the DNA at the unique site (either 5' or 3') and subsequent digestion with a second restriction enzyme to yield labelled DNA fragments of different sizes. The fragments were separated and purified by means of non-denaturing PAGE (section 2.2.4.2) as follows:

Labelled DNA fragments were prepared for electrophoresis by addition of 1/5$^{th}$ volume of agarose gel loading buffer (section 2.2.4.1). Samples from a single labelling reaction ($\approx$ 20$\mu$g) were loaded into two wells (each of length 2.5cm, height 1.0cm, width 0.3mm) of a 6.4% polyacrylamide gel. Electrophoresis was carried out for 2.5 hr at a constant 800 volts. The gel plates were separated and the gel (attached to the large plate) was covered in Saran Wrap and autoradiographed (5-10 min, room temp., no screens). The autoradiograph was then used as a guide to locate the intensely radioactive DNA bands. The bands were excised as intact gel pieces (without crushing) and placed in sterile 2ml microcentrifuge tubes. Buffer (1mM EDTA, pH 8.0) was added to cover the gel pieces completely ($\approx$800$\mu$l) and the sample incubated for 0.5hr or longer at 37°C. The tube was then centrifuged

(13,400xg, 10min), the supernatant carefully removed (400$\mu$l), monitored for radioactivity and ethanol precipitated. This procedure of incubation, centrifugation and removal of supernatant was repeated until most of the radioactivity in the gel samples had been removed. Note that the length of the fragment governs the speed and efficiency of elution - typical elutions were 1hr, 2hr, 10hr (<200 bp) and 2, 10, 20 hours for a 517bp fragment. After ethanol precipitation (where any polyacrylamide contamination would be indicated by a bulky white, insoluble precipitate), the samples were washed three times with ice-cold ethanol (70% aqueous), lyophilised and redissolved in water or buffer to a concentration giving 100 CPS/$\mu$l. Fragment solutions were stored as aliquots (100$\mu$l) at -20°C.

### 2.2.6.4 Chemical DNA Sequencing

#### 2.2.6.4.1 Maxam and Gilbert G-tracking

Guanine-specific cleavage of singly end-labelled DNA fragments was carried out by the Maxam and Gilbert DNA sequencing method (Maxam and Gilbert, 1980) using sequential base modification and strand scission reactions. For a stock of G track to be used as a marker lane for fragment electrophoresis approximately 1000 CPS of labelled DNA was used in the following protocol (enough for about 20 x 50 CPS loadings).

Solutions:

SC buffer :50mM sodium cacodylate, pH 8.0,1mM EDTA

DMS-Stop buffer :1.5M sodium acetate, pH 7.0,1M 2-mercaptoethanol, 100 $\mu$g/ml tRNA

(i) Base modification

$^{32}$P-labelled DNA (x $\mu$l) was added to SC buffer (200-x $\mu$l), the sample cooled in ice-water for 2min after which time DMS (1$\mu$l) was added. The sample was briefly vortex mixed and bench centrifuged (13,400xg, 20 sec) and then incubated at 20°C for 10 min. Ice-cold DMS-stop buffer (50$\mu$l) was added and the sample mixed gently by inversion. Ice-cold ethanol (750$\mu$l) was added, the sample mixed by inversion and incubated in a dry-ice/isopropanol bath for 15 min. After centrifugation (13,400xg, 15 min) the supernatant and pipette tip were discarded into sodium hydroxide (5M). The pellet was resuspended in 250$\mu$l sodium acetate (0.3M, pH 7.0) and the ethanol precipitation repeated. After centrifugation and removal of the supernatant (as above) the pellet was washed with ice-cold 70% aqueous ethanol (500$\mu$l). The sample was centrifuged (13,400xg, 5 min) and the supernatant discarded as above. The sample was then dried under vacuum.

(ii) Strand-scission reaction

To the base-modified, ethanol precipitated DNA sample was added freshly diluted piperidine (100$\mu$l, 1M) and the DNA dissolved by brief vortex mixing. The closed microcentrifuge tube was sealed with 3 layers of parafilm and incubated at 90°C for 30 min in a water bath. The sample was then frozen in dry-ice and lyophilised (about 70 min in a Savant speedvac). The sample was taken up in water (10$\mu$l), frozen and lyophilised (15 min) and this procedure repeated once. The sample was then either taken up in formamide gel loading buffer (10$\mu$l) and electrophoresed (for a sample of 50-100 CPS) or taken up in

this same buffer (20-50$\mu$l) and 1.0$\mu$l (~20 CPS) used per lane of G-track required (for a stock solution made from 1000 CPS). G-track lanes of 20-50 CPS (per lane) gave readable sequence with an overnight exposure of gel to preflashed film at -80°C with an intensifying screen (section 2.2.4.3).

2.2.6.4.2 Rapid chemical sequencing

Verification of DNA sequence was carried out by the rapid chemical sequencing procedures of Williamson and Celander (Williamson & Celander, 1990) designed for small, single-stranded oligonucleotides (ssDNA). Radiolabelled DNA fragments, as prepared in this section, however, were double-stranded (ds) and required conversion into substrates for these procedures by a preliminary heat denaturation step (converts ds to ss).

Solution: PF - 4% formic acid adjusted to pH 2.0 with piperidine.

Three samples of $^{32}$P-labelled DNA (200 CPS) and CT-DNA (100$\mu$M) in TE buffer (9$\mu$l) were heat-denatured (90°C, 10 min) and snap-cooled on ice. The following three reactions were then carried out on the separate samples.

G reaction: DMS (1$\mu$l) was added and the sample incubated (2 min, RT).

G + A reaction: PF (1$\mu$l) was added and the sample incubated (5 min, 65°C).

T reaction: $KMnO_4$ (1$\mu$l, 2mM) was added, the sample incubated (10 min, RT) and the reaction quenched by addition of allyl alcohol (1$\mu$l).

On completion of the above reactions the samples were immediately subjected to cleavage by adding pyrrolidine (100$\mu$l, 1.1M) and heating (15 min, 90°C) after which the samples were reduced to dryness in a speed-vac. The samples were resuspended in water (25$\mu$l) and redried twice before addition of formamide loading buffer and subsequent electrophoresis (see section 2.2.4.2).

## 2.2.7 DNA CLEAVAGE AND CHEMICAL MODIFICATION

### 2.2.7.1 Cu(II):Thiol Nuclease

Cu(II):thiol-mediated scission of radioactively labelled DNA fragments was initiated by adding an aliquot (5$\mu$l) of thiol solution (usually 0.5mM in SH groups) to sodium phosphate buffer (20$\mu$l of 12.5mM, pH 8.0) containing sonicated CT-DNA (50$\mu$M bps), end-labelled DNA fragment ($\sim$ 100 CPS, $<$ 10 pmoles bp) and $CuSO_4$ (0.25mM). Cu(II):thiol-mediated scission of plasmid DNA was carried out as above except that only the desired plasmid DNA was present (i.e. no CT-DNA or radioactive DNA was included). Both plasmid and labelled-DNA scissions were terminated by the addition of EDTA (1$\mu$l of 20mM, pH 8.0), and the labelled-DNA samples either ethanol precipitated or frozen and lyophilised before further procedures. The plasmid DNA samples were electrophoresed after addition of concentrated loading buffer (section 2.2.4.1).

2.2.7.2 Iron(II):EDTA (hydroxyl radical) nuclease

The following method was adapted from that given by Portugal and Waring (1987). The Fe(II):EDTA cleavage reagent was prepared by mixing **fresh** solutions of ferrous ammonium sulphate (0.2mM), EDTA (0.4mM), L-ascorbate (10mM) and hydrogen peroxide (0.3%) in the volume ratio 1:1:2:2, respectively. Aliquots of this solution were added to DNA samples immediately after the hydrogen peroxide had been added.

Cleavage of labelled-DNA fragments was initiated by the addition of an aliquot (5$\mu$l) of the above cleavage reagent to sodium phosphate buffer (20$\mu$l of 12.5mM, pH 8.0) containing sonicated CT-DNA (50$\mu$M bp) and end-labelled DNA fragment ( ~ 100 CPS, < 10 pmole bp). Plasmid DNA cleavage was carried out as above except that only the desired plasmid DNA was present (i.e. no CT-DNA or radioactive DNA was included). Plasmid DNA reactions were terminated by electrophoresis (section 2.2.4.1). Labelled DNA reactions were terminated by addition of thiourea (10$\mu$l, 0.1M), and EDTA (10$\mu$l, 0.2mM) and the sample ethanol precipitated with three volumes of ethanol. The samples were washed once with 70% ethanol, lyophilised and redissolved in loading buffer prior to denaturing PAGE (section 2.2.4.2).

2.2.7.3 Deoxyribonuclease I (DNAse I)

The following procedure was adapted from that given by Ford and co-workers (Ford *et al*, 1987). DNAse I cleavage of DNA was initiated by the addition of freshly diluted DNAse I (2$\mu$l of a stock solution containing 1.15 Kunitz units/ml) to a solution (18$\mu$l total volume) containing CT-DNA (50$\mu$M

bp), end-labelled DNA (~100 CPS, <10 pmole bp), sodium chloride (20mM), magnesium chloride (2mM) and manganese chloride (2 mM). For footprinting studies a DNA-binding ligand was also added to the DNA solution and the solution preincubated at an appropriate temperature prior to the addition of the enzyme. Reactions were allowed to proceed for 2, 5 and 30min at 37°C prior to freezing on dry-ice and lyophilisation. Samples were redissolved in loading buffer prior to denaturing PAGE (section 2.2.4.2).

2.2.7.4 Copper:phenanthroline nuclease

The following procedure was adapted from that given by Spassky and Sigman (1985). Fresh solutions of copper sulphate (50$\mu$M) and 3-MPA (1.25mM SH groups) were prepared. Due to low solublity in water 1,10-phenanthroline was prepared as a stock solution in absolute ethanol and diluted to a final concentration of 250$\mu$M in water. Cleavage of labelled-DNA fragments was initiated by the addition of an aliquot (5$\mu$l) of a copper sulphate: 1,10-phenanthroline solution (25$\mu$M and 125$\mu$M, respectively) to sodium phosphate buffer (20$\mu$l of 12.5mM pH 8.0) containing sonicated CT-DNA (50$\mu$M bp), end-labelled DNA fragment (~100 CPS, <10 pmole bp) and 3-mercaptopropionic acid (250$\mu$M). Reactions were terminated by addition of EDTA (1$\mu$l, 20mM), freezing on dry-ice and lyophilisation. Samples were redissolved in loading buffer prior to PAGE (section 2.2.4.2).

### 2.2.7.5 Potassium permanganate ($KMnO_4$) modification of DNA

The following procedure was adapted from that given by Jeppesen and Nielsen (1988). $KMnO_4$-mediated modification of DNA was initiated by addition of an aliquot (2$\mu$l) of freshly-prepared $KMnO_4$ (10mM) to sodium phosphate buffer (20$\mu$l of 12.5mM, pH 8.0) containing sonicated CT-DNA (50$\mu$M bp) and end-labelled DNA fragment (~100CPS, <10 pmoles bp). The reaction was carried out at room temperature (20-24°C) for times up to a maximum of 20min, after which the samples were ethanol precipitated before treatment with piperidine as in the strand-scission protocol of the Maxam & Gilbert G-track procedure (section 2.2.6.4). Samples were analysed by denaturing PAGE (section 2.2.4.2).

### 2.2.7.6 Diethylpyrocarbonate (DEPC) modification of DNA

The following procedure was adapted from that given by Jeppesen and Nielsen (1988). Modification of DNA by DEPC was initiated by addition of an aliquot (3$\mu$l) of fresh DEPC to sodium phosphate buffer (20$\mu$l of 12.5mM pH 8.0) containing sonicated CT-DNA (50$\mu$M bp) and end-labelled fragment (~100CPS, <10 pmoles bp). The sample was vigorously agitated to mix - DEPC is sparingly soluble in water - and the reaction carried out at room temperature (20-24°C) for times up to a maximum of 20min, after which the sample was ethanol precipitated. DEPC-modified samples were then treated with piperidine as in the strand-scission protocol of the Maxam and Gilbert G-track procedure (section 2.2.6.4). Samples were analysed by denaturing PAGE (section 2.2.4.2).

## 2.2.8 ASSAYS AND GENERAL PROCEDURES

### 2.2.8.1 Thiol Assay

Quantitation of thiol in solution was carried out using Ellman's reagent, DTNB, 5,5'-dithiobis(2-nitrobenzoic acid). Two methods were used, a standard method (method 1) and a "reverse" method (method 2) for use when the thiol sample was at a low concentration and/or low quantity. These procedures were adapted from those of Riddles and co-workers (Riddles *et al*, 1983). The assay follows the release of a coloured thiolate anion ($TNB^{2-}$, $\lambda_{max} = 412nm$) from a disulphide, DTNB (R'SSR'). Nucleophilic attack of the thiolate anion ($RS^-$) of a thiol on DTNB produces a mixed disulphide, RSSR', and the thiolate anion, $TNB^{2-}$. This reaction produces 1 mole of $TNB^{2-}$ for each mole of added RSH. The amount of thiol present in a solution may be calculated using the extinction coefficient for $TNB^{2-}$ ($\epsilon_{412} = 14,150 M^{-1}cm^{-1}$; Riddles *et al.*, 1983), the maximum $\Delta A_{412}$ produced on adding the thiol to a solution of DTNB and the known stoichiometry of the reaction.

Method 1 (Standard Protocol)

Aliquots of the thiol solution (< 100$\mu$l of stock solution < 10mM) were added to 1ml of DTNB solution ($1 \times 10^{-4}$M) in sodium phosphate buffer (0.1M, pH 7.0) and the increase in absorbance at 412nm followed relative to a blank of DTNB itself in buffer. The amount of thiol present in the original sample was calculated from the extinction coefficient of $TNB^{2-}$ (see above), the dilution of the stock thiol solution in the assay, and the net change in absorbance of the

solution after completion of the reaction (5-10 min). A minimum of three dilutions were measured and the $\Delta A_{412}$ values used in the following calculation to calculate thiol titre ; assuming a cuvette path length of 1cm.

$$[SH]_{stock} = \frac{1ml + \text{vol. of stock added (ml)}}{\text{vol. of stock added (ml)}} \times \frac{\Delta A_{412}}{14{,}150\ M^{-1}cm^{-1}}$$

Method 2 (Reverse)

Small volumes, or larger dilute volumes, of thiol were assayed for thiol content by the following procedure. To the thiol solution (0.89ml) was added an aliquot (0.1ml) of sodium phosphate buffer (1M, pH 7.0) and distilled water to bring the sample volume to 0.990ml. The reaction was initiated by the addition of DTNB (10$\mu$l of 10mM) in buffer (10mM sodium phosphate pH 7.0). Thiol content was determined by the net $\Delta A_{412}$ observed relative to a blank of 10$\mu$l DTNB (10mM) added to 0.990$\mu$l buffer (10mM sodium phosphate, pH 7.0) and by reference to the following equation.

$$[SH]_{stock} = \frac{1ml}{\text{vol. of stock added (ml)}} \times \frac{\Delta A_{412}}{14{,}150\ M^{-1}cm^{-1}}$$

(assuming a cuvette path length of 1cm)

### 2.2.8.2 Copper(II) Assay

The following spectrophotometric assay was developed based on (i) the reduction of Cu(II) to Cu(I) and (ii) selective chelation of Cu(I) by neocuproine to form a coloured complex (Hall *et al*, 1962; $\lambda_{max}$ = 450nm, in 10mM tris-HCl, pH 8.0, this work). An excess of GSH was added as the reductant to a solution of neocuproine in buffer containing varying amounts of the copper test solution. The blank for each determination was taken to be the solution of neocuproine in buffer (volume = 0.8ml) prior to the addition of both copper and GSH solutions (to a final volume of 1ml). The increase in $A_{450}$ observed ($\Delta A_{450}$), on addition of the copper test solution and GSH (in that order) to neocuproine in buffer, was found to be maximal and stable almost immediately after mixing and to remain so for up to at least a further 30min (at 25°C). Therefore, for convenience, readings were taken at 5min after mixing. Final assay concentrations used were: tris-HCl (10mM, pH 8.0), neocuproine (1mM), $Cu^{2+}$ (0-50$\mu$M) and GSH (0.5mM). The relationship of $\Delta A_{450}$ to concentration of copper(II) in the cuvette was found to obey the Beer-Lambert law ($A = \in .c.l$), i.e. the relationship was linear under the above conditions (see results section for standard curve).

### 2.2.8.3 Preparation of calf-thymus DNA

#### Sonicated CT-DNA

Calf thymus DNA, used to boost the quantity of DNA in DNA cleavage reactions, was sonicated prior to use. A solution of CT-DNA (1-2mg/ml) in

distilled water was sonicated on ice using an MSE Soniprep 150 fitted with a 1cm diameter probe. The machine settings were 30 seconds on, 30 seconds off and the frequency tuning at 10 microns. The DNA was sonicated for 18 cycles with 1ml aliquots of the solution removed at 8, 13 and 18 cycles for examination by agarose gel electrophoresis to determine the extent of sonication. Samples of the sonicated DNA (0.5$\mu$g) were electrophoresed in a 1% agarose gel next to a lane of molecular weight marker DNA ($\lambda$ DNA digested by EcoRI and HindIII). DNA sonicated to a size of approximately 500bp (± 200 bp) was retained, diluted to 1mM bp, and used in DNA cleavage reactions.

CT-DNA for Tm determinations

Solid CT-DNA (1-2mg/ml) was allowed to hydrate over a period of 1-2 days at 4°C in the required buffer. After this period the concentration of DNA in the solution was determined spectrophotometrically (section 2.2.3.2), the stock solution diluted to the appropriate concentration and then stored at 4°C until use. The solution was not shaken or sonicated to aid dissolution, to ensure that the DNA was present as intact polymer and not as short oligonucleotides.

### 2.2.8.4 Thermal Denaturation of DNA - Tm Determination

Thermal denaturation of DNA was studied using calf-thymus DNA in various buffers with added ligands or ions. The denaturation of the DNA was followed by the increase in $A_{260}$ of the sample while it was heated from 45°C

to 95°C at 0.5°/min (relative to an appropriate blank containing all components except the DNA). A plot was made of absorbance *versus* temperature and the temperature at which the absorbance increase was half that of the maximum increase was determined. This value (Tm) was used as a crude measure of the effect of the added ligand/ion on the stability of the DNA, *viz.*, an increase in Tm on addition of ligand or ion means that the DNA is stabilised to denaturation and *vice-versa*.

2.2.8.5 Preparation of Phenol

**CARE: PHENOL, ESPECIALLY MOLTEN, IS EXTREMELY CORROSIVE.**

Phenol crystals (AnalaR grade), enough to make 100ml liquid, were melted at 70°C using a water bath. To this was added an equal volume of buffer (1M tris, pH 8.0). The mixture was transferred to a separating funnel and thoroughly mixed. After the phases had separated (10 min), the lower (phenolic) phase was retained and the upper aqueous phase discarded. This mixing and separation procedure was repeated twice with fresh portions of buffer followed by three similar extractions with aliquots of buffer (0.1M tris, pH 8.0, 0.2% mercaptoethanol), each time the phenolic layer being retained. The resulting phenol solution was acceptable if the pH was within the range 7.6-8.2. The phenol was stored at 4°C in a brown, stoppered bottle with a layer of the 0.1M tris buffer over it, or frozen in small aliquots (0.5ml) until required for use.

2.2.8.6 Preparation of dialysis tubing

Dialysis tubing was prepared by boiling for 10 minutes in each of the following solutions in sequence:

1) sodium bicarbonate (1% w/v);

2) EDTA (1% w/v);

3) distilled water

The tubing was then rinsed in distilled water and stored in 50% aqueous ethanol at 4°C.

2.2.8.7 Preparation of Sephadex G-50 slurry

Sephadex G-50 (medium grade) was added slowly to distilled, sterile water in a 500ml bottle. Note that about 30g of Sephadex yields 500ml of slurry. Soluble dextran was removed from the swollen resin by washing the resin with distilled water several times. The resin was then equilibrated in TE (pH 7.6) by washing it in this buffer several times, autoclaved (10lb/in$^2$, 15 min) and stored at 4°C.

2.2.8.8 Siliconisation of Microcentrifuge tubes and Glass Wool

To prevent quantities of radioactive DNA adhering to the surface of microcentrifuge tubes, the tubes were treated with a reagent that made them non-stick. Clean microcentrifuge tubes (either 1.5ml or 0.5ml) were individually fully immersed in a solution of dimethyldichlorosilane (2% w/v in 1,1,1-trichloroethane), drained of fluid, dried under vacuum (1-2 hours), washed with

water (to remove any acid generated in the treatment), rinsed twice with distilled water, dried in an oven and then sterilised by autoclaving (135°C, 15 lb $in^2$, 25 min). All manipulations using radioactivity and radiolabelled DNA were carried out in tubes prepared as above.

Glass wool was thoroughly immersed in dimethyldichlorosilane (2% w/v in 1,1,1-trichloroethane) and then removed from the solution and patted dry between tissues before being dried in an oven (90°C) for 24 hours. The wool was then sterilised by autoclaving (135°C, 15 lb/$in^2$, 25 min) and was ready for use.

2.2.8.9 Autoclaving

All materials to be sterilised were placed inside a bench-top autoclave and heated to 135°C, 15lb/sq in. for 15 minutes (for buffers containing glucose) or for up to 45 min (for pipette tips, large volumes of buffers and glassware).

# CHAPTER 3 : RESULTS

## 3.1 PRELIMINARY EXPERIMENTS - PREPARATION OF PHOSPHATE BUFFER SOLUTIONS CONTAINING Cu(II)

Several experiments were carried out to check for Cu(II)-mediated pH effects or DNA denaturation and loss of Cu(II) due to possible precipitation in phosphate buffer (copper phosphate salts are insoluble). In all experiments where Cu(II) was present, $CuSO_4$ was used.

### 3.1.1 EFFECT OF Cu(II) ON pH

Addition of $CuSO_4$ (final concentration 200$\mu$M) to a solution of sodium phosphate buffer (10mM, pH 8.0) caused no significant alteration (<0.05 pH units) in the pH of the solution, both in the presence and absence of DNA.

### 3.1.2 EFFECT OF Cu(II) ON THE Tm OF CT-DNA

Addition of $CuSO_4$ (final concentration 200$\mu$M) to a solution of CT-DNA (40$\mu$M bp) in sodium phosphate buffer (10mM, pH 8.0) caused a slight increase in the mid-point of the thermal denaturation profile (Tm) compared to the DNA alone:

DNA alone, Tm = 68.3 ± 0.8°C

DNA + Cu(II), Tm = 69.5 ± 1.0°C

Both values were the means of two separate determinations. The error of reading the result from a chart recorder output was ± 0.5°C. The slightly increased Tm of the sample containing Cu(II) was therefore considered to be insignificant given the error of the determination itself.

### 3.1.3 ASSAY OF Cu(II) IN PHOSPHATE BUFFER SOLUTIONS

Copper phosphates are insoluble and so the possible removal of Cu(II) ions from solution when added to phosphate buffer solutions was examined by assaying for the copper present in the solutions. The assay was developed in this present work (section 2.2.8.2) and used (a) GSH to reduce Cu(II) to Cu(I) and (b) the production of a coloured complex ($\lambda_{max}$ = 450nm in 10mM tris-HCl buffer, pH 8.0) between Cu(I) and the Cu(I)-specific chelator, 2,9-dimethyl-1,10-phenanthroline (neocuproine).

Aliquots of a solution of Cu(II) in sodium phosphate buffer (10mM, pH 8.0) were added to a solution of neocuproine in tris-HCl buffer (10mM, pH 8.0), the Cu(II) was then reduced by addition of excess GSH ($\geq$10X [Cu(II)]) and the $\Delta A_{450}$ measured. The following results (means of two separate determinations ± deviation) were recorded:

(a) [Cu(II)] = 9.52$\mu$M, $\Delta A_{450}$ = 0.068 ± 0.003

(b) [Cu(II)] = 18.20$\mu$M, $\Delta A_{450}$ = 0.131 ± 0.002

Comparison of these experimental $\Delta A_{450}$ values to a standard curve, obtained by assay of Cu(II) in water (figure 21), produced the following values for calculated Cu(II) concentration: (a) 9.94$\mu$M, (b) 18.87$\mu$M, both ± 0.4$\mu$M. Thus, there was no significant difference in available Cu(II) present in phosphate buffer solutions compared to Cu(II) in water when assayed by this system. Thus, loss of Cu(II) by precipitation of Cu(II) as copper phosphates (or by other routes) was not detected.

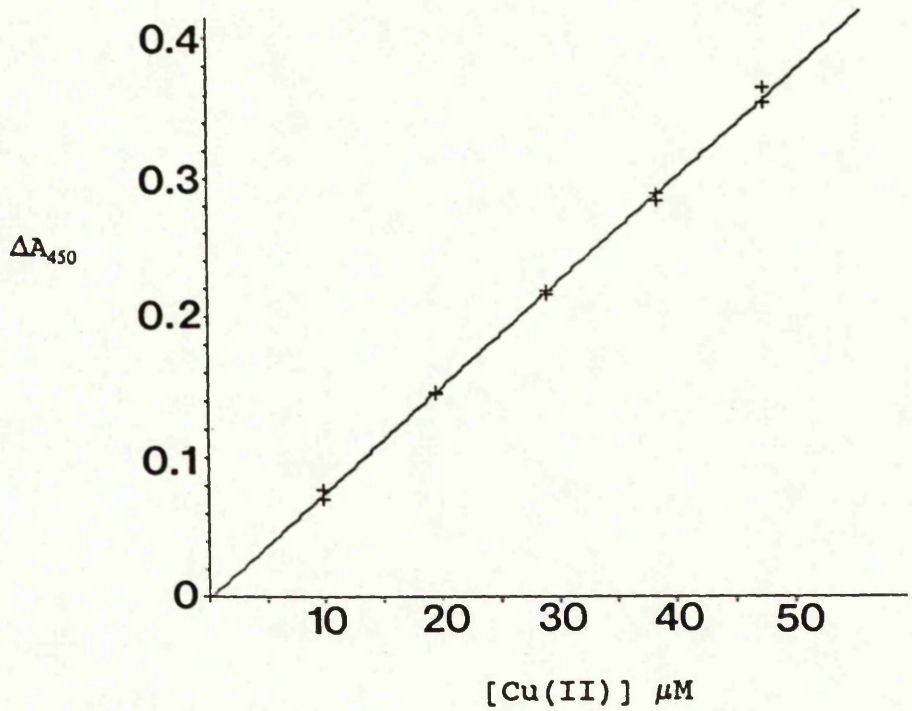



Figure 21. Standard curve of $\Delta A_{450}$ versus [Cu(II)], obtained by the method in section 2.2.8.2. Duplicate experimental points are shown corresponding to assays of Cu(II) solutions prepared in water (no buffer present). The line is a linear regression best-fit calculated from the experimental data with gradient $7.52 \times 10^{3}$ (±65.2) and intercept $-7.58 \times 10^{-4}$ ($\pm 1.90 \times 10^{-3}$), standard errors in parenthesis.

## 3.2 Cu(II):THIOL CLEAVAGE OF SUPERCOILED PLASMID DNA

### 3.2.1 TIME-COURSE

Published work (Reed and Douglas, 1989) indicated that supercoiled (SC) plasmid DNA was cleaved by relatively low concentrations of cupric ions in the presence of thiols. Those studies, however, used relatively long incubation times (up to 3 hr) at low copper and thiol concentrations. In the present studies to produce cleavage faster (<30 min), the concentrations of the reactants were altered and the extent of DNA degradation checked by agarose gel electrophoresis (Figure 22). As can be seen from figure 22, SC-DNA was cleaved by Cu(II):thiol to produce both nicked (N) and linear (L) DNA species (lanes 4 to 9). As the duration of reaction increased, the proportion of nicked DNA (N) decreased and that of the linear band increased - increased smearing toward the bottom of the gel was also seen and was due to degradation of the full-length linear plasmid DNA to smaller linear fragments. No appreciable level of DNA damage was apparent on incubation of DNA with either copper or thiol alone when compared with DNA incubated in buffer alone (compare lanes 2 and 3 with 1). Thus, under the modified reaction conditions extensive cleavage of plasmid DNA was seen after 8 to 12min of reaction. A 10min incubation time was chosen for use in further experiments.

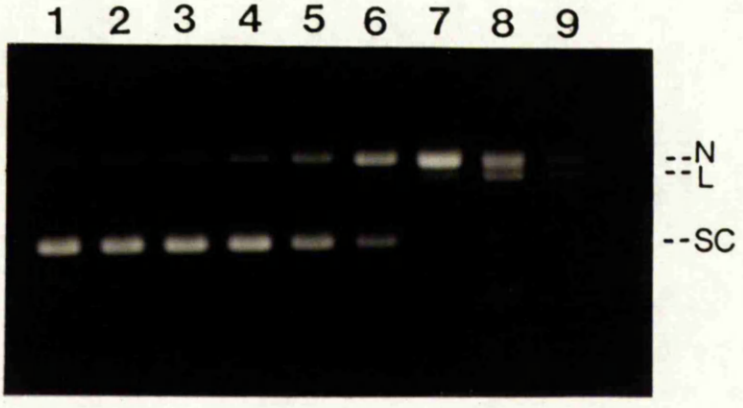



Fig. 22: Cleavage of pSP64 plasmid DNA, using the Cu(II):GSH system. Incubations contained pSP64 DNA (40$\mu$M bp) in sodium phosphate buffer (10mM, pH 8.0) with the following additions: Lane 1, none. Lanes 2 and 4-9, Cu(II) (200$\mu$M). Lanes 3-9, GSH (100$\mu$M). Incubations were carried out at 37°C for 20 min (lanes 1-3 and 9) or 1,2,4,8 and 12 min (lanes 4-8 respectively). Incubations were terminated by addition of and subsequent electrophoresis through a 0.8% agarose gel (section 2.2.4.1). DNA bands were visualised by ethidium bromide staining and polaroid photography. Positions of the various DNA forms (see text) are as indicated.


Fig. 23: Effect of metal-ion chelators on the cleavage of pSP64 plasmid DNA by the Cu(II):GSH system. Incubations contained pSP64 DNA (40$\mu$M bp) in sodium phosphate buffer (10mM, pH 8.0) with the following additions: Lane 1, none. Lane 2, EDTA (2mM). Lane 3, neocuproine (2mM). Lanes 4-10 all contained Cu(II) (200$\mu$M) plus GSH (100$\mu$M) with the following additions: Lane 4, none. Lanes 5-7, EDTA (2mM, 200$\mu$M and 20$\mu$M respectively). Lanes 8-10, neocuproine (2mM, 200$\mu$M and 20$\mu$M respectively). Incubations (10 min, 37°C) were terminated, electrophoresed and visualised as in legend to figure 22.

### 3.2.2 INHIBITION BY METAL ION CHELATORS

Published work (Reed and Douglas, 1991) had examined the effects of free radical species inhibitors and Cu(II) chelators (EDTA and citrate) on the cleavage of DNA by the Cu(II):thiol system. However, the effect of a Cu(I) chelator was not examined and therefore an experiment was carried out to compare the effects of the general divalent metal ion chelator (EDTA) and a Cu(I)-specific chelator (neocuproine) on the cleavage reaction. DNA was incubated with specific concentrations of the chelators prior to the addition of Cu(II) and thiol and the reaction allowed to proceed for 10 min. The results (figure 23) show that at EDTA or neocuproine concentrations equal to or greater than the Cu(II) concentration, the reaction was substantially inhibited (compare lanes 5, 6, 8, 9 with lane 4). At inhibitor concentrations one-tenth that of Cu(II), the reaction extent was almost indiscernible from the control sample (compare lanes 7 & 10 with 4).

These results indicate that both Cu(II) and Cu(I) are required for the DNA cleavage reaction to occur. It is likely that Cu(II) is reduced to Cu(I) by thiol and that Cu(I) is then reoxidised by molecular oxygen in solution. This reoxidation may produce oxygen-derived radicals which can cleave DNA in the vicinity of their generation.

Note: All subsequent Cu(II)-mediated cleavage reactions were terminated by addition of a 4-fold excess of EDTA.

## 3.3 Cu(II):THIOL CLEAVAGE OF LINEAR dsDNA

Cleavage of linear dsDNA fragments that had been radiolabelled at one end of one strand only (section 2.2.6) was examined by denaturing PAGE (8% acrylamide gels unless otherwise stated). The Cu(II):thiol treated samples were lyophilised before heating in formamide loading buffer to denature the strands. In this way the sites of single strand breaks on one of the duplex strands were determined. In all following results sections, labelled DNA was 3' end-labelled unless otherwise stated. The intact (non-degraded) DNA fragment appears as the uppermost (most intense) band in the following figures displaying photographs or autoradiographs of electrophoretic gels, in which the direction of electrophoresis (anode to cathode) is from the top to the bottom of the figure. Shorter lengths of DNA, i.e. degraded DNA, migrate faster and appear further down the gel (and the figure) than longer lengths of DNA.

### 3.3.1 TIME-COURSE

Treatment of the tyrT fragment (a 163bp DNA fragment derived from plasmid pKMΔ98) by the Cu(II):thiol system caused single-strand breaks to occur at all positions within the fragment (figure 24, lanes 5-9). However, it is also clear that not all positions along the fragment were cleaved with the same frequency, as judged by the variation in band intensities. There was a definite pattern of preferred sites of cleavage, a pattern which remained constant over the time-course. For analytical reasons, standard conditions for fragment cleavage were chosen to provide a limited digest of the intact DNA (i.e. where statistically, only one cut per DNA strand occurred) and therefore all further

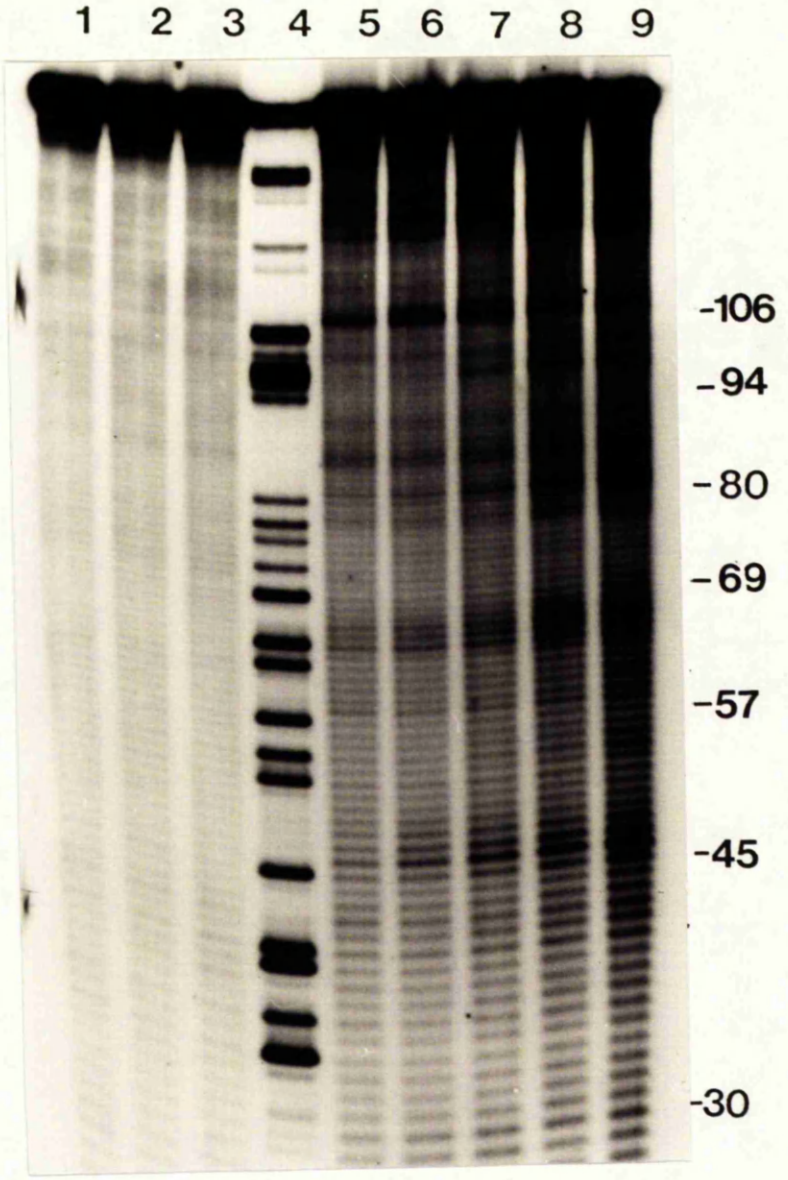



Fig. 24: Time course of Cu(II):GSH cleavage of linear tyrT DNA. Incubations contained CT-DNA (40$\mu$M bp) and 3' end-labelled tyrT DNA (<1$\mu$M bp) in sodium phosphate buffer (10mM, pH 8.0) with the following additions: Lane 1, none. Lane 2 and 5-9, Cu(II) (200$\mu$M). Lane 3 and 5-9, GSH (100$\mu$M). Incubations were carried out at 37°C for 20 min (lanes 1-3 and 9) or 2,4,8 and 12 min (lanes 5-8, respectively). All incubations were terminated, analysed by denaturing PAGE and autoradiographed as in Methods. Lane 4 was tyrT DNA cleaved specifically at guanines (G-track; see section 2.2.6.4.1). Numbering refers to that shown in fig. 16.

reactions were standardised to 10min duration under the above conditions (see legend to figure 24).

### 3.3.2 EFFECT OF THIOL STRUCTURE ON CLEAVAGE PATTERN

Figure 25 shows the effect of varying the structure of the thiol used in the Cu(II):thiol DNA cleavage reaction. The four thiols tested (i.e. D- and L-cysteine, glutathione and 3-mercaptopropionic acid) all degraded the tyr T DNA to produce closely similar DNA cleavage patterns (lanes 4 to 7). This showed that the apparent sequence preference of the Cu(II):thiol system was grossly unaffected by the nature of the thiol. The sites of preferred cleavage are tabulated in Table 2. Also apparent from figure 25 is that DTT (a dithiol) failed to produce DNA cleavage under these conditions. Further experiments using DTT and the dithiols 1,2-dimercaptopropanol and 1,3-propane dithiol, showed that DNA cleavage was obtained only after increased incubation time (results not shown).

### 3.3.3 CLEAVAGE OF VARIOUS DNA FRAGMENTS OF KNOWN SEQUENCE

To examine whether the Cu(II):thiol system was exhibiting a true sequence-dependent cleavage of DNA, additional linear DNA fragments of defined sequence were studied. Thus, two fragments of pBR322 DNA (a 167bp and 517bp fragment) were cleaved using the Cu(II):thiol system.

Figures 26 and 27 show the patterns of cleavage obtained with four different thiols and Cu(II) for the 167bp and 517bp fragments, respectively. Note that the 167bp fragment, when cleaved, exhibited a strong band at

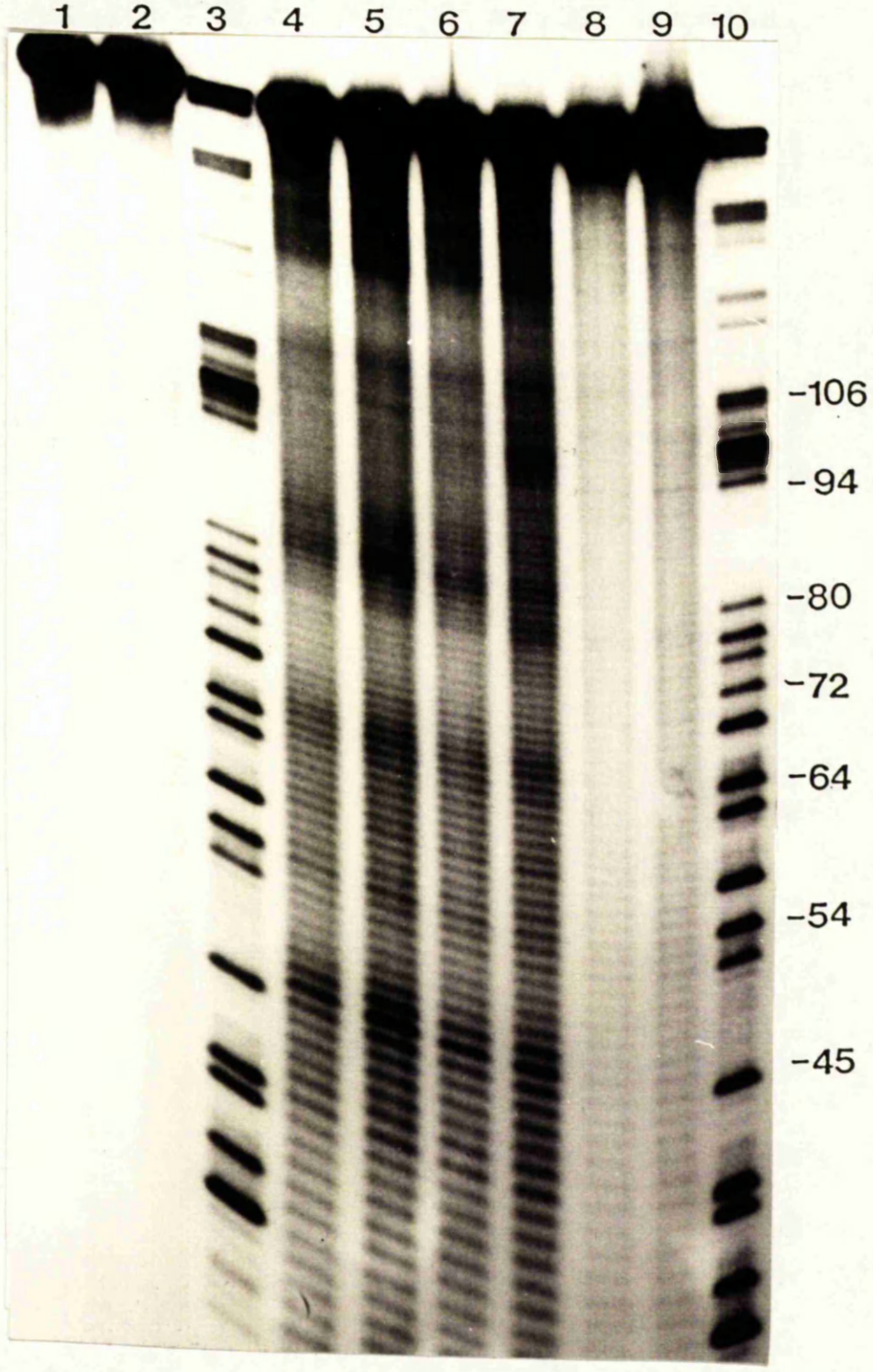



Fig. 25: Effect of varying thiol structure on Cu(II): thiol cleavage of tyrT DNA. DNA and buffer concentrations were as in legend to figure 24 with the following additions: Lanes 1, none. Lanes 2 and 4-8, Cu(II) (200$\mu$M). Lanes 4-9, D-cys, L-cys, GSH, 3-MPA, DTT and GSH, respectively (all 100$\mu$M). Incubations (10min, 37°C) were terminated, electrophoresed and autoradiographed as in Methods. Lanes 3 and 10, tyrT G-tracks. Numbering refers to that shown in fig. 16.

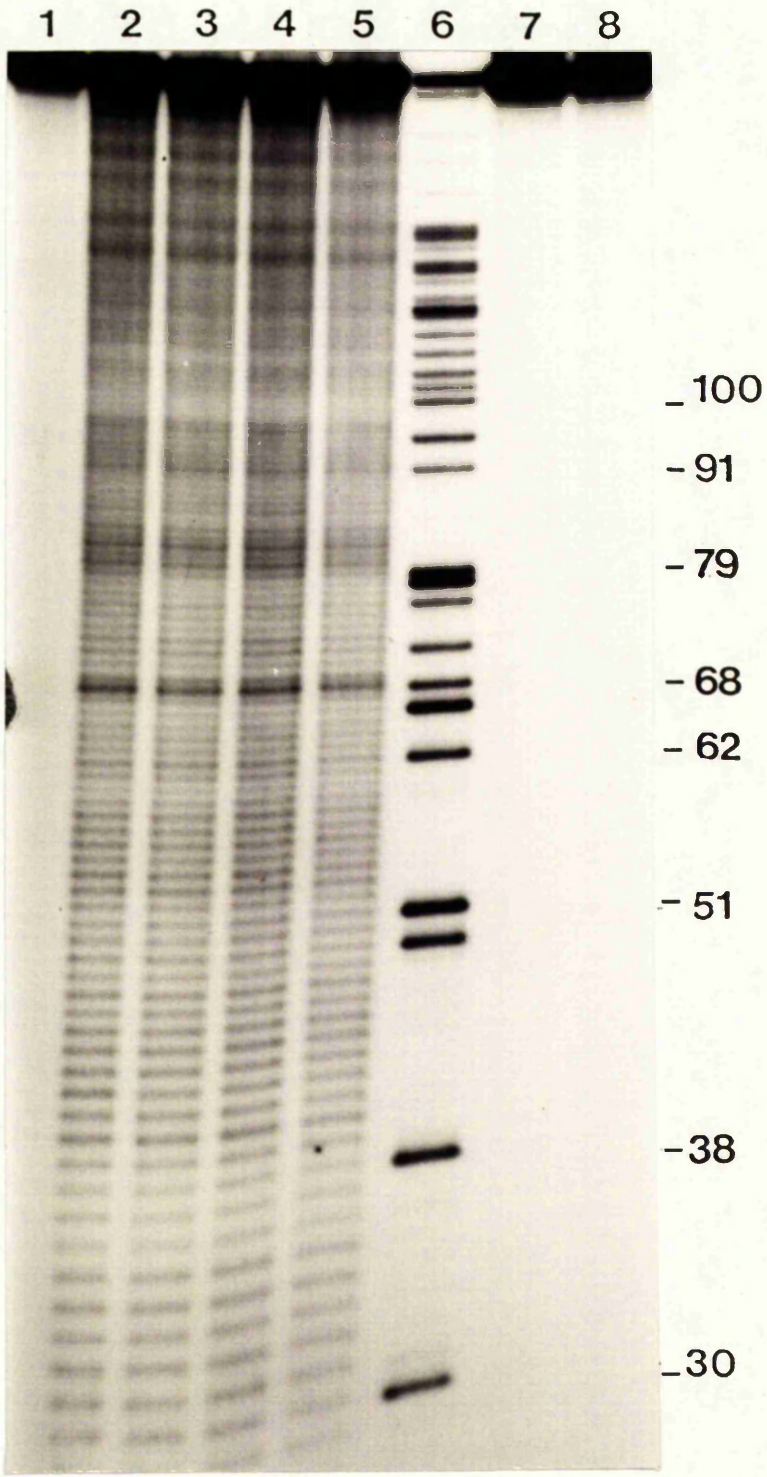



Fig. 26: Cleavage of 167bp fragment of pBR322 by Cu(II):thiol. DNA and buffer concentrations as in legend to fig.24 with the following additions: Lane 1, none. Lanes 2-5 and 8, D-cys, L-cys, GSH, 3-MPA and GSH, respectively, at 100$\mu$M. Lanes 2-5 and 7, Cu(II) (200$\mu$M). Incubations (10 min, 37°C) were terminated, electrophoresed and autoradiographed as in Methods. Lane 6, 167bp G-track (Numbering refers to that shown in fig. 17).

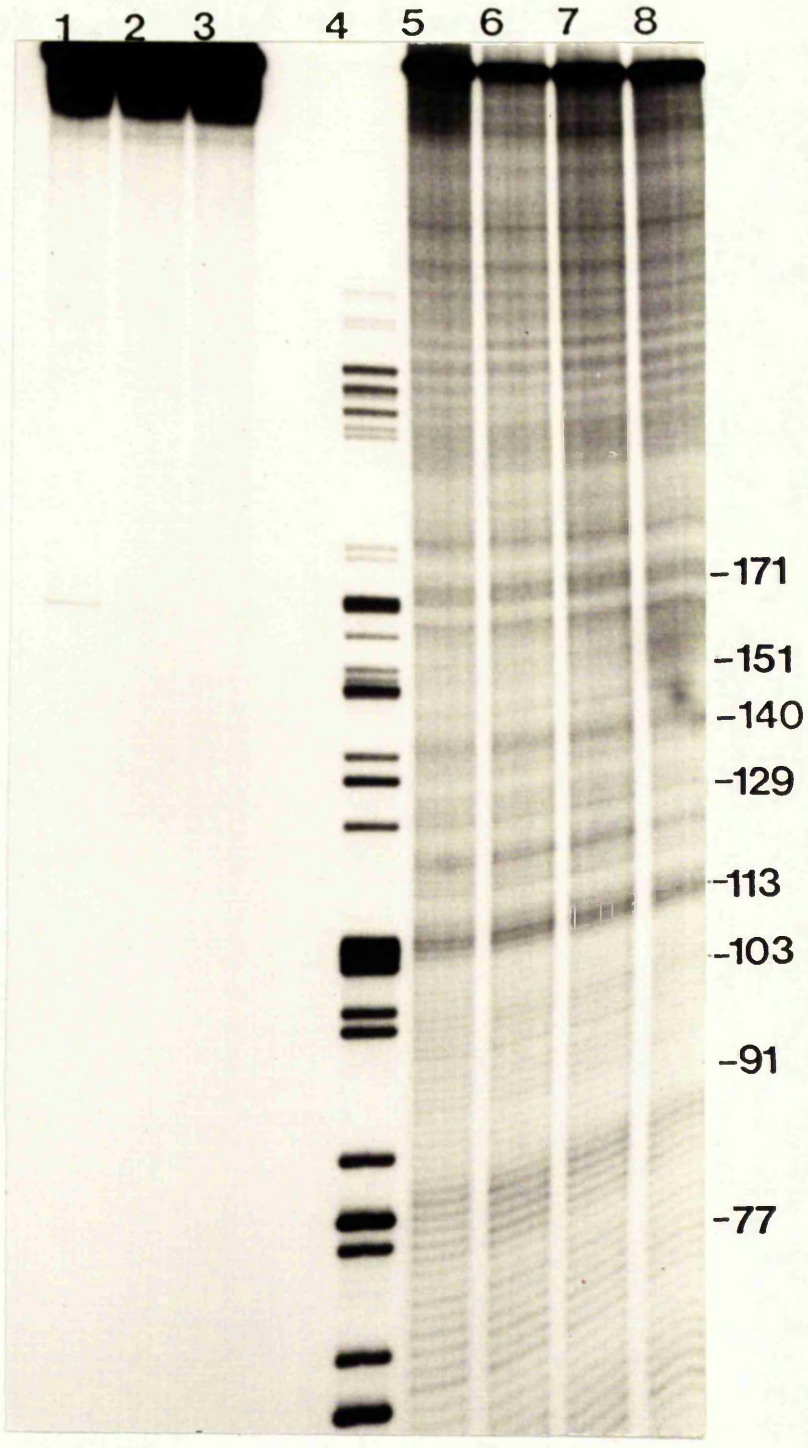



Fig. 27: Cleavage of 517bp fragment of pBR322 by Cu(II):thiol. DNA and buffer concentrations as in legend to fig.24 with the following additions: Lane 1, none. Lanes 2 and 5-8 , Cu(II) (200$\mu$M). Lanes 3 and 5-8, GSH, D-cys, L-cys, GSH and 3-MPA respectively, at 100$\mu$M. Incubations (10 min, 37°C) were terminated, electrophoresed and autoradiographed as in Methods. Lane 4, 517bp fragment G-track (numbering refers to that shown in fig. 18).

position 68/69 which was attributed to a band compression resulting in the co-migration of bands 68 and 69 (one band is missing between G68 and G72 and is therefore compressed). This compression effect is evident in published work using this DNA fragment (e.g. Harshman and Dervan, 1985) but was not commented upon. Therefore, it was concluded that this band did not correspond to a preferentially cleaved DNA site. It is clear from these figures, however, that both fragments were cleaved with different apparent sequence preferences. The sequence preference was characteristic of the piece of DNA used but occurred independently of the nature of the thiol used (c.f., tyrT, section 3.3.2). The variation in band intensities for each fragment (including tyrT, figure 25) were, however, not simply dependent on DNA sequence; comparison of preferred cleavage sites on all 3 fragments (Table 2) showed no sequence correlation.

### 3.3.4 DETERMINATION OF BASE-LABILE SITES

Damaged bases of DNA can be removed by treatment with piperidine as in the Maxam and Gilbert strand-scission protocol (section 2.2.6.4.1) - piperidine attacks C1′ of deoxyribose, displaces the altered base and strand-scission results (leaving 3′ and 5′ phosphate ends). Thus, the base damage caused by Cu(II):thiol treatment was determined by the following procedure: Cu(II):thiol treated DNA samples (section 2.2.7.1) were each split into two aliquots: one set of aliquots was lyophilised and redissolved in loading buffer; the other set was treated with piperidine, lyophilised and redissolved in loading buffer. Figures 28 and 29 show the cleavage patterns obtained for the 167bp and 517bp fragments following Cu(II):thiol treatment and Cu(II):thiol treatment

Table 2 : Preferred cleavage sites for the Cu(II):thiol system.

| DNA fragment | Cleavage site[a] | Sequence[b] (3'-5') |
|---|---|---|
| tyrT (163bp) | 45-47 | CAAGTAAAAA |
| | 64-67 | TGTGAAATGT |
| | 80-83 | GCAGTAAACT |
| | 97-99 | GCGGGGCGAA |
| | 107-109 | GGGCTATTCC |
| 167bp | 53-58 | GTCAATTTAA |
| | 79-84 | TGGCACATAC |
| | 91-97 | TAGATTGTTA |
| | 108-111 | TAGCAGTAGG |
| | 123-124 | GTGGCAGTGG |
| 517bp | 83-87 | CACCGTGAAA |
| | 111-113 | TTGGGGATAA |
| | 123-124 | AAATAAAAAG |
| | 129-134 | AAGATTTATG |
| | 171-174 | GGGGACTATT |

a - Cleavage sites on the 3' end-labelled strand (see figures 16-18 for full sequences); data taken from figures 25-27.

b - Preferred cleavage sites (underlined) and flanking sequences are shown.

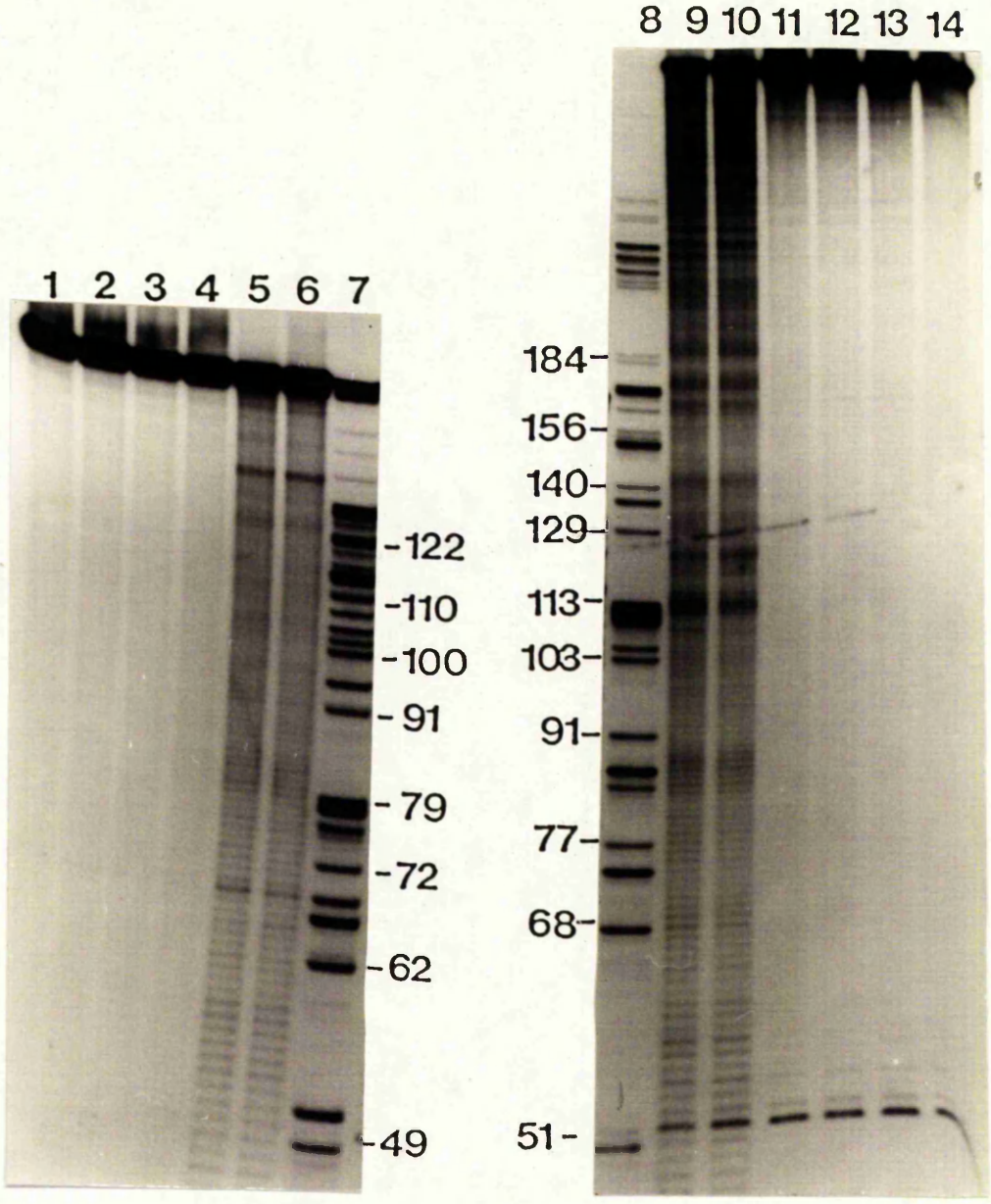



Fig. 28: Cleavage of 167bp and 517bp DNA fragments by Cu(II):reductant system. DNA and buffer concentrations as in legend to fig.24 using 167bp (lanes 1-6) and 517bp fragments (lanes 9-14). The following additions were made: Lanes 1 and 14, none. Lanes 2,5,6,9,10 and 13, Cu(II) (200$\mu$M). Lanes 3,5,9 and 11, GSH (100$\mu$M). Lanes 4,6,10 and 12, ascorbate (40$\mu$M). Incubations (10min, 37°C) were terminated, electrophoresed and autoradiographed as in Methods. The strong band at position 52 (lanes 9-14) is due to limited cleavage of the 517bp fragment during preparation. Lanes 7 and 8, 167bp and 517bp G-tracks, respectively.

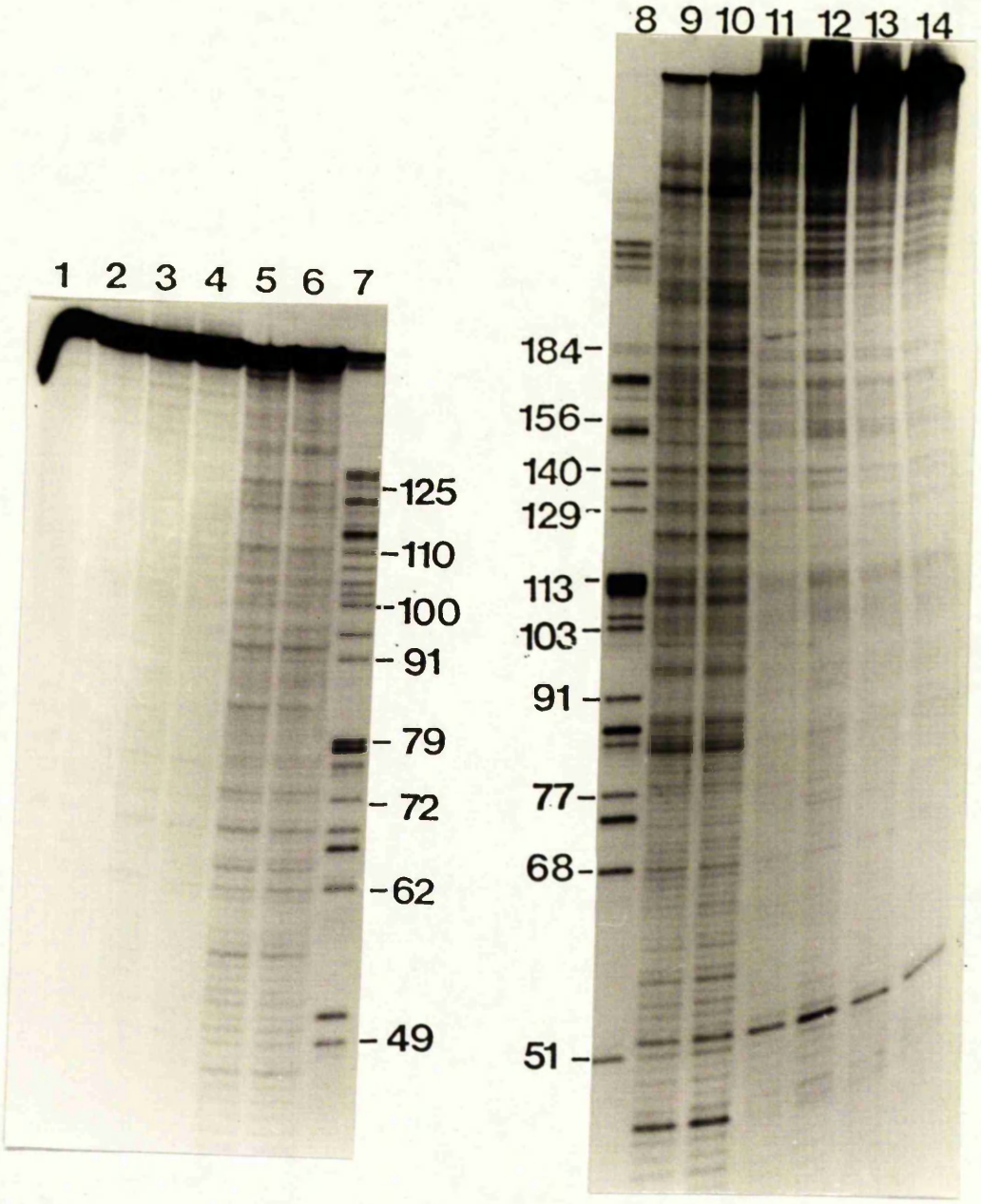



Fig. 29: Detection of base-labile sites caused by Cu(II):reductant cleavage of 167bp and 517bp DNA fragments. Samples and lane designations identical to those in fig.28 except that all samples (lanes 1-6, 9-14) were treated with piperidine (as in Methods, section 2.2.6.4.1) <u>after</u> Cu(II):reductant treatment.

plus piperidine treatment, respectively. Figure 29 shows that for the 167bp and 517bp fragments, control DNA, and DNA treated with Cu(II) or thiol alone (lanes 1-4 and 11-14), showed low levels of damaged base sites on piperidine treatment, even though strand-scission was not observed in the corresponding samples which had not been treated with piperidine (figure 28, lanes 1-4 and 11-14). Most or all of these base-labile sites were at G residues, in accord with previous literature of analogous experiments (e.g. Jeppesen & Nielsen, 1988). If this background cleavage at G residues is borne in mind then it is clear from the data presented in figures 28 and 29 (summarised in Table 3) that piperidine treatment of DNA cleaved with Cu(II):thiol detected distinct base damage occurring predominantly at T residues.

### 3.3.5 DETERMINATION OF THE NATURE OF THE DNA TERMINI GENERATED BY CU(II):THIOL CLEAVAGE

#### 3.3.5.1 End-determination (5')

To determine whether the termini of Cu(II):thiol degraded DNA possessed 5'-phosphoryl groups a sample of the 517bp fragment was treated with Cu(II):thiol (section 2.2.7.1), ethanol precipitated and then treated with calf intestinal alkaline phosphatase (CIP) which catalyses the dephosphorylation of the 5'-phosphoryl group to a 5'-hydroxyl. Since the removal of a phosphate group from the DNA reduces its overall charge it is possible to distinguish the 5'-phosphoryl and 5'-hydroxyl terminated fragments by their differences in electrophoretic mobility.

A reduction in electrophoretic mobility was clearly seen when Cu(II):thiol

Table 3 : Base-labile sites generated by the Cu(II):thiol system.

| Fragment | Cleavage site[a] | Sequence[b] (3'-5') |
|---|---|---|
| 167bp | 47 | ATA |
| | 56 | ATT |
| | 61w,62 | ACGA |
| | 64 | ATA |
| | 73 | GTC |
| | 76w,77 | CGTG |
| | 84 | ATA |
| | 91w,93 | AGATT |
| | 96 | GTT |
| | 104,105 | AGTA |
| 517bp | 57 | ATT |
| | 60 | ATT |
| | 68 | AGA |
| | 77w,78w | AGTC |
| | 80w | CCA |
| | 83,84,85w | CCGTG |
| | 87 | GAA |
| | 95,96 | CTTT |
| | 100 | ACA |
| | 102-105 | ACGCGC |
| | 108,109 | CTTG |
| | 111-113 | GGGGA |
| | 123 | ATA |
| | 129 | AGA |
| | 131 | ATT |
| | 137 | GTA |
| | 141,142 | GTTT |

a - Preferred cleavage sites on the 3' end-labelled strand (see figures 17 & 18 for full sequences); w = weak cleavage enhancement. Data from figure 29.

b - Preferred cleavage sites (underlined) and flanking nucleotides shown.

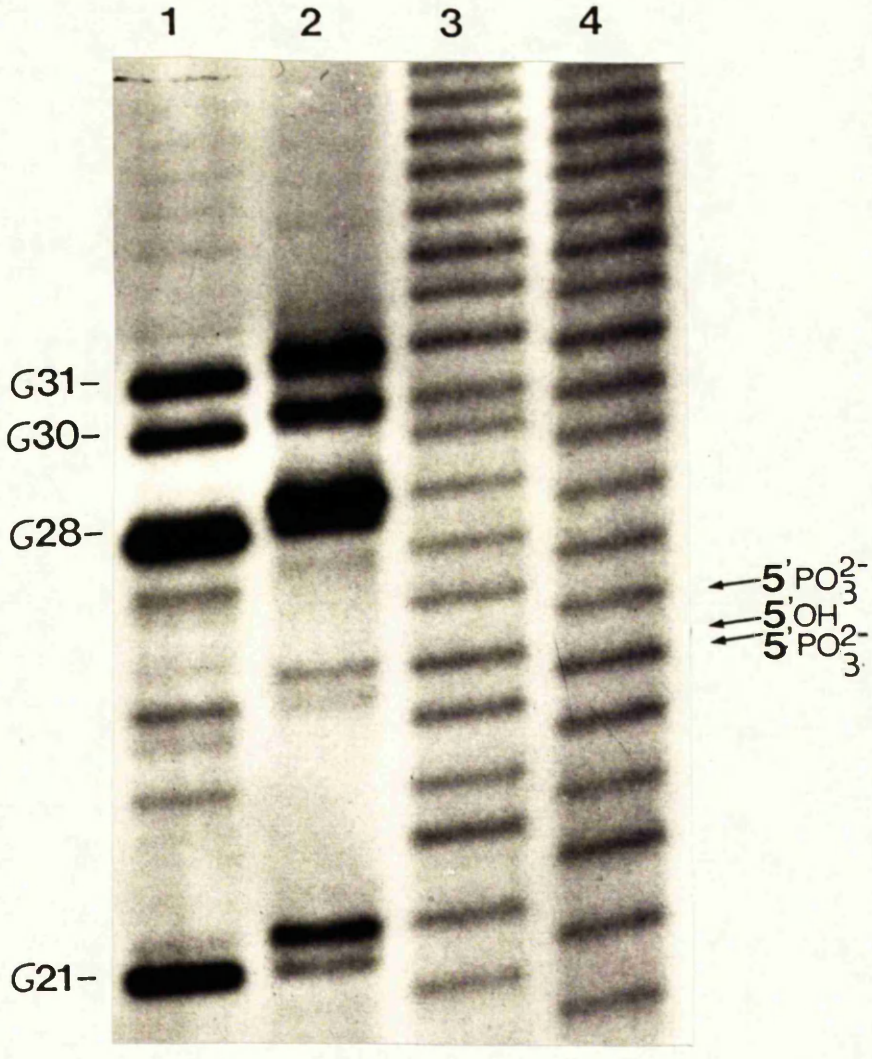



Fig. 30: 5' end-determination of Cu(II):thiol cleaved 3' end-labelled 517bp DNA fragment. Lanes 1 and 2, 517bp DNA G-tracks. Lanes 3 and 4, DNA incubated with Cu(II) (200$\mu$M) and GSH (100$\mu$M) for 10 min at 37°C under conditions given in legend to fig.24. Lanes 2 and 3, samples further treated with calf-intestinal alkaline phosphatase (see Methods section 2.2.5.2). Incubations were run on a 20% polyacrylamide denaturing gel and autoradiographed (see Methods). The lower end of the gel is shown, where maximal separation of the bands occurred (the numbering scheme is as shown in fig.18).

treated DNA was treated with CIP (c.f., lanes 3 & 4, figure 30) indicating that dephosphorylation had occurred. A G-track of the fragment (lane 1; 5′ phosphoryl ends) and a CIP treated G track sample (lane 2; 5′ hydroxyl ends) were included as controls. Thus, Cu(II):thiol digestion of 3′ end-labelled DNA produced DNA fragments bearing exclusively a 5′ phosphate group (significant bands corresponding to fragments bearing 5′ hydroxyl groups were not observed).

3.3.5.2 End determination (3′)

An analogous experiment to that of the 5′ end-determination was carried out except that 5′ end-labelled DNA was used (instead of 3′), and T4 polynucleotide kinase was used as a 3′-phosphatase (section 2.2.5.3). The apparently single, electrophoretic bands (lane 2, figure 31) formed by Cu(II):thiol degradation of 5′ end-labelled DNA were found to be made up of two separate species (lane 3, figure 31). On dephosphorylation, one of the pair of bands (the so-called "phosphatase-inert band") remained in place, the other band became reduced in mobility and co-migrated with DNAseI-digested fragment (lane 4, figure 31; 3′-hydroxyl ends). The phosphatase-inert band migrated just in front of the G-track marker fragments (lane 1, figure 31; 3′-phosphate ends), indicating a similarly charged terminal group. Thus, Cu(II):thiol digestion of 5′ end-labelled DNA produced 3′-phosphate termini and "3′-phosphate-like", phosphatase-inert termini, which probably possess a similar charge to 3′-phosphate terminated fragments.

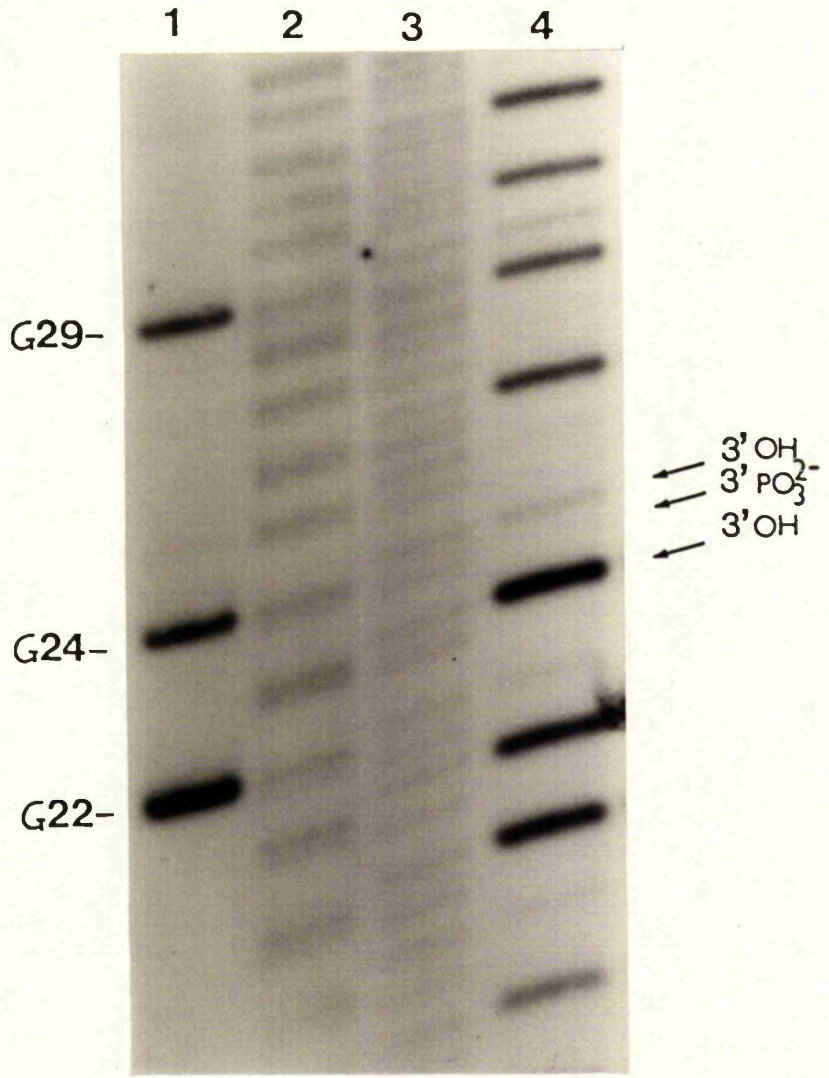



Fig. 31: 3' end-determination of Cu(II):thiol cleaved 5' end-labelled 517bp DNA fragment. Lane 1, G-track. Lanes 2 and 3, DNA incubated with Cu(II):GSH as in legend to fig.30. Lane 4, DNA incubated with DNAse I (see Methods, section 2.2.7.3) for 5 min at 37°C. Lane 3, sample further treated with T4PNK (see Methods, section 2.2.5.3). Electrophoresis and autoradiography as in legend to fig.30. The lower end of the gel is shown where maximal separation of the bands occurred (the numbering scheme is as in fig.18).

### 3.3.6 COMPARISON OF PREFERRED CLEAVAGE SITES FOR BOTH STRANDS OF A DNA DUPLEX

The 517bp fragment generated by EcoRI/RsaI double-digest of pBR322 was radiolabelled at either the 3′ or 5′ ends (section 2.2.6). Samples of fragment labelled at either the 3′ or 5′ ends were subjected, independently, to Cu(II):thiol cleavage and the products analysed by denaturing PAGE along with their corresponding G-tracks (figure 32). It is apparent that the positions of cleavage maxima were not at identical positions on both strands, but were shifted by two to four nucleotides in either the 5′ or the 3′ direction and that, in general, along the entire sequence of the fragment cleavage maxima on one strand roughly coincided with cleavage minima on the other strand (figures 32 and 33).

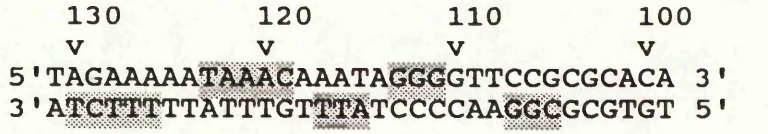



Figure 33. Comparison of major Cu(II):thiol cleavage sites on both strands of duplex DNA. Shaded boxes indicate regions of preferred cleavage (data taken from figure 32).

### 3.3.7 CLEAVAGE OF NATIVE (DUPLEX) AND HEAT-DENATURED DNA

Cu(II):thiol cleavage of tyrT DNA was carried out on both native (duplex) and heat-denatured DNA (90°C, 5min followed by snap-cooling on ice-water). Comparison of the cleavage patterns obtained (figure 34) showed that:

(i) both forms of the fragment were cleaved by Cu(II):thiol and

(ii) the cleavage patterns were non-identical with two major positions of cleavage for the heat-denatured fragment at positions 65-67 (3′AAA)

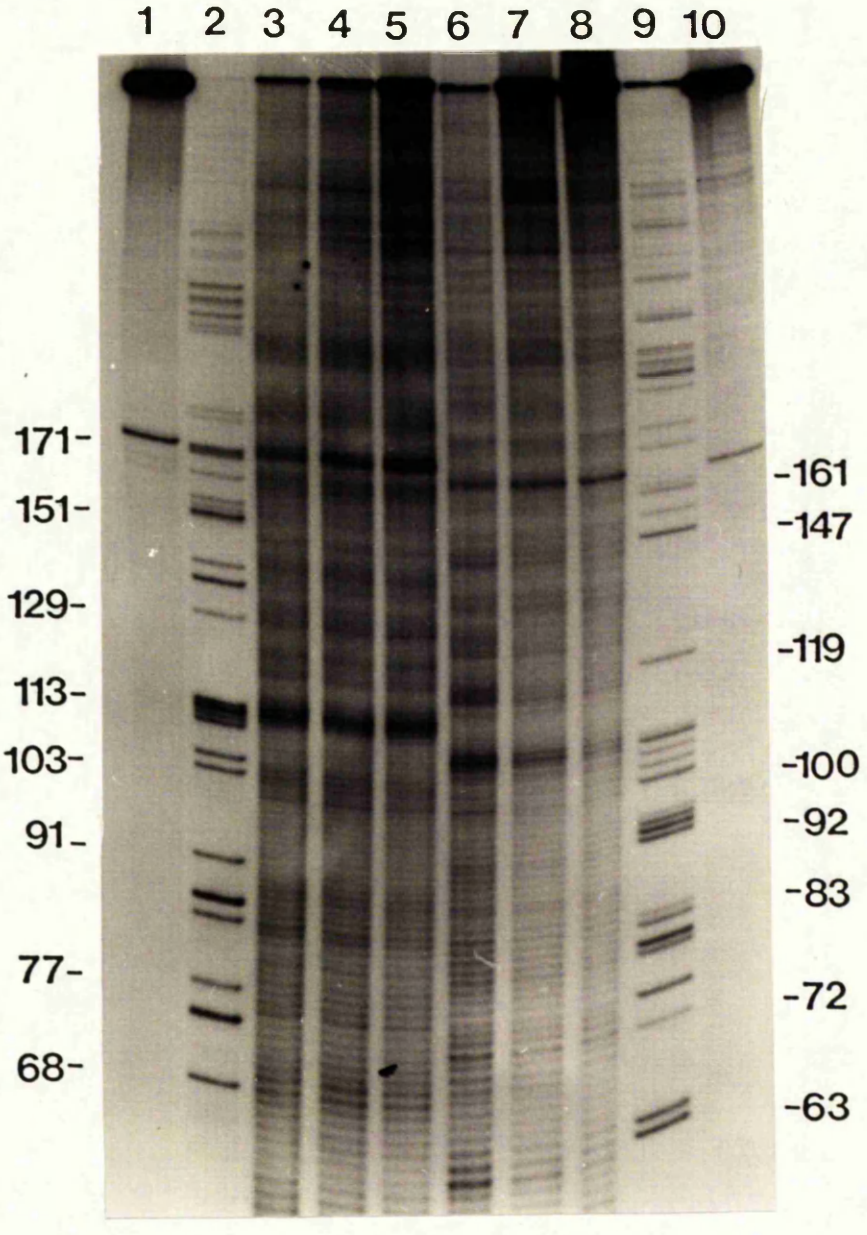



Fig. 32: Comparative cleavage of both strands of duplex 517bp fragment. Lanes 1-5 contained 3' end-labelled DNA. Lanes 6-10 contained 5' end-labelled DNA. DNA and buffer conditions as in legend to fig.24 with the following additions: Lanes 1 and 10, none. Lanes 2 and 9,G-track samples. Lanes 3-5 and 6-8, DNA incubated with Cu(II) (200$\mu$M) and GSH (100$\mu$M) for 15, 10 and 5min, respectively. Incubations were terminated, electrophoresed and autoradiographed as in Methods. The 517bp samples had a slight impurity evidenced as a band (of low intensity) migrating at approx. 170bp which was probably due to contamination by 167bp fragment. Effects of the 167bp impurity on the 517 bp degradation patterns were considered to be negligible.

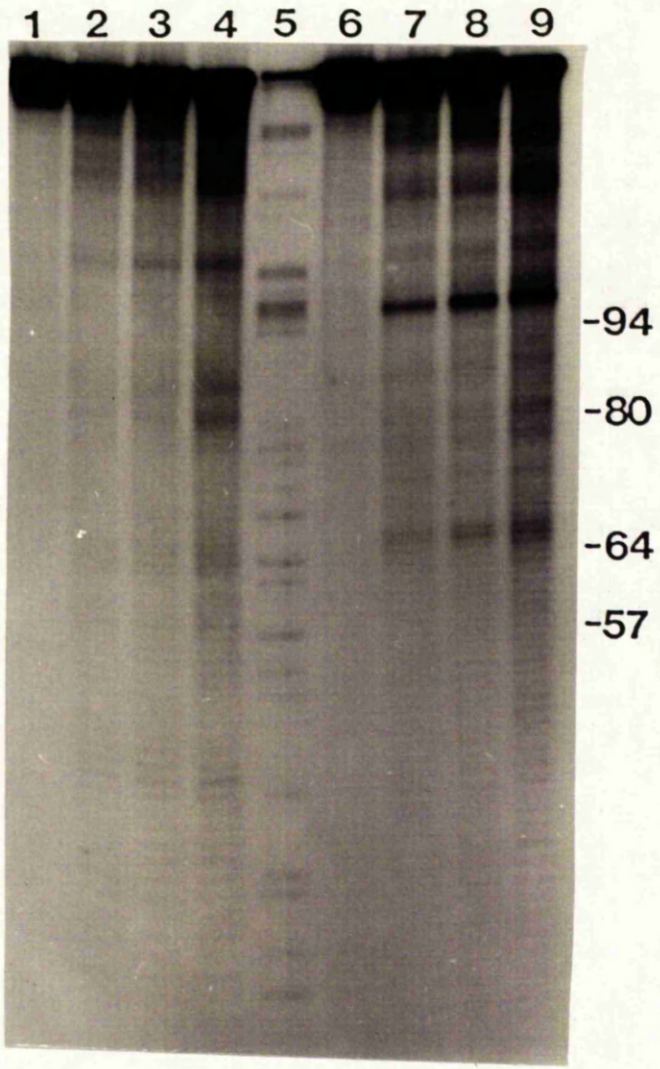



Fig. 34: Comparative cleavage of native and heat denatured tyrT DNA. DNA and buffer conditions were as in the legend to fig.24 except that lanes 1-4 contained native tyrT DNA and lanes 6-9 contained heat-denatured tyrT DNA (90°C, 5 min followed by snap-cooling on ice-water). Incubations with Cu(II) (200μM) and GSH (100μM) were carried out for 5min (lanes 2 and 7), 10min (lanes 3 and 8) or 20min (lanes 1, 4, 6 and 9) at 37°C. Termination, electrophoresis and autoradiography were as in Methods. Lane 5, tyrT G-track. (Numbering scheme is as in fig.16).

and 97-98 (3'GG) which were not present for the native fragment. The denaturation procedure was checked against alternative procedures (section 3.3.8) and the denatured fragment probed with ss-specific reagents (section 3.3.9), in order to rule out effects due to the presence of any residual dsDNA or to the presence of small regions of dsDNA on the observed cleavage patterns of denatured tyrT DNA.

### 3.3.8 DENATURATION OF tyrT DNA

The ability of the heat denaturation procedure to denature DNA to the same extent as alkali and organic solvent procedures was verified by non-denaturing PAGE of the products of various denaturation procedures (figure 35). Comparison of the mobility of heat-denatured tyrT DNA (lanes 4-6) with that of native DNA (lane 1), DNA incubated with formamide, (lanes 2 and 3), and DNA incubated with NaOH (lane 9) revealed that all three denaturation procedures gave similar extents of denaturation with the denatured DNAs migrating at similar positions in the gel. Also, figure 35 shows that addition of Cu(II), up to 0.2mM, following the heat denaturation step did not lead to any renaturation of the single strands (lanes 7 and 8). It is, however, possible that small regions of dsDNA remained undetected given the resolution of this method. Thus, probing for ss regions was carried out (section 3.3.9).

### 3.3.9 CHEMICAL PROBING OF tyrT DNA

The chemicals $KMnO_4$ and DEPC can react readily with structures of DNA that expose the thymine double bond and the N7 of purines (adenine and

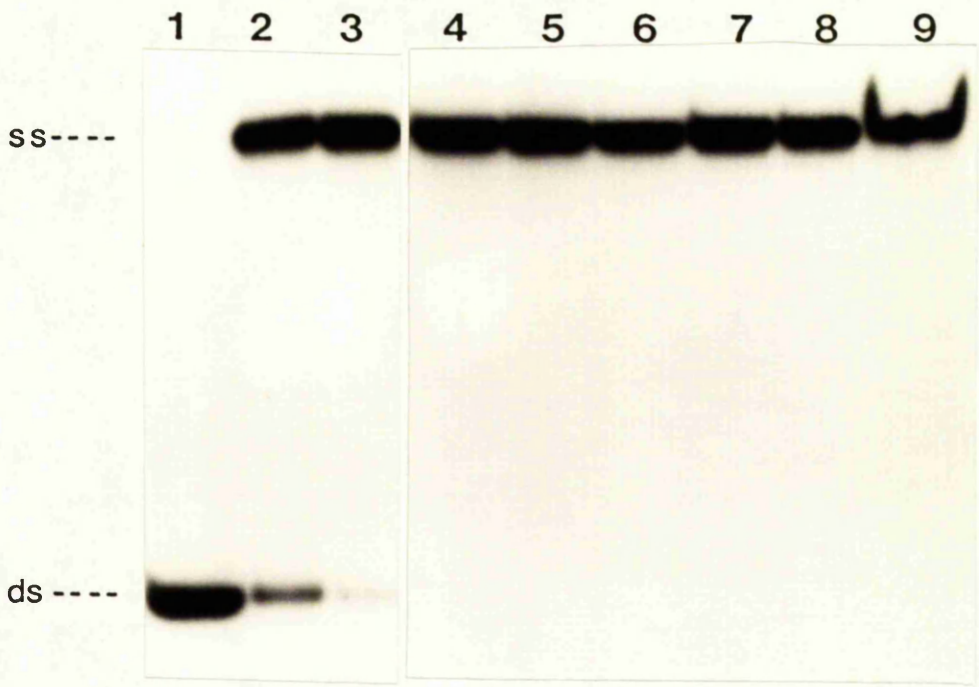



Fig. 35: Comparison of denaturation procedures. Samples of tyrT DNA (<1μM) plus CT-DNA (40μM) in sodium phosphate buffer (10mM,pH8.0) were treated as follows: Lane 1, no treatment. Lanes 2 and 3, formamide added to 40% and 74% (v/v), respectively. Lanes 4-6, heated (90°C) for 3, 5 and 10 min respectively before snap-cooling on ice-water. Lanes 7 and 8, heated as for lane 5 sample, followed by incubation (5 min, 37°C) with Cu(II) at 0.2mM and 0.1mM respectively. Lane 9, NaOH added to 0.2M. All samples (10μl) were prepared for electrophoresis by addition of agarose gel electrophoresis loading buffer (section 2.2.4.1) and electrophoresed through a 12% polyacrylamide (1:30 cross-linked) non-denaturing gel at 250V (8mA) for 16 hr before covering in Saran wrap and autoradiography (section 2.2.4.3). Positions of native (ds) and denatured (ss) DNA are as indicated.

guanine), respectively (see Section 1.3.4). Both reagents react readily with ssDNA and were used therefore to probe the heat-denatured fragment for ssDNA regions. Reaction of the probes with their respective DNA bases yields piperidine-labile sites (see section 1.3.4) - on treatment with piperidine the damaged bases (e.g. alkylated, or ring-opened) are displaced from $C_1$. of deoxyribose and a single-strand break occurs at the phosphodiester bond at the position of the nucleotide bearing the damaged base. Thus, native and heat-denatured tyrT DNA samples were treated first with $KMnO_4$ or DEPC and then with piperidine (see methods sections 2.2.7.5 and 2.2.7.6, respectively) and the resulting samples analysed by denaturing PAGE.

From figure 36 it can be seen that native DNA was fairly insensitive to these reagents giving a low level of background cleavage (lanes 1-3 and 9-11). It was clear, however, that all the relevant bases in the postulated ssDNA fragments reacted as expected with $KMnO_4$ (lanes 12-14) and DEPC (lanes 5-7) throughout the length of the fragment (see figure 37), including the regions of enhanced cleavage by Cu(II):thiol. Thus, the presence of non-denatured regions in the heat-denatured DNA was ruled out. (Probing in the presence of Cu(II) was not carried out).

## 3.4 OTHER COPPER-MEDIATED DNA CLEAVAGE SYSTEMS

### 3.4.1 COMPARATIVE CLEAVAGE OF SC DNA BY COPPER-MEDIATED SYSTEMS

Replacement of the reductant thiol by ascorbate (also a reductant) or hydrogen peroxide (usually an oxidant) can also produce DNA damage (section

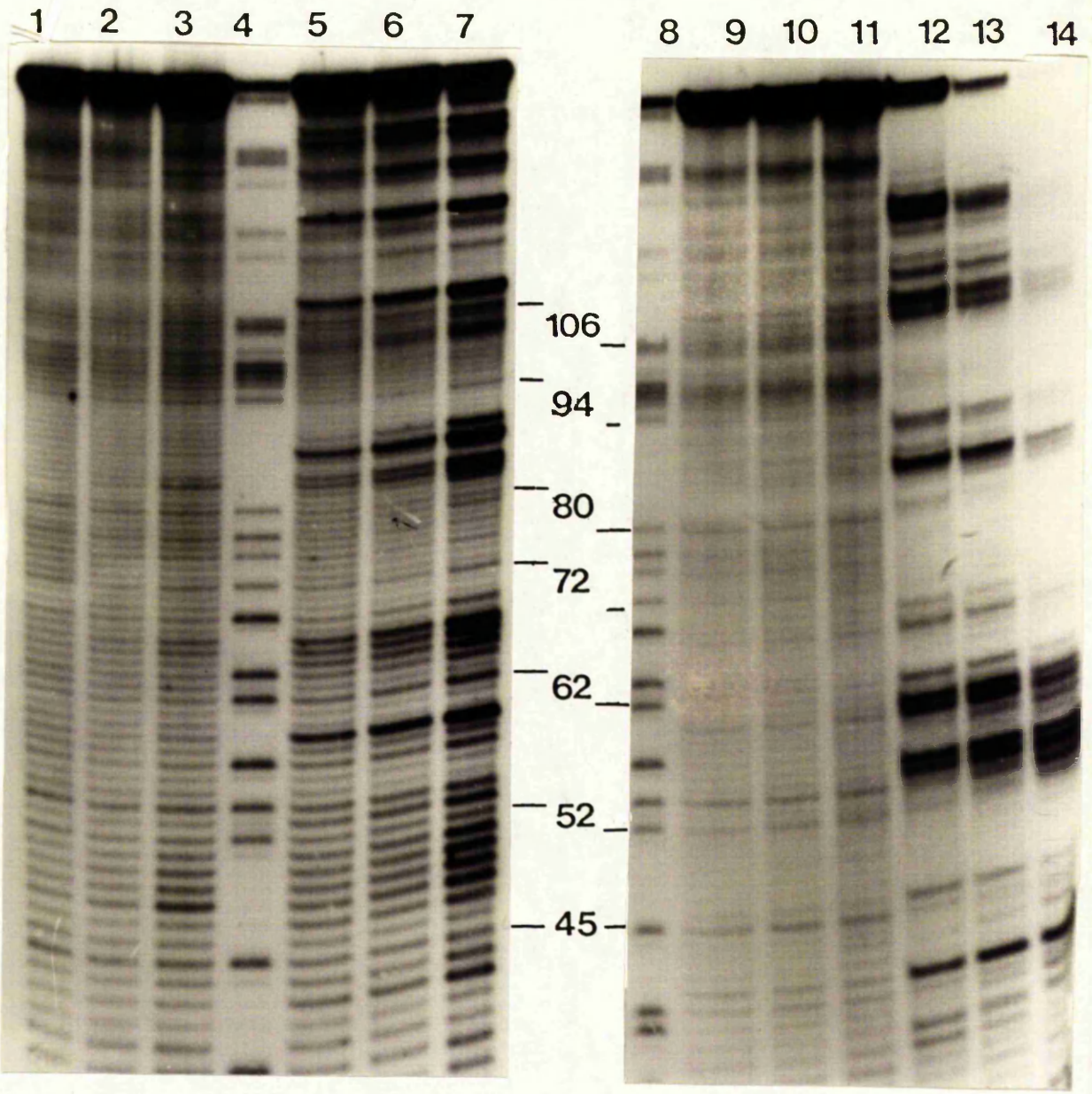



Fig. 36: Probing of DNA using DEPC and $KMnO_4$. Samples of native tyrT DNA (lanes 1-3 and 9-11) and heat denatured tyrT DNA (90°C, 5 min; lanes 5-7 and 12-14) were probed with DEPC (lanes 1-3 and 5-7) or $KMnO_4$ (lanes 9-14) under the conditions given in Methods (sections 2.2.7.5 and 2.2.7.6 respectively). Lanes 4 and 8, tyrT G-tracks. Samples were electrophoresed and autoradiographed as in Methods. (Numbering corresponds to that in fig.16).

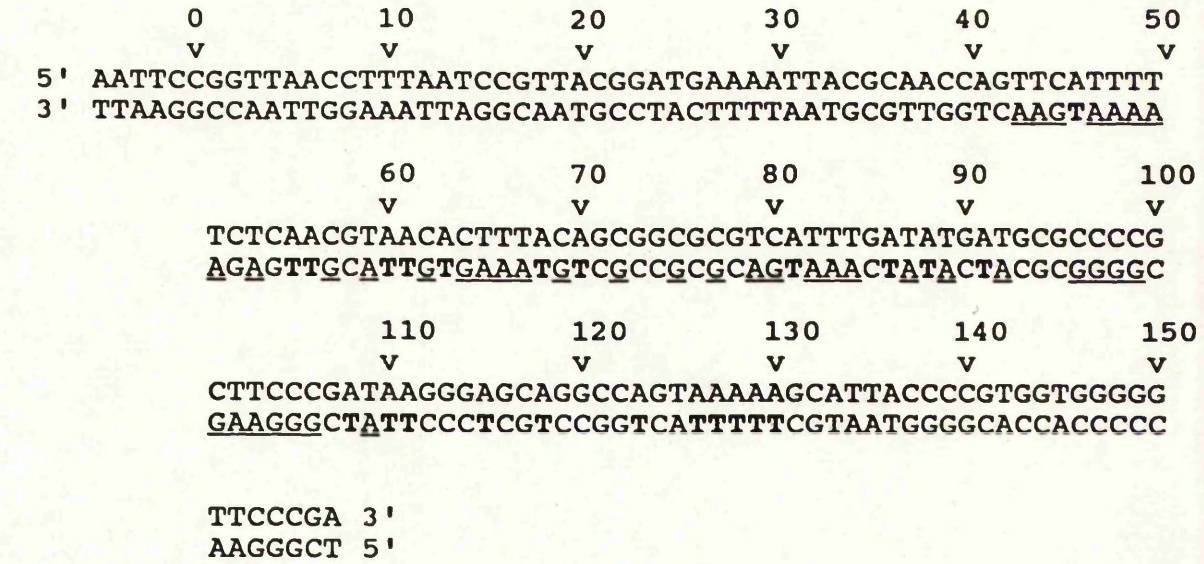



Figure 37. Nucleotide sequence of the 163bp tyrT fragment indicating the positions of piperidine-labile sites generated by modification of denatured tyrT DNA with ss-specific probes : positions modified by DEPC (underlined) and $KMnO_4$ (bold), data taken from figure 36. Modifications of the 3' end-labelled, lower strand are shown ; detection of modifications of the upper strand were not carried out.

1.3.2.2). The extent of DNA damage produced by the three systems was determined at constant copper concentration and a single time point (figure 38). The relative efficiency of the reagents (as judged by the relative amounts of cleavage at different reagent concentration) was found to be ascorbate (most effective) > GSH > $H_2O_2$. Approximate concentrations required to produce cleavage to nicked and linear DNA were ascorbate (20 $\mu$M), GSH (200 $\mu$M) and $H_2O_2$ (>0.2 mM < 2 mM). The reducing agents $\beta$-NADPH and dithionite were also tested in this system but were found to be much less effective than $H_2O_2$; typical concentrations and incubation times required to produce total conversion of sc to nicked and linear DNA were $\geq$2mM and 2hr (results not shown). Dithionite was slightly less effective than $\beta$-NADPH.

Although the ascorbate and $H_2O_2$ systems have been described previously (section 1.3.2.2 and references therein), a comparison of the sequence-dependence of cleavage by these three reagents on a single piece of DNA of defined sequence has not. A comparison of the cleavage patterns produced by all three of the copper-mediated systems was, therefore, carried out.

### 3.4.2 COMPARATIVE CLEAVAGE OF tyrT DNA BY COPPER-MEDIATED SYSTEMS

The cleavage patterns produced by cleavage of tyrT DNA with three copper-based systems were as shown in figure 39. Incubation of tyrT DNA with Cu(II) and either GSH or ascorbate (lanes 5 & 6 respectively) gave similar patterns which were different from that of the Cu(II):$H_2O_2$ system (lane 7). Thus, the species ultimately producing DNA cleavage for the Cu(II):reductant

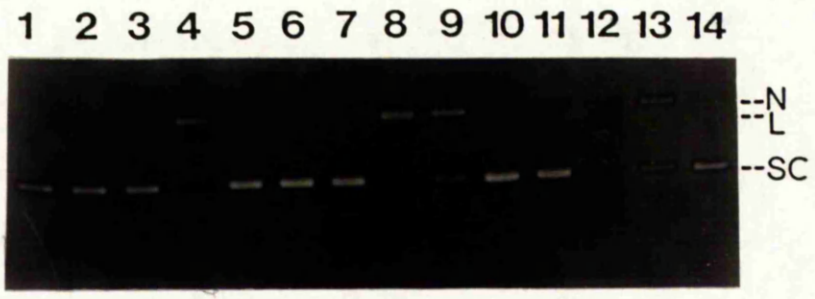



Fig. 38: Comparative cleavage of pSP64 plasmid DNA using Cu(II):reductant systems. All reactions contained pSP64 (40$\mu$M bp) in sodium phosphate buffer (10mM, pH 8.0) with the following additions: Lane 1, none. Lanes 2, 4-6, 8-10 and 12-14, Cu(II) (200$\mu$M). Lanes 3-6, ascorbate (20, 20, 2 and 0.2$\mu$M, respectively). Lanes 7-10, GSH (200, 200, 20 and 2$\mu$M, respectively). Lanes 11-14, $H_2O_2$ (2000, 2000, 200 and 20$\mu$M, respectively). All incubations (10 min, 37°C) were terminated by addition of EDTA (800$\mu$M) and agarose gel loading buffer (see section 2.2.4.1). 0.8% agarose gel electrophoresis and sample visualisation was as in Methods. The positions of the various DNA forms are as shown.

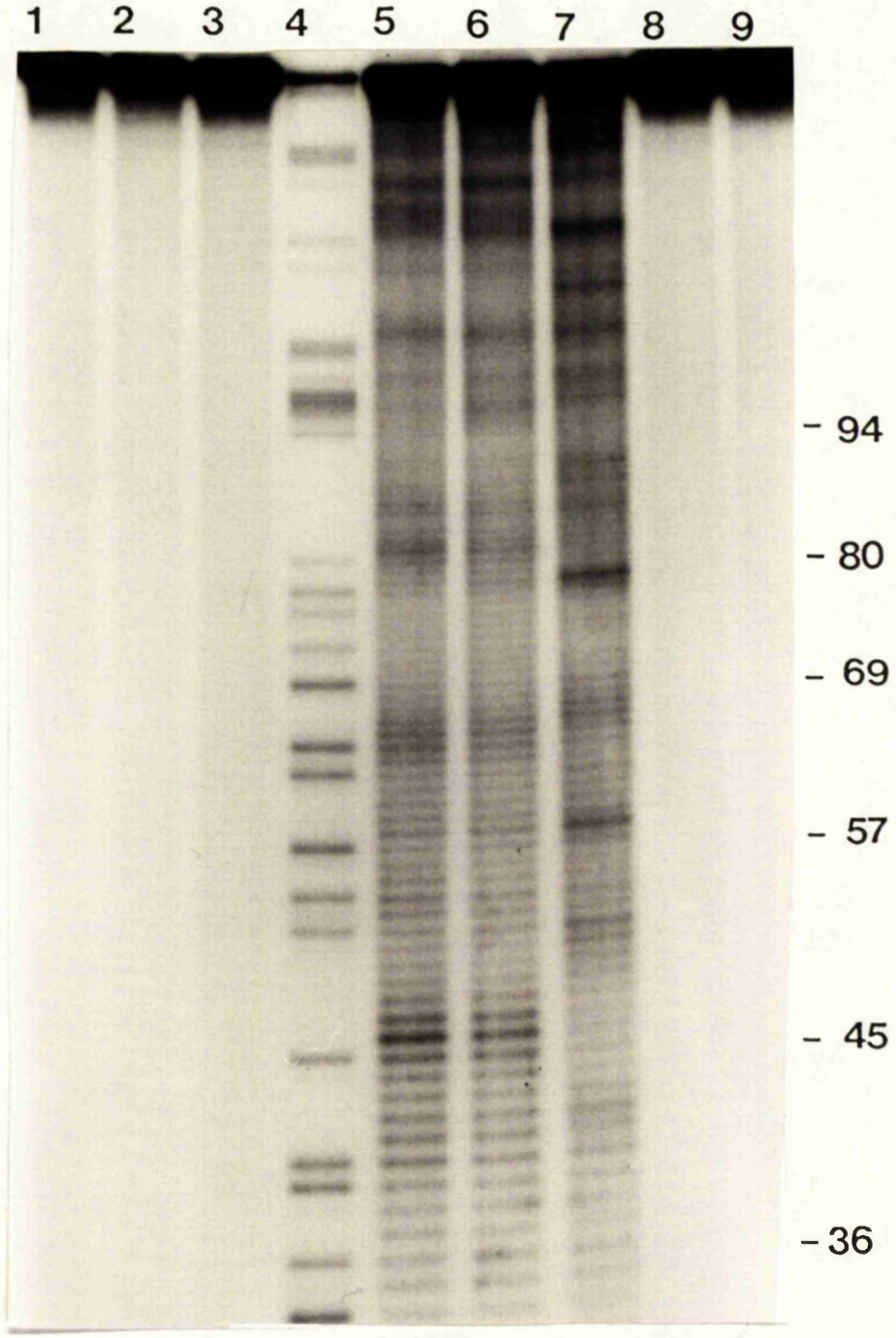



Fig. 39: Comparative cleavage of tyrT DNA using Cu(II)-based systems. All reactions contained 3' end-labelled tyrT DNA (<1μM bp), CT-DNA (40μM bp) in sodium phosphate buffer (10mM, pH 8.0) with the following additions: Lanes 1 and 5, GSH (100μM). Lanes 2 and 6, ascorbate (40μM). Lanes 3 and 7, $H_2O_2$ (2mM). Lanes 5-8, Cu(II) (200μM). Lane 9, none. All incubations (15 min, 37°C) were terminated and analysed as in Methods. Lane 4, tyrT DNA G-track (numbering corresponds to that shown in fig.16).

systems was found to be independent of the chemical identity of the reductant used. However, replacement of the reductant with an oxidant ($H_2O_2$) produced a different cleavage pattern. In particular the sites preferred by Cu(II):reductant at positions 45-47 and 80-83 appear to be absent from the Cu(II):$H_2O_2$ sample results. Also, cleavages, not seen for Cu(II):reductant samples, can be seen at positions 77-78 and 111-114 (approximately) for the Cu(II):$H_2O_2$ system.

### 3.4.3 BASE-LABILE SITES PRODUCED BY COPPER-MEDIATED SYSTEMS

Base-labile sites produced by the Cu(II):GSH, Cu(II):ascorbate and Cu(II):$H_2O_2$ systems were determined by treatment of tyrT DNA with the above systems followed by incubation with piperidine. Enhanced band intensities for both Cu(II):reductant systems were identical, occurring mainly at T residues (figure 40, lanes 5 & 6) and some of these preferred sites also occurred in the Cu(II):$H_2O_2$ sample (figure 40, lane 7), but further additional cleavage sites were apparent for the latter. The cleavage sites of all three systems are summarised in Table 4 (cf., 167bp and 517bp base-labile sites, Table 3). The differences between the reductant and oxidant systems (approximately 5 sites out of 16) occurred at sites where cytosine was cleaved, generally $^{3'}puC^{5'}$ sites. These results are not in agreement with similar experiments by Sagripanti and Kraemer (Sagripanti and Kraemer, 1989), for which only damage at polyguanosine sequences was seen after Cu(II):$H_2O_2$ reaction followed by piperidine.

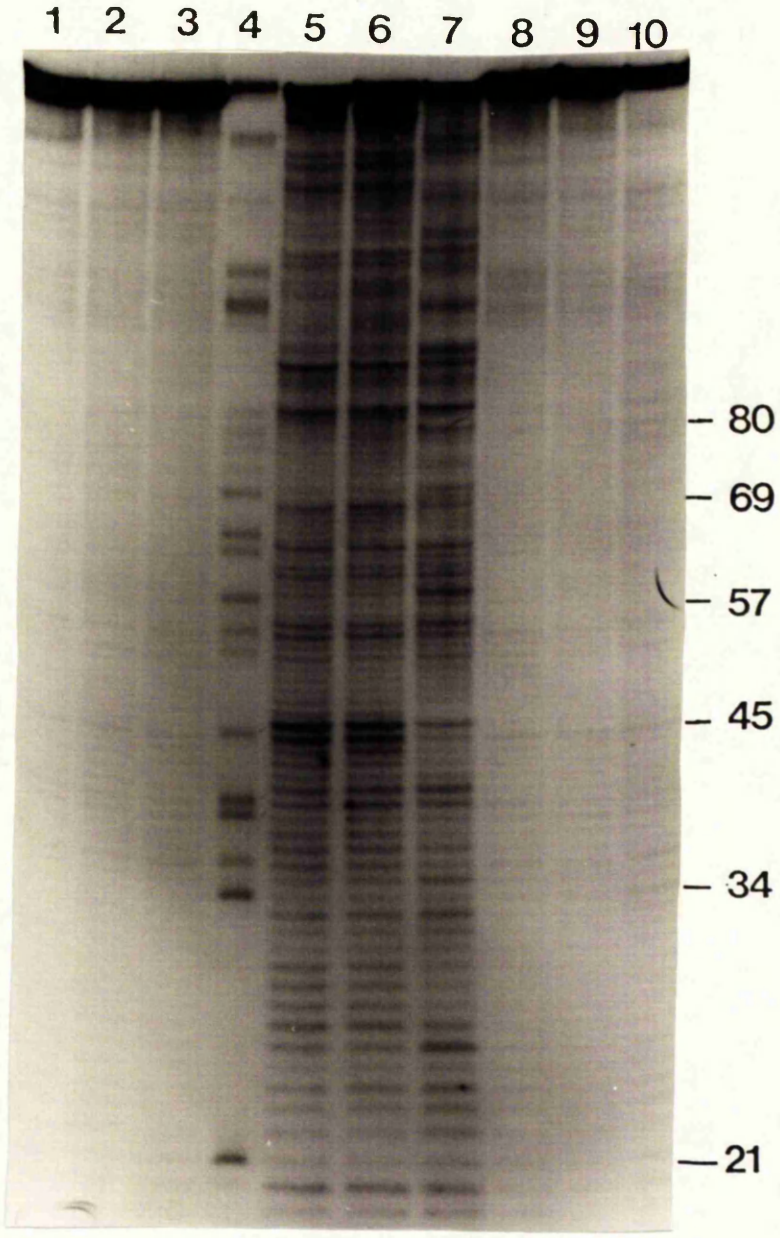



Fig. 40: Comparative cleavage of tyrT DNA using Cu(II)-based systems: determination of base-labile sites . All incubations and lane designations as in legend to fig.39 except that following termination and lyophilisation, all samples were treated with piperidine (Methods section 2.2.6.4.1) to reveal base-labile sites. Denaturing PAGE and autoradiography were as in Methods. Numbering corresponds to that shown in fig.16.

Table 4 . Base labile sites generated by Cu(II)-mediated DNA cleavage systems.

| Cleavage sites[a] | | | Sequence[b] (5'-3') |
|---|---|---|---|
| GSH | ASCORBATE | $H_2O_2$ | |
| 20 | 20 | 20 | ATG |
| - | - | 26,27w | ACTT |
| (27-30)w | (27-30)w | - | CTTTTA |
| 33w | 33w | 33w | ATG |
| 40,41 | 40,41 | 40,41 | GGTC |
| 45,46 | 45,46 | 46w | AGTA |
| 54,55 | 54,55 | 54,55 | AGTT |
| - | - | 58 | GCA |
| 61w | 60,61w | 60,61w | ATTG |
| 63 | 63 | 63 | GTG |
| 68 | 68 | 68 | ATG |
| - | - | 78 | GCA |
| 80w,81 | 80w,81 | 80w,81 | AGTA |
| 88 | 88 | 88 | ATA |
| - | - | 90,91 | ACTA |
| - | - | 96-99 | CGGGGC |

a - Base-labile sites generated by Cu(II) plus the above co-reactants detected by piperidine treatment (see text). Cleavages on the 3' end-labelled, lower strand of tyrT fragment shown (for full sequence see figure 16). Data taken from figure 40.

b - Major cleavage sites (underlined) and flanking nucleotides shown.

## 3.5 CLEAVAGE OF DNA BY OTHER CHEMICAL NUCLEASES

### 3.5.1 Fe(II):EDTA (HYDROXYL RADICAL) NUCLEASE

To check the reaction of the prepared DNA fragments toward a nuclease of known properties, three DNA fragments (167bp, 517bp and 163bp fragments) were cleaved using the hydroxyl radical-generating system, i.e. Fe(II):EDTA plus ascorbate and $H_2O_2$ (see section 1.3.2.3). In the present study cleavage of DNA by this nuclease was found to be sequence-neutral, resulting in an even intensity of cleavage at all positions in a given DNA sequence (figure 41). All three fragments reacted in this expected way toward this nuclease. Thus, from the absence of any peculiarly hyper-reactive or hypo-reactive sites within these three DNA fragments, it is concluded that the degradation patterns produced by the Cu(II) based systems (sections 3.3 and 3.4) must have resulted from reagent-specific nuclease effects and not from features (e.g. sensitive sites) of the particular preparation of DNA fragments used in this work.

### 3.5.2 Cu(I):PHENANTHROLINE NUCLEASE

The Cu(I):phenanthroline nuclease system (section 1.3.2.4.2) consists of a Cu(II) (1,10-phenanthroline)$_2$ complex which on reduction (by added thiol or superoxide) binds to DNA and cleaves DNA on further reaction with $H_2O_2$ (added, or generated from thiol). Cleavage of DNA by the metal complex is, therefore, produced by addition of thiol or thiol and $H_2O_2$. The former system shares two reactants with the Cu(II):thiol cleavage system examined in this thesis. Thus, cleavage of tyrT DNA by Cu(I):phenanthroline was undertaken to

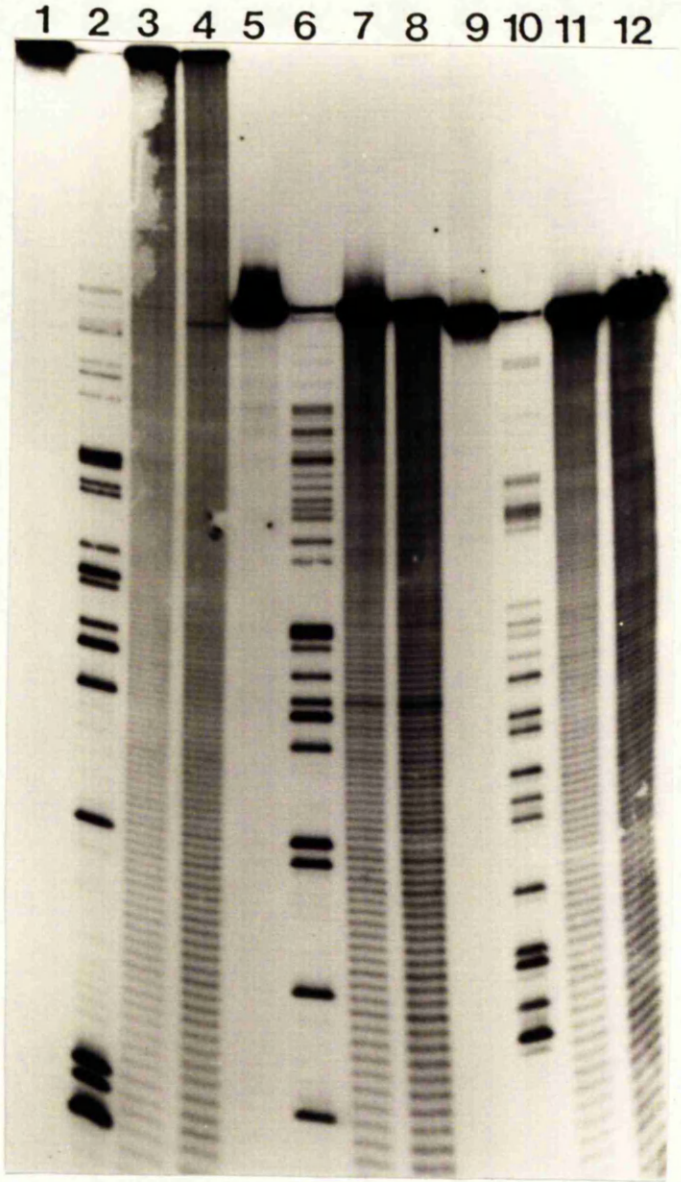



Fig. 41: Cleavage of DNA by the Fe(II):EDTA system. Three different 3' end-labelled DNA fragments were subjected to cleavage by the Fe(II):EDTA system as described in Methods (section 2.2.7.2). Lanes 1-4, 517bp fragment. Lanes 5-8, 167bp fragment. Lanes 9-12, 162bp (tyrT) fragment. Lanes 1, 5 and 9 are control lanes for untreated (intact) DNA fragment. Lanes 2, 6 and 10 are the respective G-tracks. Lanes 3, 7, 11 and 4, 8, 12 are fragments incubated with Fe(II):EDTA system for 1 and 5 min, respectively. Termination, denaturing PAGE and autoradiography were as in Methods. (Numbering of G-tracks has been omitted for clarity)

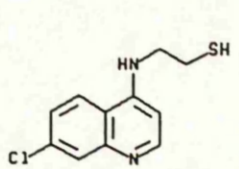
determine,(a) the reactivity of tyrT DNA preparation to the nuclease and, (b) the similarity, or otherwise, of the cleavage pattern to the Cu(II):thiol system pattern.

Cleavage of tyrT DNA by the Cu(I):phenanthroline system (figure 42) showed major preferred sites of cleavage at positions 31-32, 56-69, 86-87 and 108-110. These positions were in close agreement with published values (Drew and Travers, 1984), indicating that the prepared DNA was cleaved as expected. It was also clear, by comparing figures 25 and 42, that the cleavage patterns using Cu(II):thiol and Cu(I):phenanthroline:thiol systems were not similar.

## 3.6 EXPERIMENTS USING INTERCALATING THIOL (ISH) AND SUPERCOILED DNA

### 3.6.1 Assessment of plasmid DNA cleavage by Cu(II):ISH

The DNA-cleaving ability of a simple intercalating molecule bearing a thiol group was tested using N-(7-chloro-4-quinolinyl)cysteamine (33, ISH), a derivative of the anti-malarial agent chloroquine (35), a molecule with known DNA-intercalating ability (Hahn, 1983).

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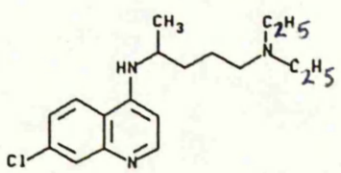


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DNA cleavage was assessed by cleavage of sc plasmid DNA (section 2.2.7.1) by addition of thiol to a DNA solution containing Cu(II). One important

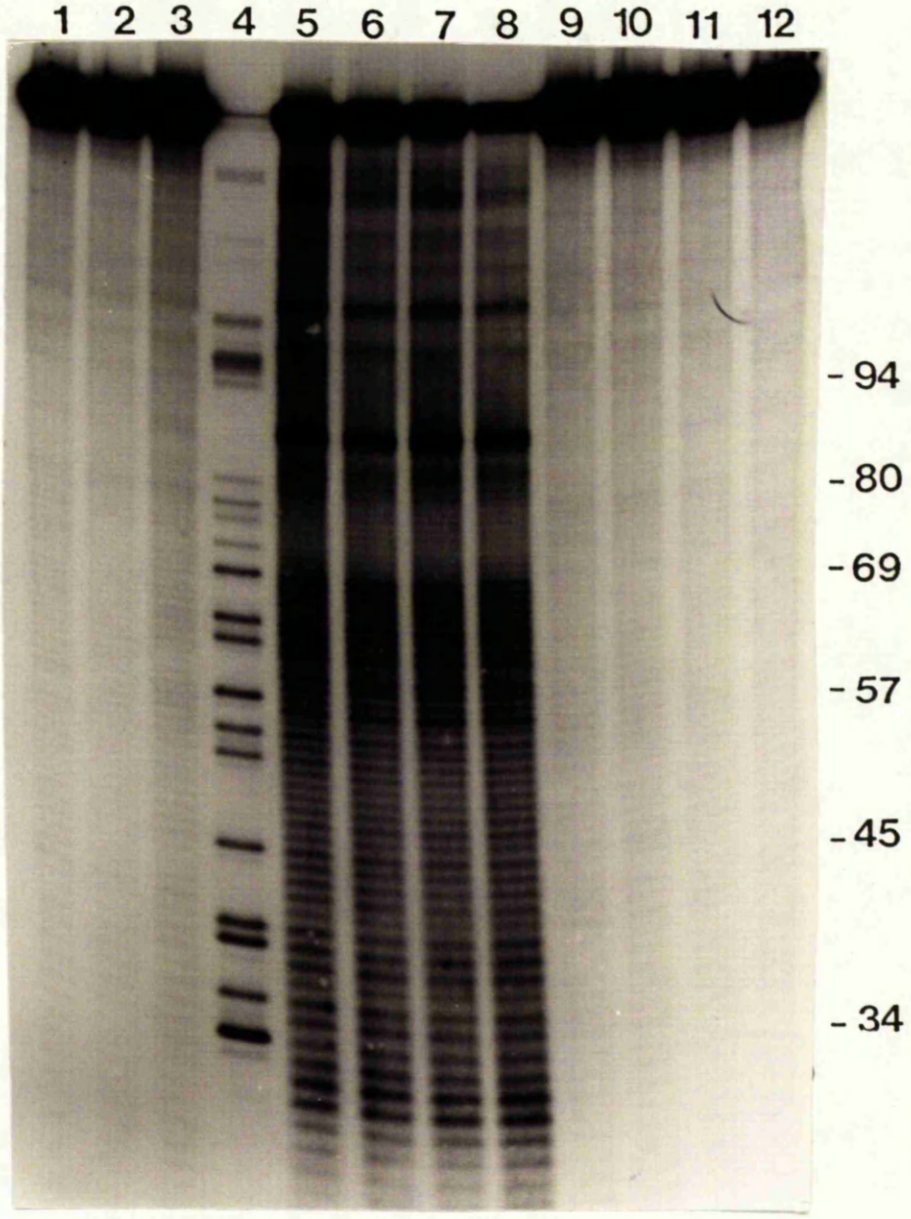



Fig. 42: Cleavage of tyrT DNA by the Cu(I):phenanthroline system. 3' end-labelled tyrT DNA was subjected to cleavage by the Cu(I):phenanthroline system as described in Methods (section 2.2.7.4). Lanes 5-8, samples incubated with Cu(I):phenanthroline system for 1, 5, 10 and 15 min, respectively. Lanes 1-3 and 9-12 were controls (15 min incubation) containing DNA and buffer as in lanes 5-8 with the following additions: Lane 1, none. Lanes 2, 10 and 12, 3-MPA (250$\mu$M). Lanes 3, 9 and 10, Cu(II) (5$\mu$M). Lanes 3, 11 and 12, phenanthroline (25$\mu$M). Lane 4, tyrT DNA G-track. Termination of reactions, denaturing PAGE and autoradiography were as in Methods. Numbering corresponds to that shown in fig.16.

difference was that as binding of ISH to DNA led to some blurring of DNA bands on electrophoresis (results not shown), a feature commonly seen with intercalator/DNA solutions (e.g. John *et al.*, 1989 and references therein), following incubation in the sc plasmid cleavage assay, ISH was removed from aqueous DNA solutions by extraction with phenol/chloroform/butan-2-ol solution (section 2.2.3.3). This procedure was previously shown to remove the DNA-binding phthalocyanine, cuprolinic blue, from DNA solutions (John *et al.*, 1989). This treatment of ISH/DNA solutions led to production of reproducible, well-defined plasmid bands with no blurring (see below) where relative amounts of the various DNA forms were easily assessed. The results indicated that no cleavage of DNA above background levels occurred with ISH as the thiol (lanes 11-13, figure 43) whereas GSH provided a positive control for DNA cleavage under identical conditions (lanes 4-6, figure 43). Note that under these conditions (see figure 43 legend), increasing [RSH] above [Cu(II)] caused a decrease in the amount of DNA cleavage (compare lane 6 with lanes 5 and 4) probably due to the increased scavenging effect of excess thiol groups on the DNA-damaging radicals produced by Cu(II):thiol. When [RSH] $\leq$ [Cu(II)], increasing [RSH] leads to increased DNA cleavage (see figure 38, lanes 7 to 10). To determine whether the order of addition of the two DNA-binding reactants, Cu(II) and ISH, to a DNA solution was the factor causing this null effect, the experiment was also carried out by addition of thiols to the DNA solution prior to addition of Cu(II). The results for GSH (lanes 7-9) and ISH (lanes 15-17) were identical to those where thiols were added after Cu(II). Thus, order of addition of the reactants was not responsible for the observed lack of DNA cleavage by the Cu(II):ISH system.


Figure 43. Comparative cleavage of plasmid pSP64 using Cu(II):GSH and Cu(II):ISH systems. All incubations (25$\mu$l) contained pSP64 DNA (40$\mu$M bp) in sodium phosphate buffer (10mM,pH8.0) with the following additions: Lanes 2,4-9,11-13 and 15-17, Cu(II) (0.1mM). Lanes 3,4 and 7, GSH (1.6mM). Lanes 5 and 8, GSH (0.4mM). Lanes 6 and 9, GSH (0.1mM). Lanes 10,11,14 and 15, ISH (1.6mM). Lanes 12 and 16, ISH (0.4mM). Lanes 13 and 17, ISH (0.1mM). Cu(II) was added to DNA in buffer either before (lanes 4-6 and 11-13) or after (lanes 7-9 and 15-17) addition of thiol. All reactions (30min, 37°C) were terminated by addition of EDTA (800$\mu$M) and kept on ice. Samples in lanes 10-17 were then split into two equal aliquots, one aliquot of each pair was then boosted to 100$\mu$l with TE buffer and phenol extracted twice and chloroform extracted once (see Methods), the aqueous phase being retained and lyophilised before taking the dried sample up in TE buffer. All samples were electrophoresed through a 0.8% agarose gel and the gel stained and photographed (see Methods). The positions of the various DNA forms are as indicated.

### 3.6.2 FURTHER STUDIES ON ISH

The stability of the thiol group of ISH in buffer only as well as in buffer containing DNA, the rate of disappearance of ISH thiol titre in the presence of buffer and Cu(II), and the rate of disappearance of ISH thiol titre in buffer containing Cu(II) and DNA were examined by determination of the thiol concentration at various time intervals after addition of ISH or Cu(II) (see figure 44). Identical control reaction mixtures were set up and monitored but with GSH in place of ISH.

Both ISH and GSH titres decreased only slightly (~5%) after 30min of incubation at 37°C in the presence or absence of DNA. GSH was, however, very rapidly degraded by Cu(II) (0% thiol left after 10min) both in the presence and absence of DNA. In contrast, ISH was much less rapidly degraded than GSH in the presence of Cu(II) (~55% thiol remaining after 30min) but was more rapidly degraded when Cu(II) and DNA were both present (30% thiol left after 30min).

An additional experiment, designed to determine whether the thiol of the postulated intercalated ISH molecule was relatively inaccessible or unreactive for reaction with Cu(II), was carried out in the following way: plasmid pSP64 DNA was added, to various final concentrations, to identical samples of ISH (50$\mu$M) in sodium phosphate buffer (10mM, pH 8.0) and an aliquot (200$\mu$l) of each solution was then assayed for SH content after 5min incubation at room temperature (total assay volume = 1.2ml). It was found that there was no significant difference in the thiol titres for ISH incubated in the absence or the presence of DNA up to a 12-fold molar excess (Table 5, absorbance values are the means of two separate determinations $\pm \leq 0.008$ absorbance units)

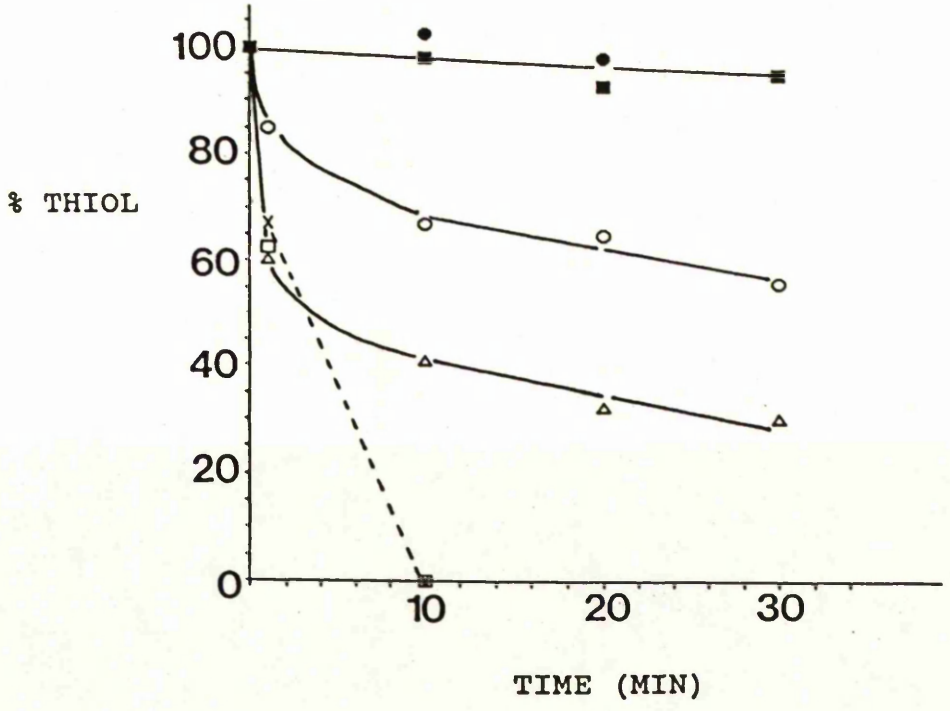



Figure 44. Plot showing time-dependent loss of thiol titre from solutions of GSH or ISH containing various added reagents. Incubations (1ml) in sodium phosphate buffer (10mM,pH8.0) contained, where appropriate, RSH (100$\mu$M), Cu(II) (70$\mu$M) and CT-DNA (35$\mu$M bp). All samples were assigned a thiol titre of 100% prior to addition of co-reactants and/or start of incubation. Thiol assays were carried out on an aliquot (200$\mu$l) of thiol solution under Method 1 conditions (section 2.2.8.1). Initial assays, where an aliquot was removed immediately after addition of co-reactants, were assigned an incubation time of 1min. All incubations were at 37°C. Note that thiol titres for GSH and ISH alone were identical at 30min. Points are the means (≤5%) of two determinations. Lines are notional to assist in visualisation.

Key to symbols :
- ▪ - GSH alone
- • - ISH alone
- □ - GSH plus Cu(II)
- o - ISH plus Cu(II)
- x - GSH plus Cu(II) and CT-DNA
- Δ - ISH plus Cu(II) and CT-DNA

TABLE 5: Determination of thiol titre in solutions of ISH containing increasing amounts of DNA.

a Molar ratio of [DNA] (in bp) to [ISH] prepared as a DNA/ISH mixture in sodium phosphate buffer (10mM, pH8.0).

b The maximum change in $A_{412}$ ($\Delta A_{412}$ max) observed on adding 200ul of DNA/ISH solution to 1ml of DTNB solution (see Methods section 2.2.8.1; method 1).

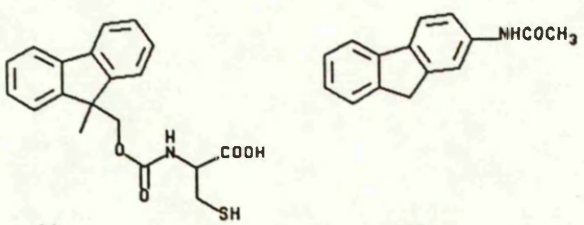
c Calculated thiol concentration present in DNA/ISH solution determined using $\Delta A_{412}$ max and equation in section 2.2.8.1.

Table 5.

| Ratio [DNAbp]/[ISH][a] | $\Delta A_{412}$ max[b] | [SH],$\mu$M[c] calculated |
|---|---|---|
| 0 | 0.118 | 49.8 |
| 0.4 | 0.121 | 51.3 |
| 4.0 | 0.116 | 49.2 |
| 12.0 | 0.116 | 49.2 |

### 3.6.3 OTHER INTERCALATOR-BORNE THIOLS

Very preliminary work was undertaken on two other thiol-bearing molecules: Fmoc-cysteine (36), a molecule which is extremely unlikely to intercalate due to the substitution at position 9 (from data on acetylamino fluorene (37) and derivatives; Kriek, 1974), and an anthraquinone derivative (38) which is likely to exhibit similar intercalative ability to mitoxantrone (39) and other 5-substituted anthraquinones (Kapuscinski *et al.*, 1981).

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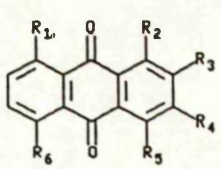
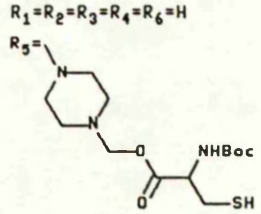



39 $R_2=R_5=NH(CH_2)_2NHCH_2CH_2OH$
$R_1=R_6=OH, R_3=R_4=H$

38 $R_1=R_2=R_3=R_4=R_6=H$

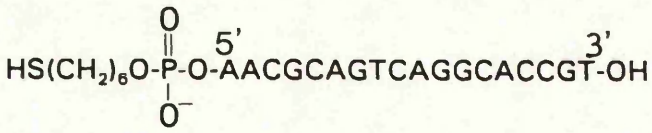
$R_5=$



Preliminary experiments showed that, compared to Cu(II):GSH system, the Cu(II):Fmoc-cysteine system caused comparable levels of DNA cleavage whereas cleavage by Cu(II):anthraquinone derivative was not detected (results not shown), although it was observed that the latter system was prone to self-reduction presumably caused by reaction of the thiol with the quinone moiety.

## 3.7 ATTEMPTED SEQUENCE TARGETTING OF Cu(II):THIOL CLEAVAGE USING AN OLIGONUCLEOTIDE-THIOL MOLECULE

Synthetic oligonucleotides selectively modified with a thiol group at the 5' end (oligo-SH) may be prepared by modification of the standard methods of oligonucleotide synthesis. Targetting of the Cu(II):thiol nuclease activity was attempted using such a thiol attached to an oligonucleotide of complementary sequence to a part of the 167bp fragment of pBR322 DNA. In this case the modified oligonucleotide (oligo-SH) was the 18mer (34).

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This oligonucleotide sequence was complementary to residues 64-81 of the lower, 3' end-labelled strand of the pBR322 167bp fragment (figure 17). The experimental strategy used was similar to previously described protocols for oligo-linked free radical generators (Chu and Orgel, 1985; Dreyer and Dervan, 1985; Chen and Sigman, 1986) and is shown as method 1 in figure 14:

(i) the dsDNA fragment carrying the target DNA sequence was denatured

to single strands,

(ii) the oligo-SH was allowed to anneal to the strand bearing its complementary sequence,

(iii) Cu(II) was added to activate the cleavage reaction,

(iv) the reaction products were denatured and analysed by denaturing PAGE.

### 3.7.1 CHECK OF OLIGO-SH THIOL CONCENTRATION

Oligo-SH thiol concentration was checked by titration with Ellman's reagent (thiol assay: method 2, section 2.2.8.1). Lyophilised oligo-SH (approx. 2nmoles) was redissolved in deaerated water (50$\mu$l) and an aliquot (10$\mu$l) removed and retained. The remaining oligo-SH solution was made up to 1 ml with sodium phosphate buffer (0.1M, pH 7.0) and assayed for thiol titre as above. The aliquot which had been removed was then diluted accordingly and used in the annealing and cleavage procedures. The time taken to establish a steady absorbance reading at 412nm for solutions of oligo-SH was considerably longer than for GSH, cysteine and the other thiols of this study ($\simeq$45min, compared to $<$10min).

### 3.7.2 CHECK OF ANNEALING CONDITIONS

The two methods used for annealing the oligo-SH to its target DNA were:

(a) Incubation of target dsDNA with oligo-SH (90°C, 5min) followed by cooling of the sample at room temperature for 30min (Chen and

Sigman, 1986) and

(b) incubation of target dsDNA with oligo-SH (70°C, 5min) and then allowing the solution to cool slowly to room temperature (~ 1hr) - this procedure is analogous to that used in sequencing of DNA by enzymatic methods (Maniatis *et al*, 1982).

Oligo-SH was used in excess (> 100 fold molar excess) over the target DNA and the products of the annealing reactions checked by non-denaturing PAGE. Typical results are shown in figure 45. Both annealing methods produced a band of DNA migrating at a position intermediate to that of the controls for ds and ssDNA (compare lanes 1-3 with lanes 5,6,8 and 9). This band was ascribed to the complex between oligo-SH and the target 167mer strand. It was also apparent that this band had much lower intensity than the renatured 167bp fragment (dsDNA) itself and thus the majority of denatured 167mer target DNA (ssDNA) had reassociated to form 167bp dsDNA rather than complexing with oligo-SH. This problem has been commented upon by other authors (e.g. Chen and Sigman, 1986) but did not seem to impair their results in subsequent procedures and so the quantity of annealed product was therefore considered acceptable for further procedures in this present work. Conditions giving the maximum proportion of annealed oligo-SH:target DNA product (i.e. procedure (b), molar excess oligo-SH:target DNA = 400) were used in all subsequent procedures where annealed product was required.

### 3.7.3 CLEAVAGE OF TARGET DNA BY OLIGO-SH

Oligo-SH was annealed to target DNA under the conditions described in section 3.7.2. Cu(II) was added to initiate possible Cu(II):thiol reaction at

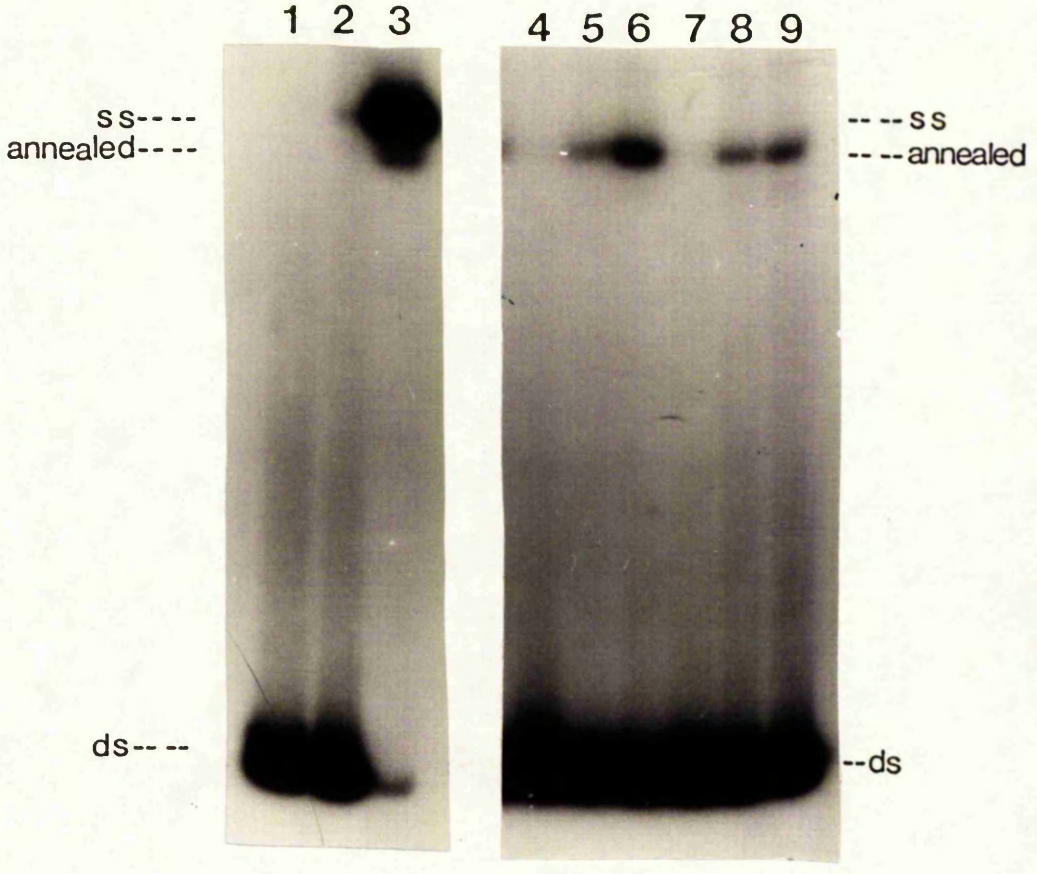



Fig. 45: Non-denaturing PAGE of annealing procedure products. All samples contained 3' end-labelled 167bp fragment (<1$\mu$M bp), CT-DNA (40$\mu$M bp) in sodium phosphate buffer (10mM, pH 7.4) with the following additions: Lanes 2, 3, 6 and 9, oligo-SH (0.4$\mu$M). Lanes 5 and 8, oligo-SH (0.1$\mu$M). Samples were then treated as follows: Lanes 1 and 2, no treatment. Lane 3, heated (5 min, 90°C), snap-cooled on ice-water. Lanes 4-6 and 7-9 heated and annealed as in procedure (b) or procedure (a) (section 3.7.2), respectively. All samples were then prepared for electrophoresis by addition of agarose gel sample loading buffer (section 2.2.4.1) and analysed by non-denaturing PAGE (section 2.2.4.2) through a 12%, 1:30 cross-linked gel run at 800V (15mA) for 7 hr. Autoradiography was as in section 2.2.4.3. Positions of the various DNA forms (see text) are as shown.

sites on the target DNA strand proximal to the position of the thiol group of the annealed oligo-SH. Typical results are shown in figure 46. Note that specific cleavage would have been evident as band(s) of increased intensity in the region of residues 60 to 68 (marked on figure 46). Lanes 1-4 and 7-10 show that no specific cleavage of the target DNA occurred under the conditions employed (note that the DNA sample used was slightly degraded even in the controls). Variations in the experimental protocol such as adding DTT to the sample to be annealed (in order to maintain the oligo-SH as free SH) were also unsuccessful (see lanes 11-14). Further variations included (i) addition of oligo-SH after denaturation of the ds target DNA (in order to avoid loss of SH at elevated temperatures), followed by mild heating (65°C, 5 min) and fast cooling (as for procedure (a) section 3.6.1), (ii) addition of oligo-SH to denatured target DNA followed by incubation with Cu(II), (iii) changing of buffer solution to 50mM tris, pH 7.4, 50mM NaCl (used by Chen and Sigman, 1986 and Dreyer and Dervan, 1985), and (iv) piperidine teatment of reaction products such as those in figure 46 to detect any base modifications. All of these variations proved unsuccessful in causing site-specific cleavage of DNA (results not shown).

## 3.8 CLEAVAGE OF DNA BY Cu(II):THIOL IN THE PRESENCE OF $Mg^{2+}$ AND $Na^{+}$ IONS

Cleavage of tyrT DNA by Cu(II):thiol in the presence of $Mg^{2+}$ was assessed using $Na^{+}$ as a control for ionic strength effects (figure 47). Apart from the redissolving and electrophoretic problems encountered with the sample of $Mg^{2+}$ at highest concentration (lane 4), cleavage of tyrT DNA by

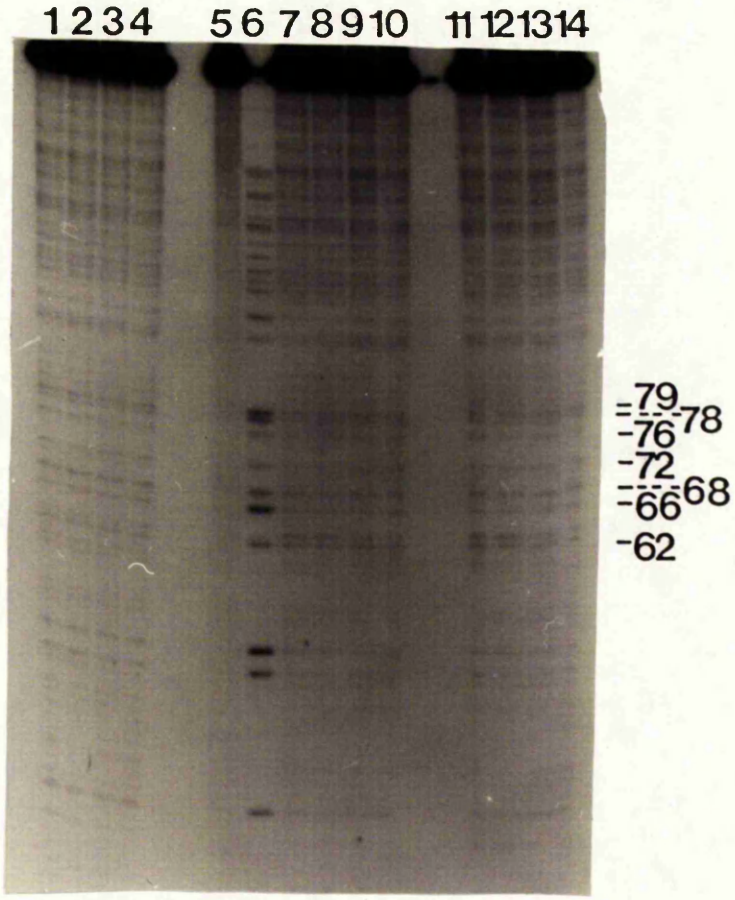



Fig. 46: Reaction of Cu(II) with oligo-SH annealed to denatured 167bp fragment. All incubations contained 3' end-labelled 167bp fragment (<1$\mu$M bp) and CT-DNA (40$\mu$M bp) in sodium phosphate buffer (10mM, pH 7.4) with the following additions: Lanes 7-10 and 11-14, oligo-SH (0.4$\mu$M). Lanes 11-14, DTT (10$\mu$M). Lanes 1, 7 and 11 samples treated by annealing procedure (b) (section 3.6). Lanes 2, 8 and 12 treated by annealing procedure then incubated (60 min, 37°C). Lanes 3, 9, 13 and 4, 10, 14 all treated by annealing procedure (b) and further incubated for 20 or 60 min with Cu(II) (200$\mu$M), respectively. Lanes 5 and 6 intact, untreated DNA and G-track, respectively. Incubations were terminated by addition of EDTA (800$\mu$M) and lyophilised.Denaturing PAGE and autoradiography were as in Methods.

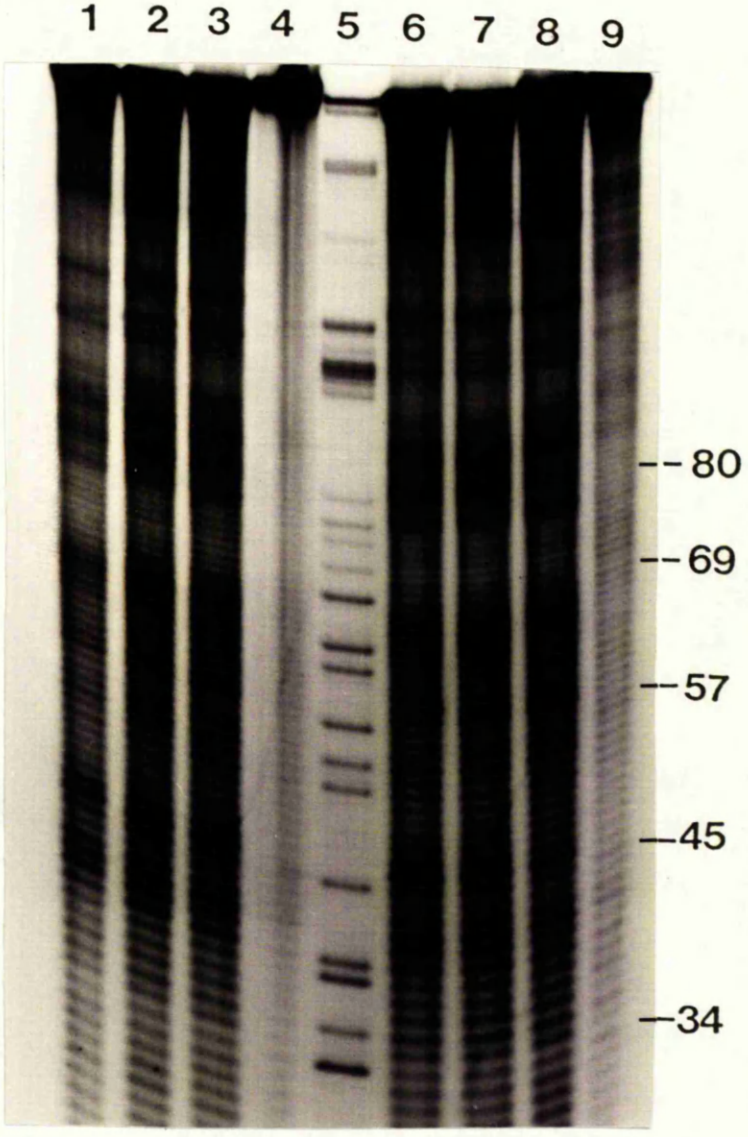



Fig. 47: Cleavage of tyrT using the Cu(II):thiol system in the presence of $Mg^{2+}$ or $Na^{+}$. Samples of 3' end-labelled tyrT DNA were incubated(30min, 37°C) with Cu(II):GSH under standard conditions (section 2.2.7.1) with the following additions: Lane 1, none. Lanes 2-4, $MgCl_2$ at 0.33mM, 3.3mM and 33.3mM. Lanes 6-8, NaCl at 1mM, 10mM and 100mM. Incubations were terminated by freezing on dry-ice and lyophilisation before redissolving in formamide buffer and denaturing PAGE (see Methods). Lane 5, tyrT DNA G-track. Lane 9, DNA incubated in the absence of Cu(II):GSH. Numbering is as shown in fig.16.

Cu(II):thiol in the presence of up to 3.3mM $Mg^{2+}$ (lanes 2 and 3) occurred with a similar pattern to that of the control sample (no $Mg^{2+}$ present, lane 1). It was also apparent that the cleavage reaction was unaffected by the increased ionic strength of the solutions incorporating $Mg^{2+}$ or $Na^{+}$ as a control. At 3.3mM [$Mg^{2+}$] or 0.1M [$Na^{+}$] no protection against Cu(II):thiol cleavage was observed. Thus, in this limited concentration range neither specific (metal ion-binding) nor non-specific (ionic strength) effects of $Mg^{2+}$ (or $Na^{+}$) were observed - these effects would, if present, probably manifest themselves as a change in the cleavage pattern and/or an inhibition of the reaction, respectively.

## 3.9 Cu(II):THIOL CLEAVAGE OF FRAGMENTS CONTAINING REPEATING DINUCLEOTIDE SEQUENCES

The plasmids pRW1011 and pRW1015 are chimaeric molecules consisting of pBR322 DNA with a section deleted (base numbers 44-2435), a polylinker region (containing many restriction enzyme sites) and specific, synthetic DNA sequences inserted at the BamHI site (see section 2.1.4). The most important feature of these plasmids is the nature of the inserts which are $(TG)_{12}$ and $(TG)_6(AC)_6$ in pRW1011 and pRW1015, respectively. Under various conditions of salt concentration and in supercoiled plasmids (i.e. covalently closed circular DNA), these sequences form Z-DNA and non-Z, left-handed DNA respectively (McLean and Wells, 1988). Previous work has determined that the Cu(II):thiol system does not specifically cleave these plasmids (or several others containing different inserts) at the insert sequences (Reed and Douglas, 1991). It was, therefore, appropriate to examine this system further

for any more subtle sequence effects which might be more apparent using end-labelled linear DNA analysis.

Samples of the plasmids pRW1011 and pRW1015 were cleaved with EcoRI, radiolabelled at their 3' ends (section 2.2.6.1) and cleaved a second time with HindIII. This procedure generated fragments of approximately 70bp containing the inserted DNA sequences (see figures 19 and 20). After purification (as in section 2.2.6.3), these fragments were cleaved by the Cu(II):thiol system under standard conditions (section 3.2.1). The results were as shown in figure 48.

Three important features are apparent from the cleavage patterns:

(i) severe compression of bands occurred in the region of positions 37-48 (lanes 4, 5 & 6) of the pRW1015 derived 70bp fragment,

(ii) for both fragments, a regularly alternating pattern of band intensities (light-dark-light, etc.) was seen in the region of the inserted sequences (positions 19-42, lanes 9, 10 & 11 and positions 19-28, lanes 3-6),

(iii) the cleavage patterns showed no other distinguishing features at the remaining residues of the fragment sequences.

Detailed inspection revealed that the most intense bands of the alternating patterns were at positions 18, 20, 22, 24, 26, 28, 30, 32, 34, 36, 38 and 40 of pRW1011 and positions 18, 20, 22, 24, 26, 28 and 30 of pRW1015. These positions correspond to the underlined residues in the following sequences:

(pRW1011) 5'TGTGTGTGTGTGTGTGTGTGTG3'
3'ACACACACACACACACACACACAC5'

(pRW1015) 5'TCTGTGTGTGTGTG3'
3'AGACACACACACAC5'

Cleavages in the region 32-42 of pRW1015 were obscured by the compression

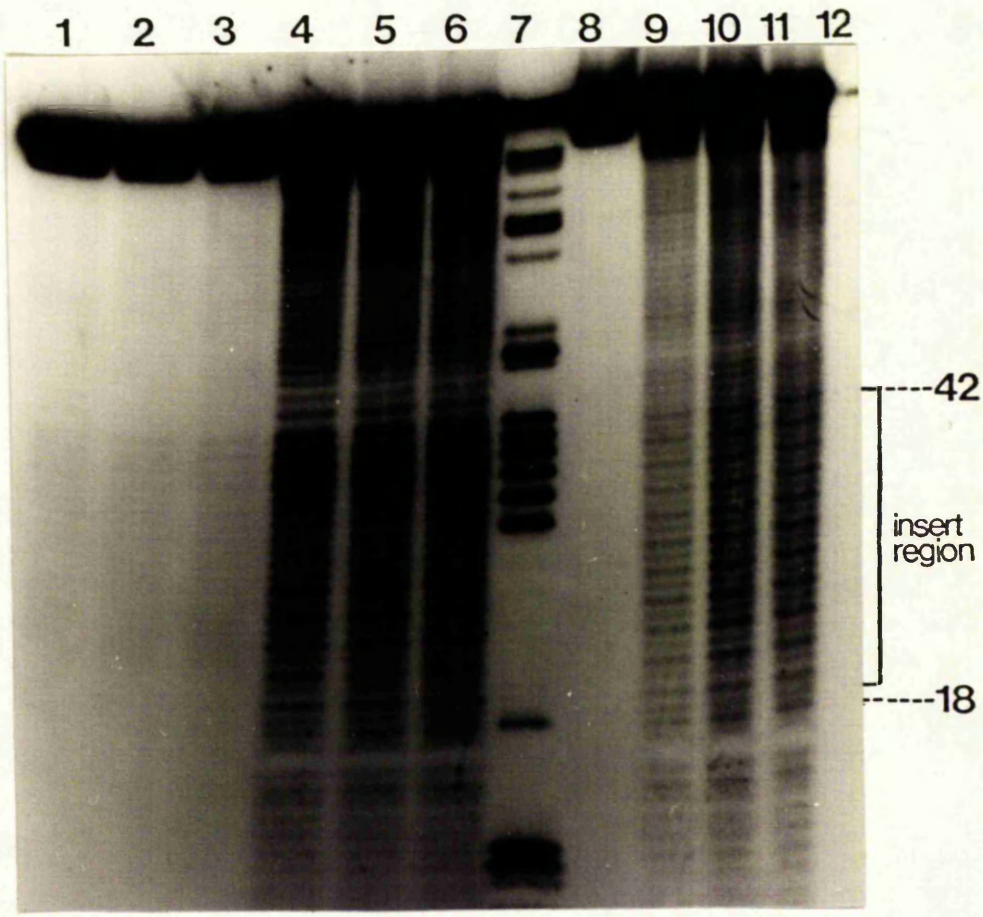



Fig. 48: Cu(II):thiol cleavage of 70bp fragments derived from pRW1011 and pRW1015. All incubations contained 3' end-labelled 70bp fragment (<1$\mu$M bp) and CT-DNA (40$\mu$M bp) in sodium phosphate buffer (10mM, pH 8.0). Lanes 1-7 contained pRW1015 derived 70bp fragment. Lanes 8-12 contained pRW1011 derived 70bp fragment. The following additions were made: Lanes 1 and 8, none. Lane 2, Cu(II) (200$\mu$M). Lane 3, GSH (100$\mu$M). Lanes 4-6 and 9-11, Cu(II) (200$\mu$M) plus GSH (100$\mu$M). Incubations were for 10 min (lanes 4 and 9), 20 min (lanes 5 and 10) or 40 min (lanes 1-3, 6, 8, and 11), all at 37°C. Lanes 7 and 12, G-track samples. Sample in lane 12 was weak but guanine positions were determined from a separate, over-exposed, autoradiograph. Denaturing PAGE and autoradiography was as in Methods. Insert sequence region is marked and numbering is as shown in figures 19 and 20.

of bands. Resolution of such band compressions can be achieved by electrophoresis in denaturing gels containing 30% formamide in addition to urea (Shafer *et al.*, 1989). Use of this technique showed that the alternating band pattern did not extend into the previously unresolved section (results not shown). Cleavage sites on the complementary strands have not been determined.

## 3.10 FOOTPRINTING STUDIES

### 3.10.1 COMPARATIVE FOOTPRINTING OF HOECHST 33258 USING Fe(II):EDTA AND Cu(II):THIOL NUCLEASES

Footprinting of the minor groove-binding ligand Hoechst 33258 (4) was carried out on the 167bp fragment of pBR322 DNA (3' end-labelled) for ease of comparison with literature results (e.g. Harshman and Dervan, 1985). Reactions were carried out in sodium phosphate buffer (10mM, pH 8.0) containing 50mM NaCl. Reactions were effected by pre-incubation of DNA fragment, carrier CT-DNA and ligand (30min, 37°C) before addition of the chemical nuclease. The reaction (15min, 37°C) was stopped by freezing the sample on dry ice and lyophilisation, as preparation of the sample for analysis by denaturing PAGE.

Fe(II):EDTA footprinting (see section 2.2.7.2) produced easily visible areas of cleavage protection caused by the ligand at concentrations as low as 1$\mu$M (lanes 6-8, figure 49). The strongest binding sites for Hoechst 33258 appeared at positions 55-58 and 86-89 of the fragment corresponding to $^{3'}$ATTT$^{5'}$ and $^{3'}$CTTT$^{5'}$ sequences. These positions of strong protection agree

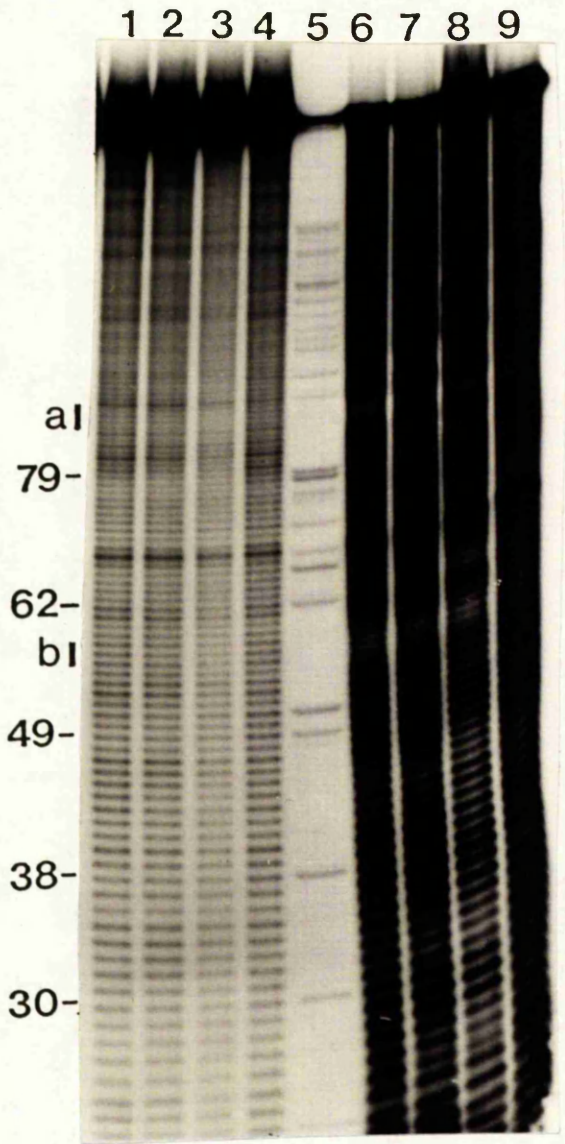



Fig. 49: Footprinting of Hoechst 33258 binding sites on 167bp fragment - comparison of Fe(II):EDTA and Cu(II):GSH systems. All incubations contained 3' end-labelled 167bp fragment (<1$\mu$M bp) and CT-DNA (40$\mu$M bp) in sodium phosphate buffer (10mM, pH 8.0) with the following additions: Lanes 1-3 and 6-8, Hoechst 33258, 1$\mu$M, 5$\mu$M and 20$\mu$M respectively. Lanes 4 and 9, none. Reactions 1-4 and 6-9 were preincubated (dark, 37°C, 30min) before addition of Cu(II):thiol (lanes 1-4) or Fe(II):EDTA (lanes 6-9) under standard conditions as in Methods. Incubations (10min, 37°C) were terminated by freezing and lyophilisation and analysed by denaturing PAGE. Strongest Hoechst 33258 binding sites are marked a and b. Numbering is as shown in figure 17.

well with those observed in footprinting studies using MPE-Fe(II) as the nuclease (Harshman and Dervan, 1985), although the protection at positions 43-48 in the above work was not observed in the present work. The reasons for this anomaly are unclear. At higher ligand concentrations further areas of protection were seen, e.g. 94-96 ($^{3'}$TGT$^{5'}$), 62-64 ($^{3'}$GAT$^{5'}$). It was also determined that the footprints observed in sodium phosphate buffer (10mM, pH 8.0) containing 50mM NaCl were identical to those observed in the same buffer at pH 7.0, pH 7.5 and without added NaCl (results not shown).

Footprints of Hoechst 33258 were not obtained when the Cu(II):RSH nuclease was used (Cu(II) 0.2mM, GSH 0.4mM, final concentrations) although a slight reduction in cleavage extent was observed on increasing the Hoechst 33258 concentration from 1$\mu$M to 20$\mu$M (lanes 1-4, figure 49). Altering the conditions of the reaction did not produce footprints (results not shown) and alternative conditions tried included (i) pre-incubation of DNA, ligand and Cu(II) for 30min at 37°C before addition of the reductant and (ii) increased Hoechst 33258 concentrations, reduced Cu(II) concentration and increased reaction times.

### 3.10.2 ATTEMPTED FOOTPRINTING OF A PROTEIN BOUND TO DNA: HINDIII BOUND TO 167bp FRAGMENT

Of the fragments used in this thesis, one had a restriction enzyme site ($^{5'}$AAGCTT$^{3'}$) located within its sequence, namely the pBR322 167bp fragment which has a HindIII site at residues 27-32, (figure 17). Thus, in the absence of its essential cofactor ($Mg^{2+}$) it was expected that the enzyme would bind to, but not cleave, this DNA (by analogy with EcoRI studies; Kuwabara *et al.*,

1986) and enable a 'footprint' of the enzyme bound to DNA to be obtained. Incubation of the HindIII:167bp complex with Cu(II):thiol was expected to yield a footprint but results (figure 50) revealed that no footprint was produced by this system. Proof of HindIII binding in the absence of $Mg^{2+}$ was afforded by the bands evident at the restriction site corresponding to cleavage of the DNA by the enzyme using adventitious metal ions (or perhaps added Cu(II)). At higher enzyme concentrations reduction in the overall cleavage by Cu(II):thiol was seen; DNA bands became fainter showing that less DNA cleavage was occurring. This was possibly due to chelation of Cu(II) and/or thiol by the amino acid residues of the protein. Even at lower enzyme concentrations, however, no footprint was apparent either in the restriction site region or anywhere else.

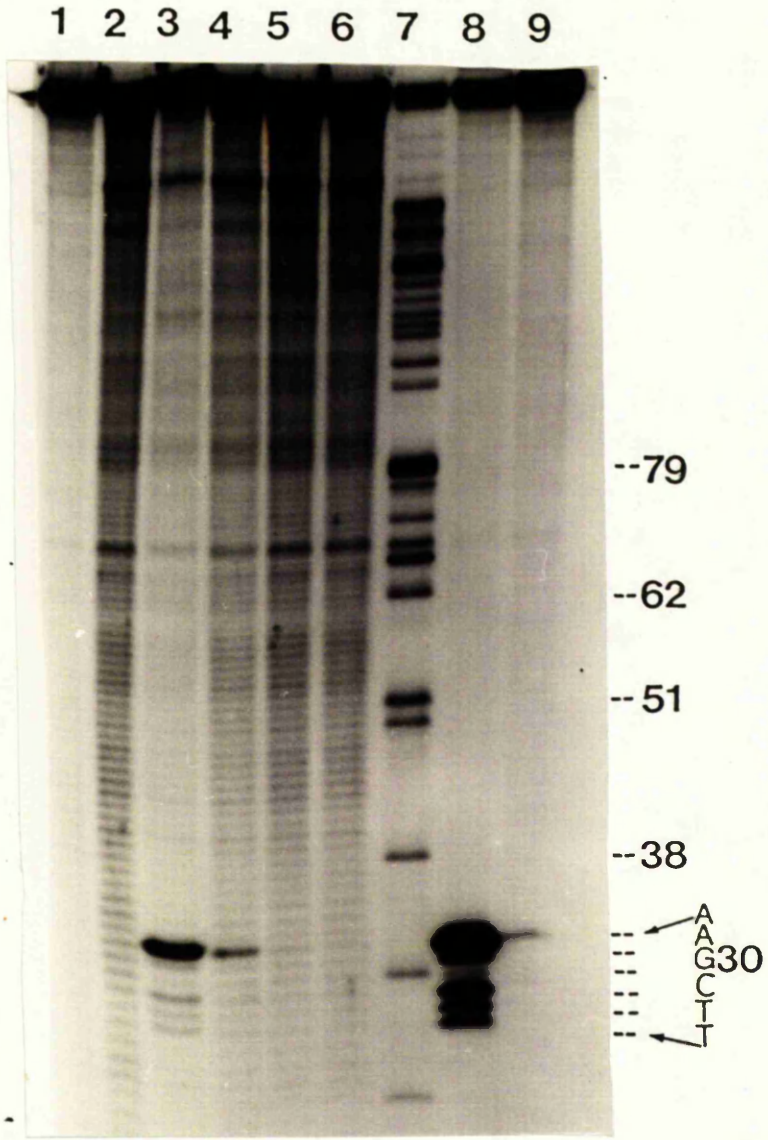
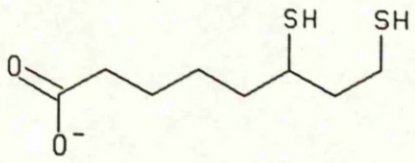



Fig. 50: Attempted footprinting of HindIII bound to 167 bp DNA fragment using Cu(II):thiol system. All incubations contained 3' end-labelled 167 bp fragment (<1$\mu$M bp) and CT-DNA (40$\mu$M bp) in sodium phosphate buffer (10mM, pH 8) with the following additions. Lane 1, none. Lanes 3-6, HindIII enzyme 2.4, 0.24, 0.024, 0.0024 U/$\mu$l respectively. Lanes 2-6 pre-incubated at 37°C for 30 min before addition of Cu(II) (200$\mu$M) and GSH (100$\mu$M) and further incubation (25 min, 37°C) before termination by addition of EDTA (0.8mM) and lyophilisation. Lanes 8 and 9, HindIII enzyme (0.096 U/$\mu$l). Lane 8, $Mg^{2+}$ (10mM). Lanes 1, 8 and 9 incubated for 25 min (37°C) and terminated as for lanes 2-6. All samples were resuspended in formamide loading buffer and electrophoresed on denaturing PAGE (see Methods). Lane 7, G-track (numbering as in fig 17).

# CHAPTER 4 : DISCUSSION

## 4.1 GENERAL DISCUSSION

In the presence of Cu(II) at neutral pH, previous workers have shown that thiols can cause single-strand breaks in supercoiled plasmid DNA (Reed and Douglas, 1989). Of the metal-ions tested only Cu(II) was effective for the thiol-induced DNA cleavage reaction. In contrast, Cd(II), Co(II), Cr(III), Fe(III), Fe(II), Ni(II), Mn(II) and Zn(II) were ineffective. The relative ability of thiols to cleave DNA in this system was found to be independent of the structure of the thiol molecule itself for many thiols, e.g. dihydrolipoic acid (40), 3-mercaptopropionic acid (41) and glutathione (42) all cleaved plasmid DNA with similar efficiency although the dithiols 1,3-propanedithiol (43) and 1,2-dimercaptopropanol (44) were less effective than the above. Oxidised thiols (45) and S-blocked thiols (46) were unable to produce DNA cleavage indicating that a free SH group was required in the reaction. It was also shown that removal of dissolved oxygen (by $N_2$-scrubbing of solutions) inhibited the cleavage reaction, as did several scavengers of oxygen-derived species (Reed and Douglas, 1991). The Cu(II):thiol system was also found to be effective in cleaving single-stranded DNA. In addition those studies showed that the Cu(II):thiol system did not appear to cleave supercoiled DNA at special structural features of plasmids containing certain insert sequences, e.g. Z-DNA inserts, cruciform structures or left-handed (but non-Z) DNA regions. Base damage was detected, together with some DNA 5'OH and 3'OH groups, as well as 2-thiobarbituric acid-reactive species (probably base propenals) as products of the cleavage reaction. Thus, the work of Reed and Douglas (1989, 1991) provided information on the main features of the Cu(II):thiol DNA cleavage reaction. It was clear that the reaction required Cu(II), a molecule

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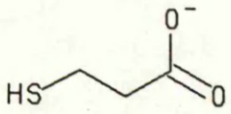


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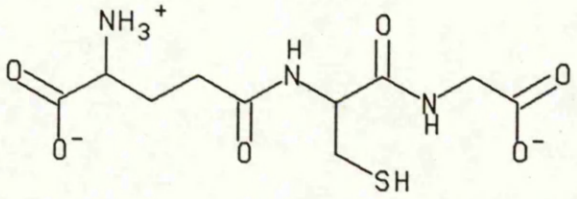


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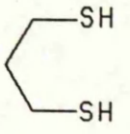


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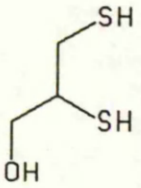


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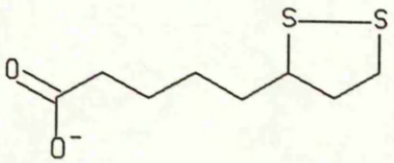


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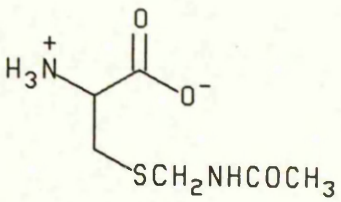


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bearing a free thiol group and the presence of dissolved molecular oxygen. A simple summary of this is shown as a possible reaction scheme (Fig. 51).

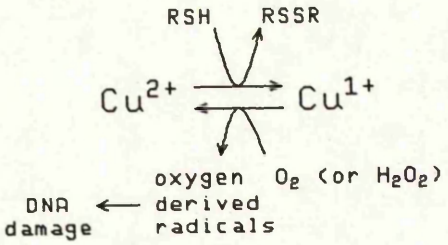



Figure 51. Scheme for Cu(II):thiol reaction in the presence of $O_2$.

In the work described in this thesis, the Cu(II):thiol cleavage reaction was further examined in detail (at single nucleotide resolution), using radioactively labelled linear double-stranded restriction fragments of DNA. In agreement with Reed and Douglas (1989) it was found that the Cu(II):thiol system caused single-strand cleavages within the linear ds-DNA substrate (assessed by denaturing PAGE) and that the extent of DNA cleavage was independent of the nature of the thiol used (the thiols used were D- and L-cysteine, GSH and 3MPA; see section 3.3.2). No cleavage was observed when either partner of the Cu(II):thiol system was incubated in the absence of the other partner. In addition, and surprisingly at first, it was observed that the Cu(II):thiol induced degradation of DNA did not occur equally at every nucleotide position in any given DNA fragment. Rather there was an apparent sequence preference for cleavage, some regions of the DNA fragments being cleaved at a greater rate than others. This apparent sequence preference of the DNA cleavage caused by the Cu(II):thiol system was then found to be independent of the nature of the thiol - the preferred (most intense) sites of cleavage (figure 25) were the same for cysteine, glutathione (an N-blocked

cysteine) and for 3-MPA (cysteine without the $\alpha$-amino group). Thus, the cleavage was independent of the structure and charge distribution of the thiol. Moreover, the reaction was also independent of the stereochemistry of the thiol molecule as D- and L-cysteine showed identical sequence preference. These results indicate that the DNA was cleaved by a common reaction product of Cu(II):thiol interaction and not by either a copper-thiol complex itself or by a Cu(II)-derived thiol species (such as a thiyl radical, $RS^{\cdot}$).

At this point a most important observation was made by carrying out studies of the same DNA fragments as used for Cu(II):RSH cleavage but activating cleavage using Cu(II):ascorbate (a known DNA-cleaving system; Chiou, 1983). Replacement of the thiol by a different reducing agent, namely ascorbate (a structurally unrelated molecule with no thiol group), led to DNA degradation with the same sequence preference as the Cu(II):RSH system (section 3.4.2). Degradation of pBR322-derived DNA fragments by the Cu(II):ascorbate system has previously been shown (Chiou *et al.*, 1985). However, direct comparison with preferred cleavage sites formed in the present work was not possible due to the use of different restriction fragments. Moreover, the analysis of major cleavage sites for the Chiou *et al.*, study concentrated on cleavages within the first 20bp of each fragment (this aspect is further discussed in section 4.2). Data from the above work, however, listed preferred sites as 3′ of dG, 3′ or 5′ of dA and least preferred sites as pyrimidine clusters (e.g. TTCTC). Late on in the studies of this thesis we found literature on DNA cleavage produced by D-glucosamine and D-glucosamine-6-phosphate in the presence of Cu(II) ions (Watanabe *et al.*, 1990). These systems also showed sequence preference, although the DNA fragments used were again different from those used here so a direct comparison of cleavage sites was

not possible from the published data. Analysis of piperidine-labile sites generated by this system, however, showed preferential damage at C and T residues, a result similar to the Cu(II):thiol system (this work, section 3.4.3). It is important to note that cleavages by the Cu(II):reducing sugar system were achieved only after extended incubation (3hr, 37°C) and high concentration of co-reactants (D-glucosamine-6-phosphate 0.1M, Cu(II) 1mM) and thus this system is less efficient in producing DNA damage than Cu(II):thiol.

In this thesis the apparent sequence preference caused by Cu(II):thiol was shown to be identical to that caused by Cu(II):ascorbate (and perhaps the Cu(II):D-glucosamine-6-phosphate systems also gives similar cleavage preference as the damaged base sites were similar to those caused by the former two systems). Detailed analysis of the Cu(II):glucosamine cleavage results of Professor Watanabe's study showed identical cleavage to the present Cu(II):thiol and Cu(II):ascorbate systems (K. Watanabe, personal communication to K.T. Douglas). Therefore, it can be concluded that the common DNA cleavage reaction in all these systems must be mediated by a reaction product whose structure is independent of the reductant used. Cleavage of DNA by Cu(II) plus $\beta$-NADPH or dithionite was also observed (using supercoiled plasmid DNA) although these were the most ineffective reductants used (section 3.4.1). Of the reducing agents tested in this thesis the order of DNA cleaving activity with Cu(II) was: ascorbate > thiol >> $\beta$NADPH $\simeq$ dithionite. This order agrees with literature results for the related MPE-Fe(II):reductant system (ascorbate > thiol >> $\beta$-NADPH; Hertzberg and Dervan, 1984). It is likely that the order of reactivity of these reductants in association with Cu(II) reflects the relative ability of the reductants to participate in reactions such as those of equations 13-15 (see below) which

ultimately lead to DNA cleavage.

On replacing the reductant with an oxidant such as $H_2O_2$ (which is known to produce DNA cleavage in association with Cu(II); Chiou, 1983 and Sagripanti and Kraemer, 1989) it was found in this thesis (figure 39) that the apparent sequence preference was no longer the same as that found using Cu(II):reductant (section 3.4.2). The base-labile sites generated by Cu(II):reagent degradation of DNA were non-identical when Cu(II):reductant and Cu(II):$H_2O_2$ systems were compared (section 3.4.3). In the latter case the results were also in disagreement with those of Sagripanti (Sagripanti and Kraemer, 1989) where specific sites ofbase damage were only seen at polyguanosine sequences. This aspect will be discussed later in more detail (section 4.6).

Taken as a whole, these observations of the DNA degradation caused by reducing agents in the presence of Cu(II), indicate that intermediates or products of the Cu(II):reductant reaction, which are independent of the nature and structure of reductant used, are responsible for the DNA-cleaving activity of such systems. On reduction of Cu(II) to Cu(I) it is most likely that Cu(I) is rapidly reoxidised by dissolved molecular oxygen or oxygen-derived species (Cu(I) disproportionates to Cu(0) and Cu(II) in solution) (Russell, 1981). Modulation of such reactions is expected to occur in the presence of copper-chelating ligands such as the phosphate groups and bases of DNA, which may make the reduction to Cu(0) less favoured and the reoxidation to Cu(II) more favoured (Russell, 1981).

From the inhibition of the cleavage reaction by (mainly) hydroxyl radical scavengers (Reed and Douglas, 1991), the known DNA-cleaving ability of such radicals (Tullius, 1989) and the documented hydroxylation of aromatic

molecules by Cu(II):ascorbate (Udenfriend *et al.*, 1954), it is reasonable to postulate that the hydroxyl radical is the major species responsible for DNA cleavage by Cu(II):reductant systems.

Studies on the Cu(II):$H_2O_2$ system (Chiou, 1983; Sagripanti and Kraemer, 1989) implicated hydroxyl radical in its mechanism of DNA cleavage, together with singlet oxygen ($^1O_2$). However, the dissimilarity of the patterns of DNA cleavage presented for the Cu(II):reductant and Cu(II):$H_2O_2$ systems (figure 39) indicates that the two systems are significantly distinct in the exact nature of the DNA-cleaving species produced. The generation of $^1O_2$, in addition to hydroxyl radical, by the Cu(II):$H_2O_2$ system (Chiou *et al.*, 1983) cannot alone explain the differences in cleavage patterns of this and the Cu(II):reductant systems. Preferential damage at guanine bases is caused by $^1O_2$ (Kawanishi *et al.*, 1986) but this is not the difference observed for the two systems. The origin of this apparent contradiction is further complicated by published reaction schemes which show the Cu(II):reductant and Cu(II):$H_2O_2$ systems as essentially equivalent! For example, for Cu(II) ascorbate:

$$\text{ascorbate} + 2Cu^{2+} \longrightarrow \text{dehydroascorbate} + 2Cu^{+} + 2H^{+} \qquad \text{(eqn. 13)}$$

$$\text{ascorbate} + O_2 \longrightarrow \text{dehydroascorbate} + H_2O_2 \qquad \text{(eqn. 14)}$$

$$Cu^{+} + H_2O_2 \longrightarrow Cu^{2+} + {}^{-}OH + {}^{\bullet}OH \qquad \text{(eqn. 15)}$$

Equations 13 to 15 (Chiou, 1983) suggest that the ultimate oxidant of $Cu^+$ may be $H_2O_2$ resulting in generation of $^{\bullet}OH$. Similar reactions may be written for RSH as the reductant (e.g. Munday, 1989):

$$RS^{-} + Cu^{2+} \longrightarrow RS^{\bullet} + Cu^{+} \qquad \text{(eqn. 16)}$$

$$Cu^{+} + O_2 \longrightarrow Cu^{2+} + O_2^{-} \qquad \text{(eqn. 17)}$$

$$RS^{-} + RS^{\bullet} \longrightarrow R\overline{S}\overset{\bullet}{S}R \qquad \text{(eqn. 18)}$$

$$R\overline{S}\overset{\bullet}{S}R + O_2 \longrightarrow RSSR + O_2^{-} \qquad \text{(eqn. 19)}$$

$$RSH + O_2^{-} + H^{+} \longrightarrow RS^{\bullet} + H_2O_2 \qquad \text{(eqn. 20)}$$

$$Cu^{+} + H_2O_2 \longrightarrow Cu^{2+} + {}^{\bullet}OH + {}^{-}OH \qquad \text{(eqn. 21)}$$

Note that equations 15 and 21 are essentially equivalent; both reductant systems produce hydroxyl radicals *via* the same final step and the generation of $H_2O_2$ from RSH catalysed by Cu(II) is well documented (e.g. Ehrenberg *et al.*, 1989). Possible reaction schemes for Cu(II):$H_2O_2$ are:

$$Cu^{2+} + H_2O_2 \longrightarrow Cu^{+} + O_2^{-} + 2H^{+} \qquad \text{(eqn. 22)}$$

$$Cu^{+} + H_2O_2 \longrightarrow Cu^{2+} + {}^{\bullet}OH + {}^{-}OH \qquad \text{(eqn. 23)}$$

Thus, although the Cu(II):reductant and Cu(II):$H_2O_2$ systems may both cleave DNA and both generate $^{\bullet}OH$ radicals *via* a Cu(I):$H_2O_2$ reaction, the observed differences in the sequence preferences for DNA cleavage indicate that specific DNA cleavage must be due to different reactive products. The dissimilarity between sequence-preferential cleavage of DNA by a Cu(II):reductant system and the sequence-neutral cleavage of DNA by the Fe(II):EDTA system which is known to generate hydroxyl radical is a further issue to be resolved. With a view to resolving such apparent anomalies further details of the DNA cleavage caused by Cu(II):reductant systems are now discussed in relation to other, well-documented DNA cleaving systems (section 4.2).

## 4.2 DETAILS OF Cu(II)-MEDIATED CLEAVAGE OF LINEAR DNA

Detailed examination of the DNA degradation caused by Cu(II):thiol (and other Cu(II)-based systems) was carried out by means of the cleavage of $^{32}P$ end-labelled restriction fragments of known sequence (sections 3.3-3.5). Analysis of the products of cleavage by denaturing PAGE provided details of the cleavage sites to the level of single-base resolution. The major results are listed below and then discussed in relation to each other and other cleavage systems described in the literature:

i) the degradation of DNA by Cu(II):thiol was not sequence-neutral, an apparent sequence preference for cleavage was observed;

ii) for any given DNA fragment this apparent sequence preference was independent of the structure of the thiol compound used;

iii) cleavage of three different DNA fragments of non-identical sequences showed three distinct cleavage patterns with no clear consensus sequence for sites of preferred cleavage being detectable;

iv) base-labile sites generated by the Cu(II):thiol system for all three DNA samples were predominantly thymine residues;

v) the cleavage of ds-DNA produced 5′-phosphoryl, 3′-phosphoryl and 3′ phosphatase-inert termini at the point of cleavage;

vi) independently produced cleavage patterns of each strand of a duplex showed that sites of cleavage maxima and minima were at non-identical nucleotide positions being displaced by 3 or 4 bases in either direction;

vii) both Cu(II):thiol and Cu(II):ascorbate systems produced identical

DNA cleavage patterns and base-labile sites;

viii) replacement of Cu(II):thiol or Cu(II):ascorbate systems by the Cu(II):$H_2O_2$ reagent produced a different cleavage pattern and some different base labile sites;

ix) the Cu(II):thiol DNA cleavage reaction was not affected by increasing ionic strength ($\Delta\mu$ = 0.1) or by addition of metal ions ($Na^+$ up to 0.1M or $Mg^{2+}$ up to 3.3mM).

x) the Cu(II):thiol system caused cleavage of a heat-denatured, single-stranded DNA fragment with a greater sequence preference than that observed with dsDNA fragment.

Control experiments were carried out to check that the particular preparations of DNA fragments used in these procedures reacted as expected with known DNA-cleaving systems in order to eliminate the possibility that any peculiarly hyper- or hypo-sensitive sites were present in the DNA fragments. Reactions with the Fe(II):EDTA system (section 3.5.1) showed that the fragments were cleaved in a "sequence-neutral" (i.e. a sequence-independent) manner, as expected from literature reports (e.g., Schultz and Dervan, 1984). Study of one of these fragments (the tyrT fragment) with the Cu(I):phenanthroline system (section 3.5.2) confirmed that the fragment was cleaved in the expected, sequence dependent manner (Drew and Travers, 1984). Thus it was concluded that, for the DNA preparations used, there was no evidence of abnormal reactivity in the two standard chemical cleavage systems and therefore the results observed for cleavage of these fragments by Cu(II):thiol were not caused by an inherent abnormal reactivity of the DNA preparations.

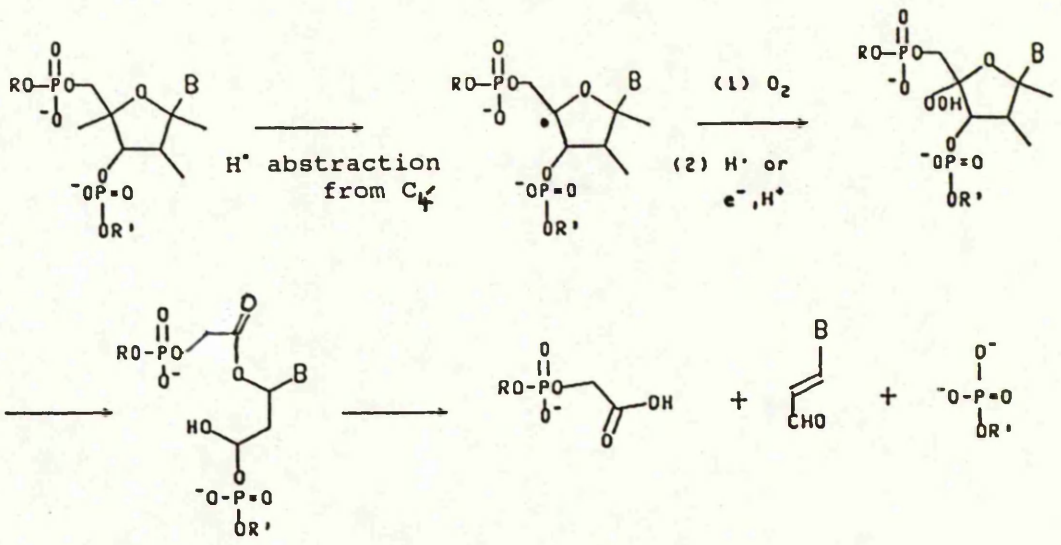
The presence of identical cleavage patterns for DNA digested by Cu(II) plus a variety of thiols as well as by copper(II):ascorbate (sections 3.3.2 and 3.4.2, respectively) suggests that the DNA cleavage was mediated by a product of the reaction of Cu(II) and reductant and not by a Cu(II):reductant complex itself. (The latter has been reported to be the case for Cu(I):phenanthroline cleavage; reviewed by Sigman and Chen, 1989). The reactive species generated by Cu(II):reductant in this oxygen-dependent reaction (Reed and Douglas, 1991) is most likely to be formed on re-oxidation of Cu(I) by oxygen or oxygen-derived species (see section 3.2.2 for proof of the Cu(II) and Cu(I) requirement) and is probably therefore an oxygen-derived radical species. The hydroxyl radical, which is a well-documented DNA-cleaving species (reviewed by Tullius, 1989), is the most likely candidate for reasons discussed in section 4.1. Equations detailing the reactions of Cu(II):ascorbate and Cu(II):thiol respectively which lead to the production of $^{\bullet}OH$ are given in section 4.1. Work further to that of Reed and Douglas (1991) on identifying the radical species causing DNA cleavage was not undertaken, although inferences from the determination of the nature of the DNA termini generated by Cu(II):thiol treatment (section 3.3.5) may be made regarding the likely nature of the radical.

Determination of the nature of the DNA termini generated at the point of Cu(II):thiol cleavage (section 3.3.5) produced results identical to those determined for the hydroxyl radical-generating, MPE:Fe(II) system (Hertzberg and Dervan, 1984) namely, production of 5'-phosphoryl, 3'-phosphoryl and 3'-"phosphatase-inert" termini (the last two products in equal proportions). Although for the MPE:Fe(II) system the 3' phosphatase-inert species was further identified to be a 3'-phosphoglycolate species, the 3' phosphatase-inert

species generated by Cu(II):thiol has not yet been further identified in this current work. It is appropriate to note that hydroxyl radical-mediated DNA damage produced by either $^{60}$Co $\gamma$-irradiation or MPE:Fe(II) treatment both lead to production of equivalent proportions of 3'-phosphoryl and 3'-phosphoglycolate termini (Kuwabara *et al.*, 1986; Hertzberg and Dervan, 1984, respectively) whereas unequal proportions are produced by Cu(I):phenanthroline cleavage (3'-phosphoglycolate is a minor product; Kuwabara *et al.*, 1986) and BLM cleavage (3'-phosphoglycolate is the primary stable product; Stubbe and Kozarich, 1987). This possibly indicates that the Cu(II):thiol DNA cleavage mechanism is similar to that of the hydroxyl radical- generating systems as both systems produce similar products in similar proportions. Thus, by comparison to the MPE:Fe(II) and $^{60}$Co $\gamma$-irradiation systems (Hertzberg and Dervan, 1984; Kuwabara *et al.*, 1986, respectively) the 3' phosphatase-inert species produced by Cu(II):thiol cleavage of a 5' end-labelled fragment (section 3.3.5.2) can be inferred to be a 3'-phosphoglycolate species. The reactions producing this pattern of cleavage products are equal attack of hydroxyl radical at C1' and C4' of deoxyribose (see figure 52).

Previous work by Reed and Douglas (1991) showed the presence of 5'-OH and 3'-OH termini in plasmid DNA digested with Cu(II):thiol, but detection of 3' and 5'-phosphoryl termini was not carried out. In this present work, however, no 3'- or 5'-OH termini were detected (section 3.3.5), but would have been observed if present in significant quantities. Thus, it is concluded that DNA fragments bearing 3' or 5'-hydroxyl termini are likely to be very minor products of Cu(II):thiol DNA cleavage, only detected in the plasmid system by the very sensitive assay used by previous workers (Reed and Douglas, 1991). Note that although 5' and 3' end-determinations were not

SCHEME 1




SCHEME 2

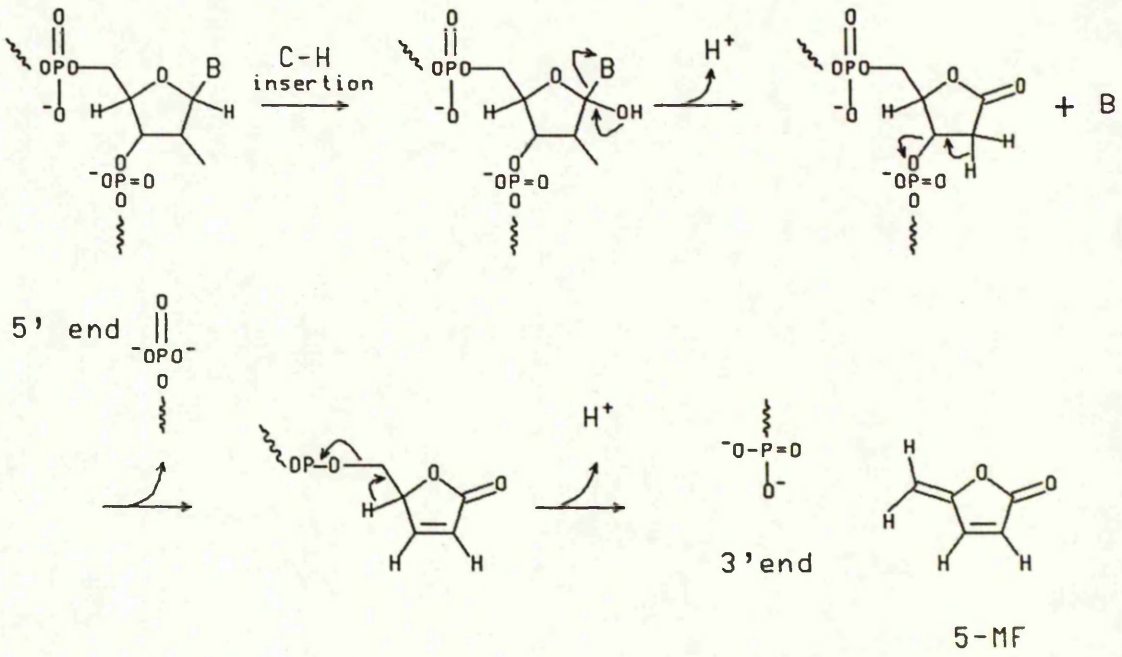



Figure 52. Possible reaction pathways for the Cu(II):thiol mediated DNA cleavage involving attack of •OH equally at C4' (scheme 1) and C1' (scheme 2) of deoxyribose. Scheme 1 (see also figure 11) accounts for formation of 5'-phosphoryl and 3'-phosphoglycolate termini (observed products) and also base propenals (presence not determined). Scheme 2 (see also figure 12) accounts for 3'-phosphoryl and 5'-phosphoryl termini (observed products) and 5-methylene-2-furanone (5-MF, presence not determined).

carried out for Cu(II):$H_2O_2$, the co-migration of G-track marker fragments (5'-phosphoryl termini) and 3' end-labelled fragments digested with Cu(II):$H_2O_2$, indicates that Cu(II):$H_2O_2$ cleavage generates fragments with 5'-phosphoryl termini. The 3' termini were not determined in this work and could not be assessed from the published data.

The observation of an apparent sequence-specificity seems to group the Cu(II):thiol nuclease with other nucleases that produce a sequence-dependent cleavage, e.g. DNAse I (figure 31), Cu(I):phenanthroline (figure 42) and (to some extent MPE:Fe(II) (Harshman and Dervan, 1985). All three of the above systems bind to DNA and this binding produces variations in cleavage extent dependent on the precise DNA sequence (although the extent of such sequence-dependence is most marked for DNAse I but only slight for MPE:Fe(II)). A cleaving system that does not bind to DNA is the Fe(II):EDTA system. This system produces sequence-independent, sequence-neutral DNA cleavage, i.e. it gives approximately equal cleavage at all points in a DNA sequence (except where the DNA assumes altered conformations, e.g. as in the study by Shafer *et al.*, 1989). Comparison of Cu(II):thiol, Fe(II):EDTA or Cu(I):phenanthroline cleavage of tyrT DNA, (figures 25, 41 and 42, respectively) reveals that the extent of sequence preference shown by Cu(II):thiol is somewhere between those exhibited by the other two systems. Further it can be seen that Cu(II):thiol cleavage resembles that of Fe(II):EDTA more closely than that of Cu(I):phenanthroline and so might perhaps be classed as a non-DNA-binding cleavage system. However, it is well known that divalent metal ions such as Mg(II), Co(II), Ni(II), Zn(II), Cd(II) and Cu(II) can bind to DNA (e.g. Eichorn and Shin, 1968) *via* interactions with both phosphate and base sites. Copper(II) ions are known to destabilise the double-helix by means of

interactions with the bases, lowering the Tm of DNA. Cu(II) also has the greatest affinity for DNA bases, as opposed to phosphates, of all the metal ions in the above list. However, all the experiments on the effects of these metal ions (e.g. Eichorn, 1962; Eichorn and Shin, 1968; Forster *et al.*, 1979; reviewed by Pezzano and Podo, 1980) were conducted without buffering compounds present to minimise the effects of metal ion-binding to buffering compounds (which are usually chelating compounds). In this present study, however, buffered solutions were used (10mM sodium phosphate, pH 8.0) and thus it was likely that metal ions added, would partition between DNA and the phosphate buffer components. Therefore, checks were made to determine the possible effects of Cu(II) in this buffered system (e.g. pH effects, DNA denaturation).

Effects of the added Cu(II) ions on the pH of the buffered solution were found to be negligible (section 3.1.1). Effects of the added Cu(II) on the Tm of DNA in buffered solution (section 3.1.2) were also found to be negligible (DNA Tm is substantially lowered by Cu(II) in non-buffered solution; Eichorn and Shin, 1968). Thus, the apparent sequence preference of the copper(II):thiol system was not likely to be due to Cu(II)-related pH effects or gross alterations, such as denaturation, in the dsDNA structure.

The possibility of the cleavage patterns being due to adsorption of DNA onto copper-phosphate microcrystals was also checked for in the light of literature reports of Fe(II):EDTA cleavage of DNA adsorbed onto calcium-phosphate microcrystals (Tullius and Dombroski, 1985). In these studies of microcrystals, marked variations in cleavage intensity were observed for nucleotide positions according to whether they were bound or not bound to the surface of the crystals, which were present as a suspension. However,

experiments (section 3.1.3) established that a similar phenomenon was unlikely to be occurring in the present study of the Cu(II):thiol system since there was no detectable loss of Cu(II) from solution by precipitation (or any other route). It was also established that increasing the ionic strength of the incubation solution had no observable effect on Cu(II):thiol cleavage of DNA (section 3.8). The reaction was not inhibited in the presence of added NaCl up to 0.1M ($\Delta\mu$ = 0.1), in agreement with results of supercoiled plasmid experiments (Reed and Douglas, 1991). In addition the observed sequence preference was not altered at this higher ionic strength. Reactions were also carried out with added $Mg^{2+}$ ions (as $MgCl_2$) but due to difficulties in redissolving the lyophilised DNA pellet, following the cleavage reaction, only results for incubations containing up to 3.3mM $MgCl_2$ ($\Delta\mu$ = 0.01) were obtained. Higher levels of $MgCl_2$ led to a reduction in recovery of DNA. However, at such low levels Mg(II) had no effect on the Cu(II):thiol cleavage preference or on the extent of reaction. These preliminary results may indicate that Cu(II) if bound to DNA, is not bound in the same manner or at the same sites as $Na^+$ or $Mg^{2+}$ ions.

Cleavage of ss-DNA by Cu(II):thiol has been shown previously using M13 DNA (Reed and Douglas, 1991) although this was not studied at the nucleotide level. Consequently, heat-denatured tyrT dsDNA was used as a ssDNA substrate for the Cu(II):thiol reaction (section 3.3.7). It was cleaved preferentially at two sites in a reaction that yielded a much more specific cleavage pattern than that observed using the native tyrT DNA (figure 34). Proof of the effectiveness of the denaturation procedure was provided by comparison with other harsher denaturing procedures (section 3.3.8) and reaction of the heat-denatured DNA with ss-specific chemical probes (section 3.3.9). Such probing of the denatured DNA (in the absence of Cu(II)) using

$KMnO_4$ and DEPC showed that around the two preferred Cu(II):RSH cleavage sites, and throughout the rest of the DNA fragment, the DNA reactivity was characteristic of non dsDNA (i.e. ssDNA). Thus, it is unlikely that the preferred cleavage sites of Cu(II):thiol on denatured tyrT DNA were due to, for example, some local renaturation of complementary strands or formation of local double-stranded sites by intramolecular base-pairing of a single strand creating sites for preferred Cu(II):thiol reaction. The cause of these preferred cleavage sites has not been determined.

## 4.3 CLEAVAGE OF DNA CONTAINING INSERTS OF REPEATING DINUCLEOTIDE SEQUENCES

Previous workers (Reed and Douglas, 1991) had examined the Cu(II):RSH cleavage of supercoiled plasmids containing specific inserts which produced Z-DNA, cruciform or left-handed non-Z-DNA structures when under supercoil stress. Cleavages occurred throughout the whole plasmid DNA sequence and not specifically (or preferentially) at the sites of the non-B-DNA insert sequences. Thus, Cu(II):RSH showed no ability to act as a probe for these non-B-DNA structures unlike, for example, chiral tris-phenanthroline complexes (section 1.3.2.4.3 and references therein). In the present study, further examination of Cu(II):thiol cleavage of such inserts was undertaken, this time as components of linear restriction fragments. The purpose was to determine if subtle differences in cleavage were occurring at the nucleotide level, a feature which could not be probed as minutely using the protocols available for plasmid DNA.

Unless specific conditions are used, such as the presence of micromolar

concentrations of polyamines (e.g. spermine, 16$\mu$M; Thomas and Messner, 1986), the presence of cytosine methylation and addition of $Co(NH_3)_6^{3+}$ (Behe and Felsenfield, 1981), the presence of [tris(2-aminoethyl)amine]zinc(II) chloride (Woisard and Fazakerley, 1986) or the presence of NaCl at high concentration (e.g. 5M; Klysik *et al.*, 1981), there is no evidence for structural transitions of B to Z-DNA occurring in linear restriction fragments (where the supercoil induced structural transition cannot occur). Thus, any effects observed on cleavage of these fragments containing the potential Z-forming inserts would not be ascribable to the presence of Z-DNA.

DNA fragments containing 24 bp insert sequences of $(TG)_{12}$ and $(TG)_6(AC)_6$, which can adopt Z-DNA and non Z-DNA, left handed forms, respectively (McLean and Wells, 1988) were cleaved using the Cu(II):thiol system (section 3.9). Results showed that within the insert sequences there was a definite preference for cleavage at cytosines in the 3'$(AC)_n$5' sequence (only 3' labelled, lower strand examined; figure 48). On considering cleavage intensities at all nucleotide positions of the DNA fragments it was clear that not all cytosines were preferentially cleaved, but that cleavage within the insert sequences was relatively enhanced at cytosines compared to intervening adenines. Cleavage preferences for the complementary strands were not examined. In the absence of the special conditions required for restriction fragments to form Z-DNA and, given that the presence of Cu(II) at 0.2mM is unlikely to effect any DNA conformational change (e.g. Cu(II) has no effect on DNA Tm, see section 3.1.2; Cu(II) is unable to produce the B to *H-DNA transition in supercoiled plasmids, Bernués *et al.*, 1990), then the cleavage patterns observed for the insert sequence regions must be due to some other factor(s) (e.g. local helical parameters affecting binding of Cu(II) to DNA at

3'(pu.pyr)$_n$ sequences). Studies of buffered DNA solutions containing Cu(II) are not evident in the literature and so detailed knowledge of the binding, or otherwise, of Cu(II) to DNA in this situation is lacking. Conclusions on these Cu(II):thiol cleavage results are therefore difficult to make. However, it is appropriate to note that the sequence-neutral, non-DNA-binding, Fe(II):EDTA nuclease shows small variations in cleavage intensity at certain sequences of DNA, e.g. at adenine tracts in kinetoplast DNA (Burkhoff and Tullius, 1987), which has been interpreted to arise from narrowing of the minor groove from one end of the adenine tract to the other - this subtle sequence variability in cleavage extent probably reflects the accessibility and reactivity of hydroxyl radicals to deoxyribose moieties (Tullius, 1990) but has also been attributed to weak binding of the coordination complex to DNA (Jczewska *et al.*, 1990). Similarly, the preferential reaction of Cu(II):thiol with the bases of the insert regions of these 70bp fragments may also be dependent on sequence-determined factors, although not on sequence itself *per se*.

## 4.4 ATTEMPTED DNA CLEAVAGE WITH DNA-BINDING THIOL SYSTEMS

Attempts were made to target the Cu(II):thiol reaction to occur in the vicinity of DNA by using various classes of DNA-directed thiol groups. Thus, thiols linked to DNA-binding molecules such as intercalators or oligonucleotides were studied. By juxtaposing the thiol and DNA it was envisaged that subsequent reaction of thiol with Cu(II) would result in production of reactive species close to the DNA itself and therefore yield more effective cleavage of DNA than was possible by thiols free in solution.

4.4.1 Intercalator-borne thiols

Incubation of DNA with a thiol-bearing intercalator molecule (ISH, 33) and Cu(II) ions, produced no detectable DNA cleavage under conditions which produced cleavage of sc to nicked and linear DNA by GSH plus Cu(II) (section 3.6.1). Thus, instead of increasing the amount of DNA cleavage, as was anticipated, the DNA cleaving ability was abolished. Several factors were explored in order to try to explain these results:

a) the reactivity of ISH with Cu(II) in the presence and absence of DNA, and

b) the reactivity of ISH with a thiol-specific reagent (DTNB) in the presence and absence of DNA.

Factor (a) was studied to compare the relative reactivities of ISH and GSH with Cu(II). The results (section 3.6.2) showed that, whereas the loss of GSH thiol titre on addition of Cu(II) was very fast (0% left after 10 min; in agreement with Ehrenberg *et al.*, 1989), loss of ISH titre occurred much more slowly (65% left after 10 min; in the absence of DNA). In addition, the loss of ISH thiol titre was greater when both Cu(II) and DNA were present (40% left after 10 min). Under these conditions there was no detectable difference in the rate of GSH loss when DNA was absent or present.

As was shown earlier (section 4.1), reaction of Cu(II), RSH and $O_2$ produces oxygen-derived free radicals which react with DNA to cause cleavage and also lead to loss of RSH as it is not regenerated in the system. It is reasonable, therefore, to correlate the rapid loss in thiol titre observed for GSH in the presence of Cu(II) with DNA cleaving ability. Other thiols tested, e.g. L-cysteine and 3-MPA, showed similar rapid loss of thiol titre (results not

shown). On this basis, the slower, but significant, loss of ISH thiol titre would seem to indicate that cleavage of DNA would be expected for Cu(II):ISH systems (albeit at a reduced rate compared to "simple" thiols).

These results also indicate the free radical nature of the Cu(II):thiol generated DNA cleavage reagent: a time course of plasmid DNA cleavage using higher Cu(II) concentrations than in the loss of thiol titre study (section 3.6.2), showed that DNA damage increased with the time of incubation from 1 to 20min (figure 22, section 3.2.1). Under these conditions (RSH, 100$\mu$M; Cu(II), 200$\mu$M) loss of thiol titre is expected to have occurred more rapidly than that shown for reactions in figure 44 (RSH, 100$\mu$M; Cu(II), 75$\mu$M) due to the higher Cu(II) concentration in the former. Thus, how does DNA cleavage proceed for up to 20min when all the GSH may have reacted within less than 5 min? The answer is that the products generated by reaction of Cu(II), thiol and $O_2$ (a reaction which leads to the loss of GSH thiol titre) are involved in subsequent reactions which produce the ultimate, DNA-cleaving species. Thus, when all the GSH has "disappeared" from the solution, the DNA cleavage reaction will continue.

The slow reaction of ISH with Cu(II) probably indicates slower production of Cu(I) and the free radicals required for DNA cleavage and thus makes ISH an extremely inefficient DNA cleavage reagent. Experiments using longer incubation times (not carried out) would perhaps show DNA cleavage by Cu(II):ISH, although cleavage to the extent shown by Cu(II):GSH is extremely unlikely in view of the "tailing off" of the thiol titre loss observed for ISH (figure 44). The reason for the low reactivity of ISH with Cu(II) in the absence of DNA, is not known, although in the presence of DNA, factors such as DNA-ISH binding interaction may also serve to reduce the ISH thiol

reactivity.

Also apparent from figure 44, is that the initial drop in thiol titre was greater for the ISH sample in the presence of DNA than for ISH alone (60% thiol remaining compared to 85%) and this difference remained even after 30min reaction. Thus, the addition of DNA reduced the initial thiol titre, but subsequent loss of thiol occurred at roughly comparable rates in the presence or absence of DNA. To examine whether the addition of DNA led to a concentration-dependent reduction in ISH thiol titre, increasing amounts of DNA (up to a 12.5 fold molar excess) were added to solutions of ISH and the thiol titre checked. No difference in the thiol titres for ISH in the presence or absence of DNA were observed (Table 5). This seems to indicate that even in the presence of excess DNA (where a large proportion of ISH would be expected to intercalate) the thiol was 100% titratable, and therefore reactive towards the thiol reagent DTNB (i.e. remained as free thiol itself). Therefore, the initial differences in thiol titre observed for solutions of ISH in the presence of Cu(II) and ISH in the presence of Cu(II) plus DNA, cannot be explained by loss of titratable thiol by binding of ISH to DNA. Also it is noted these results preclude the possibility that proximally intercalated ISH molecules may dimerise while bound to DNA, thereby creating a loss in thiol titre. The explanation of the lower value of thiol titre observed for Cu(II):ISH solution in the presence of DNA may therefore be due to a Cu(II):DNA-related phenomenon. For example, binding of Cu(II) by DNA may increase its reactivity toward ISH, leading to a more rapid loss in thiol titre compared to reaction of Cu(II) with ISH in the absence of DNA. Although, if the above is the case then the almost parallel "tailing off" of the ISH titres is less easily explained as a more rapid loss of thiol titre in the presence of DNA would be expected. Full explanation of these

results will, however, require further kinetic work as yet not undertaken.

Preliminary results using a thiol postulated Fmoc-cysteine (36), and a thiol-derivatised anthraquinone derivative (38, a postulated intercalator-thiol) showed that the non-intercalator molecule cleaved DNA to the same extent as glutathione for example, whereas the anthraquinone derivative was unable to effect DNA cleavage. From these, albeit very preliminary, results both examples of thiol-derivatised molecules containing known intercalating moieties failed to produce DNA cleavage. In contrast, Fmoc-cysteine with a large fluorene chromophore but probably poor intercalating ability because of substitution at the 9-position rather than the 2 position, was able to produce DNA cleavage as effectively as other "free" thiols. Hence, it is proposed that intercalative binding of the intercalator-thiol molecules may lead to a non-covalent deactivation of the thiol moiety, e.g. by hydrogen bonding to DNA. Further work will be needed to confirm this, although, data for intercalation of chloroquine (O'Brien *et al.*, 1966) has shown that the positively-charged terminal substituted amine functional group (linked by a 5 atom linker to the 4-position of the ring system), is present within the molecular dimensions of the DNA helix. Thus, the terminal SH group of ISH (linked by a 3 atom linker to the 4-position of the ring system) must also be within the dimensions of the DNA-helix and therefore may be able to hydrogen bond with the bases of DNA. Synthesis of an ISH derivative with a longer linker chain between the intercalating moiety and the thiol may produce a more active DNA cleaving molecule by positioning the thiol outside the DNA helix.

### 4.4.2 Oligonucleotide-borne thiols

Targetting the Cu(II):thiol reaction to a specific sequence of DNA by means of a modified oligonucleotide bearing a thiol moiety at its 5′ end (oligo-SH, 34) also failed to yield DNA cleavage (section 3.7). Neither DNA strand cleavage nor base damage (as assessed following piperidine treatment) was observed. Unlike the intercalator-thiol experiments the DNA target in these reactions was single-stranded except at the region where the oligo-SH was bound to DNA by base-pairing with its complementary sequence. In this case the lack of positive cleavage results might be ascribed to any (or all) of the following:

a) hydrogen bonding of DNA-bound thiol moiety to the bases of the ssDNA strand rendering RSH unreactive to Cu(II),

b) lack of binding of Cu(II) to ssDNA in the region of the annealed oligo-SH thiol moiety preventing Cu(II):RSH reaction,

c) insufficient oligo-SH:target DNA complex, leading to insufficient cleavage above background levels,

d) loss of thiol prior to initiation of cleavage reaction.

Point (b) is unlikely as Cu(II):thiol has been shown to cleave ss M13 DNA (Reed and Douglas, 1991) and point (c) is also unlikely as the presence of a significant amount of annealed oligo-SH:target DNA was detected (section 3.7.2). Loss of thiol is likely to occur in the samples annealed for periods of up to 45min, and may therefore, explain the lack of DNA cleavage observed in these samples. However, samples of oligo-SH plus target DNA that were not subjected to an annealing procedure before initiation of the Cu(II):RSH reaction

(see section 3.7.3) also failed to show DNA cleavage and thus point (d) is also unlikely to be the cause of the observed lack of DNA cleavage. Thus, point (a) is considered to be the most likely cause of the observed results.

With hindsight, targetting of the Cu(II):thiol reaction to a specific sequence of DNA should be attempted using an oligo-SH molecule (with either a polypurine or polypyrimidine sequence only) which is capable of binding to dsDNA, in the major groove, to create a triple helix (see section 1.4.2 and references therein). In this case there would be no need for the denaturation of target dsDNA, or the annealing procedure (used in section 3.7.2). However, it is noted that even this approach may not be successful because if Cu(II) were to bind mainly in the minor groove then reaction with oligo-SH thiol (bound in the major groove) would be impossible.

## 4.5 USE OF Cu(II):THIOL FOR FOOTPRINTING

Attempts to use the Cu(II):thiol system as a footprinting reagent for DNA binding molecules suchas small drug-like molecules (section 3.10.1) or for proteins (section 3.10.2) did not yield footprints.

### 4.5.1 Hoechst 33258 as minor groove directed ligand

Control reactions (section 3.10.1) using the Fe(II):EDTA system produced clear footprints of Hoechst 33258 (a minor groove binding ligand). The protected regions of sequence were in close agreement with data in the literature (e.g. Harshman and Dervan, 1985). Reactions similar to those above but using the Cu(II):thiol system instead of Fe(II):EDTA did not display

observable differences in the DNA fragment cleavage patterns in the presence or absence of Hoechst 33258, that is, no footprint was produced. However, it was noted that the efficiency of the Cu(II):thiol cleavage reaction decreased with increasing concentration of Hoechst 33258 a feature shared with other "nucleases", e.g. DNAse I, MPE:Fe(II), and Fe(II):EDTA. This is normally attributed to the increasing non-specific binding of drug molecules found at increasing [drug]:[DNAbp] ratios; the majority of DNA sites, not just those with ligand specifically bound, become inaccessible to the "nuclease" and so cleavage is prevented. Indeed, during optimisation studies of the Fe(II):EDTA footprinting reaction (results not shown) it was found that there was very little cleavage of the fragment at Hoechst 33258 levels at or above 50$\mu$M [drug]:[DNAbp] ratio $\geq$1.25), i.e. no Fe(II):EDTA footprint of Hoechst 33258 was apparent under such conditions. As the levels of Hoechst 33258 used in the Cu(II):thiol footprinting experiments were identical to those used in the optimised Fe(II):EDTA experiment, it is clear that the lack of footprint and the presence of inhibition of the Cu(II):thiol cleavage reaction cannot be attributed to inappropriate drug:DNA ratios. Other possible factors to explain the lack of footprinting activity of the Cu(II):thiol system may be:

a) interference of Hoechst binding by the presence of Cu(II) ions,

b) reaction of the cleavage system with Hoechst 33258,

c) cleavage of DNA by Cu(II):thiol at DNA sites not involved in the drug:DNA interaction.

Referring to point (a), it is possible that the slight increase in ionic strength ($\Delta\mu$ = 0.8 x $10^{-3}$M) and/or the presence of Cu(II) ions may disturb the binding of the drug to DNA by competition of Cu(II) ions (200$\mu$M) and the drug (1-20$\mu$M) for binding to anionic sites on the DNA (e.g. minor groove). It should

be noted, however, that 50mM NaCl had no effect on the footprint of Hoechst 33258 given by Fe(II):EDTA (section 3.10.1, results not shown). Thus, the increase in ionic strength caused by adding Cu(II) to 0.2mM is not expected to lead to disruption of Hoechst 33258 binding to DNA. Although Hoechst 33258 was present at concentrations 10-200 fold lower than that of Cu(II), this concentration difference is not thought to be relevant as the relative DNA-binding affinities of Cu(II) and the drug are widely different - literature values for Hoechst:DNA, $K_{assoc} \simeq 10^6$-$10^9$ $M^{-1}$ (Loontiens *et al.*, 1990) and Cu(II):DNA, $K_{assoc} \simeq 0.1$-$2.5 \times 10^4$ $M^{-1}$ (Sorokin *et al.*, 1987).

Reaction of either Cu(II) or thiol with Hoechst 33258 (point (b)) seems unlikely, e.g. chelation of Cu(II) by the drug *via* a pair of its nitrogens is unlikely given the distance separating any two nitrogens and the rigidity of the molecule keeping them too far apart to form an efficient Cu(II) chelator. It is conceivable that the DNA cleaving species generated by Cu(II):thiol might react with the drug and therefore lead to drug molecules with modified (or no) DNA binding abilities. At the low concentration of drug used, however, it is apparent that this cannot be the major reaction occurring in the presence of DNA as the DNA is itself cleaved. Moreover, the Fe(II):EDTA system is capable of footprinting Hoechst 33258 even though it is a very efficient hydroxyl radical generator and may modify drug molecules by reaction with the DNA cleaving. Thus, this factor is not considered responsible for the lack of footprinting by Cu(II):thiol.

Point (c) is very important as it has been proved for at least one chemical nuclease (i.e. Cu(I):phenthanroline; Kuwabara *et al.*, 1986) that the binding of the nuclease in the minor groove of DNA (required for its DNA cleaving activity) precludes this nuclease from footprinting major groove

binding molecules, such as the EcoRI restriction enzyme. Other nucleases that do not bind to DNA, e.g. Fe(II):EDTA, can produce footprints of both major and minor groove ligands (e.g. Tullius and Dombroski, 1986; Laughton *et al.*, 1990). Thus, it may be possible that the Cu(II):thiol system produces its DNA damage *via* reactions with DNA in regions other than the minor groove (e.g. major groove, phosphate backbone or bases) and so does not produce footprintings of minor groove binding drugs (e.g. Hoechst 33258) since it cannot detect their presence in a drug:DNA complex. Thus, point (c), although unproven, is the most likely explanation of the inability of Cu(II):thiol to footprint Hoechst 33258.

### 4.5.2 A major groove ligand as footprinting target

Footprinting of a major groove binding molecule was attempted using the HindIII restriction enzyme as the DNA-binding ligand. The contacts between a DNA-binding protein and DNA, in the majority of systems, occur in the major groove of DNA - as proven for EcoRI restriction enzyme (McClarin *et al.*, 1986) and numerous other DNA binding proteins (section 1.2.2 and references therein). As all the DNA fragments used routinely in this thesis had been produced by initial EcoRI digestion of plasmid DNA, the footprinting of EcoRI was not feasible. However, one of the fragments, the 167bp (pBR322) fragment, contained a HindIII restriction site within its sequence and so could be used for footprinting HindIII:DNA interactions (even though the binding of HindIII to DNA has not yet been fully characterised).

Footprinting of HindIII bound to the 167bp fragment was carried out using Cu(II):RSH in the absence of the enzyme cofactor, $Mg^{2+}$, which is

required for DNA cleavage, but not for DNA binding (section 1.3.1.1 and references therein). The results offered no evidence of a footprint for HindIII using this Cu(II):RSH cleavage system (section 3.10.2). No specific areas of protection from Cu(II):RSH cleavage were apparent in or around the region of the HindIII restriction-site sequence. Although Fe(II):EDTA footprinting of this system was not carried out (and may have shown a footprint), evidence exists in figure 50 to prove that the protein had bound specifically to the DNA fragment, *viz.*, the emergence of a background level of specific DNA cleavage (within the enzyme recognition sequence) caused by addition of excess enzyme solution. Thus, lack of protein binding was probably not the reason for the observed, null footprinting result. Factors, such as those described for Hoechst 33258 footprinting (section 4.5.1) may also be involved in the inability of Cu(II):RSH to footprint this enzyme:DNA complex. However, addition of Cu(II) to 0.2mM is less likely to perturb the enzyme:DNA complex than the Hoechst:DNA complex as protein:DNA complexes involve many protein-DNA contacts and protein-protein contacts produced only on binding of the protein to DNA, whereas in contrast, a ligand such as Hoechst 33258 forms only a limited number of ligand-DNA contacts (mainly van der Waals) and is therefore inherently less stable than the former type of complex. It is also clear that the enzyme molecule may chelate Cu(II) (and possibly RSH) *via* amino acid side chains and other groups e.g. imidazole, primary amine, carboxylate and thiol. Thus, the presence of increasing concentrations of protein may lead to increasing inhibition of the Cu(II):thiol DNA cleavage reaction (as was observed). Chelation of Cu(II) may also affect the cleavage and/or binding specificity of the enzyme although evidence that such effects must have been minimal was provided by the fact that specific background cleavages were

observed within the restriction enzyme cleavage site. These background cleavages may have been due to use of Cu(II) instead of Mg(II) as co-factor in the restriction cleavage reaction.

As in the case of the Hoechst 33258 footprinting experiments, it may be postulated that lack of Cu(II) binding, and therefore of Cu(II):thiol DNA cleavage, in the region where HindIII:DNA contacts are made (probably the major groove) may account for the lack of footprint. This is the preferred (although unproven) explanation.

## 4.6 SUMMARY AND CONCLUSIONS

Many features of the Cu(II):thiol system have now been detailed and it is appropriate to summarise the main findings.

The cleavage of DNA using thiol is catalysed most effectively by Cu(II) under neutral pH conditions (Reed and Douglas, 1989) in a reaction that involves Cu(II) and Cu(I). Initial reduction of Cu(II) can be performed by various reductants including ascorbate, thiol, $\beta$-NADPH or dithionite, although with varying efficiency, (ascorbate is the most effective, dithionite the least). The preferred DNA cleavage sites caused by the Cu(II):reductant system are independent of the nature of the reductant, whether it be thiol or ascorbate. This demonstrates that the cleavage preference is not due to reaction of either the Cu(II):reductant complexes themselves or of oxidised forms of reductant (e.g. thiyl radical) with the DNA but is due to a common reaction product that bears no structural resemblance to the reductant used. Cleavage of DNA causes both strand breaks and base damage (the latter occurs before detectable strand cleavage). Products include oligonucleotides bearing 5'-

phosphoryl, 3'-phosphoryl and 3'-phosphoglycolate-like termini (this work), minor amounts of 5'-hydroxyl and 3'-hydroxyl termini, and malondialdehyde (Reed and Douglas, 1991). The cleavage reaction requires dissolved oxygen and is inhibited by hydroxyl radical scavengers, catalase and SOD (Reed and Douglas, 1991) all of which indicate the intermediacy of oxygen derived species (e.g. $^{\bullet}OH$, $H_2O_2$, $O_2^{\bullet-}$) in the cleavage reaction. Both ds and ssDNA are degraded by Cu(II):thiol, and Z-DNA sequences are not preferentially degraded (Reed and Douglas, 1991) indicating no real secondary structure preference.

The similarity of the products of Cu(II):reductant DNA cleavage and those of hydroxyl radical cleaved DNA, together with the inhibition by hydroxyl radical scavengers strongly suggests hydroxyl radical as the major DNA cleaving species. However, the Cu(II):$H_2O_2$ system is also suspected to cleave DNA *via* hydroxyl radicals (Chiou, 1983; Sagripanti and Kraemer, 1989), and yet produces different patterns of DNA cleavage to those of Cu(II):reductant, although base-labile sites generated by both systems are largely similar. The reasons for this apparent contradiction are not clear, but the exact nature of the radicals produced by Cu(II):reductant and Cu(II):$H_2O_2$ systems cannot be identical. It should also be noted that the DNA base damage caused by Cu(II):$H_2O_2$ (section 3.4.3) gave contradictory results to those of Sagripanti and Kraemer (1989), who found cleavage specifically at polyguanosine sequences. The explanation provided by Sagripanti and Kraemer (originally mooted by Minchenkova and Ivanov, 1967) involved Cu(II) binding specifically to guanine, with consequent reaction of Cu(II) perhaps by proton transfer involving guanine, to yield the piperidine-sensitive base sites. This seems unlikely from the results presented in section 3.4.3 as several polyguanosine sites present in the tyrT fragment were not specifically cleaved or damaged.

The origin of the discrepancy in these results and those of Sagripanti and Kraemer is not known although the use of higher concentrations of Cu(II) and $H_2O_2$ may play a part in their results - [Cu(II)] levels were kept low in the studies of this thesis to minimise any possible deleterious effects of Cu(II) binding to DNA in footprinting experiments. From the very low sequence dependence of Cu(II):reductant cleavage of DNA it is more likely that the majority of DNA-damaging radical production occurs either *via* Cu(II) bound to DNA non-specifically and/or *via* Cu(II), free in solution, and from which the cleaving radical may diffuse to react with DNA (as is the case for Fe(II):EDTA).

The Cu(II):reductant system has significant differences from known hydroxyl radical-generating systems. For example, the former system is incapable of footprinting a minor groove binding drug, and does not cleave DNA in a sequence-neutral manner. However, the very low sequence-preference of the cleavage reaction caused by Cu(II):reductant may indicate that cleavage of DNA by hydroxyl radical at least accounts for the majority of the observed DNA cleavages.

As detailed earlier (section 4.2 and references therein), existing data on Cu(II) binding to DNA relates mainly to non-buffered solution studies, a medium which bears little resemblance to the cleavage reaction conditions presented in this thesis. The major conclusions from such data on Cu(II):DNA interactions (reviewed by Pezzano and Podo, 1980) are that preferred sites of Cu(II) binding are (a) $N_7$ of guanine bases, (b) between G-C base pairs (by forming a complex with $N_7$ and $O_6$ of guanine and $N_3$ and $O_2$ of cytosine) and perhaps (c) between adjacent guanosines of the same DNA strand (with the guanosine $N_7$ nitrogens acting as electron donors and Cu(II) acting as an acceptor in a charge-transfer complex). However, in the present situation the absence of the denaturing

effect of Cu(II) and of preferred cleavage sites at the above Cu(II) binding sites argue against the existence of such specific Cu(II):DNA interactions and hence, their role in causing the observed apparent sequence preference.

Influence of a DNA parameter such as sequence-dependent minor groove-width variation is unlikely to cause the observed cleavage patterns for two reasons, (i) groove-width narrows from the 5′ to the 3′ end of adenine tracts (Burkhoff and Tullius, 1987) and no clear relationship exists between preferred sites of Cu(II):reductant cleavage and such tracts (e.g. section 3.3), and (ii) the Fe(II):EDTA nuclease, which responds to such variations in groove width by modulations in cleavage efficiency (Burkhoff and Tullius, 1987), produces no such variations in cleavage of the same DNA fragments which Cu(II):reductant systems cleave with an apparent sequence dependence. Thus, unless the Cu(II):reductant system is inherently more sensitive than Fe(II):EDTA to dimensional variations in DNA structure then such variations must be unimportant in determining the preferred sites of DNA cleavage by the former reagent.

The similar appearance of Cu(II):thiol and Cu(II):ascorbate DNA cleavage patterns rules out DNA-binding of Cu(II):reductant complexes or of compounds structurally related to the reductants as being possible causes of the observed sequence preference. On the other hand the inequivalent nature of Cu(II):reductant and Cu(II):$H_2O_2$ patterns of DNA would indicate that the apparent sequence preference lies not with Cu(II) but with either its co-reactant (ruled out above), or the products of Cu(II):co-reactant reaction and/or subsequent reactions of such products (i.e. oxygen-derived free radicals).

The situation is unfortunately complex, and conclusions on this aspect of the cleavage of DNA by Cu(II):reductant and related systems are limited to

the following:

- Cu(II):reductant systems cause oxidative degradation of DNA producing similar products to those of hydroxyl radical cleaved DNA.
- The apparent sequence preference exhibited by Cu(II):reductant and Cu(II):$H_2O_2$ cleavage of DNA is not explained by either the behaviour of the radical species postulated to be produced by the above systems or by the expected preferred DNA-binding sites of Cu(II). The factors causing such a preference remain obscure at the present.
- It is suspected that these, as yet unknown, factors also determine the observed unsuitability of Cu(II):reductant as a DNA footprinting reagent and perhaps contribute to the ineffectiveness of DNA-bound thiols in causing DNA cleavage.

## 4.7 FURTHER EXPERIMENTS

The lack of footprinting ability shown by the Cu(II):thiol system was disappointing and yet not entirely unexpected. The most useful footprinting reagent to date is the Fe(II):EDTA chemical nuclease which, as a consequence of not being able to bind to DNA (the metal complex is anionic $[Fe(II):EDTA]^{2-}$ and is repelled by the phosphate backbone), generates cleavage of DNA without disrupting the binding interactions of the particular DNA-binding molecule under test. DNA-binding nucleases such as DNAseI (a protein) and MPE:Fe(II) (an intercalator) run the risk of perturbing the DNA-bound molecule in order to produce DNA cleavage in close proximity to it. They also can suffer from long-range alterations in DNA structure which may be induced by certain DNA-binding molecules, resulting in larger regions of protection where nuclease

cleavage has been disturbed at sites remote from the ligand binding site (although this feature may prove actively useful in procedures other than footprinting). Thus, Cu(II), which has some DNA-binding ability, is not perhaps the best basis for a successful DNA cleavage reagent. However, as has been shown by Chiou's work (Chiou *et al.*, 1983) the complex of Cu(II):diglycylhistidine is an effective nuclease (although sequence-specific aspects have not yet been explored) and Cu(II) in this chelated form, has been attached to a protein to provide site-specific DNA cleavage (Mack *et al.*, 1988). Thus, chelation of Cu(II) to prevent binding of the metal to DNA may solve the problems encountered in the footprinting experiments of this thesis, although choice of chelate is limited as many Cu(II) chelators are redox inactive. In conclusion, therefore, simple DNA nucleases such as Cu(II):reductant, in the absence of metal ion chelation, are not suitable for footprinting purposes. The simplest, most useful chemical nucleases involving metal ions must, therefore, consist of metal ions chelated by a non-DNA binding molecule (e.g. Fe(II):EDTA) in order to serve as footprinting reagents.

It is possible, however, that the binding of Cu(II) to DNA may be exploited to probe DNA structural variations. The experiments using DNAs with specific sequence inserts (section 3.9) show that, perhaps by Cu(II) binding preferences, the Cu(II):thiol system distinguishes between neighbouring nucleotides in a certain sequence. The feature of these sequences that the Cu(II):thiol reaction responds to is not yet known but further experiments of a similar nature on more DNA sequences/structures will possibly enable evaluation of Cu(II):mediated DNA-cleavage/modification as a probe of DNA structure. The possibility that 0.2mM Cu(II) may be able to cause formation of Z-DNA in these fragments is not known, however, it is perhaps worth

investigating as the fragment degradation patterns seen in section 3.9 resemble those obtained for some other systems, e.g. the mithramycin-induced structural change in $(AT)_n$ sequences flanking mithramycin binding sites (Cons and Fox, 1990) and the B to *H-DNA transition of supercoiled DNA induced by metal ions (Bernues *et al.*, 1990). Limited Cu(II):thiol treatment of supercoiled DNA molecules bearing structural inserts (such as Z-form DNA), where base modification rather than strand cleavage is the predominant reaction, followed by restriction digestion, radiolabelling, restriction digestion and piperidine treatment (similar to procedures of McLean and Wells, 1988, for example) may yield preferred base modified sites within the insert sequences - normally such structures are probed using molecules that covalently react with DNA (e.g. DEPC) by modifying bases preferentially in non-B-DNA structures.

Modification of the oligo-SH sequence-directed DNA cleavage experiment (as detailed in section 4.4.2) by use of an oligo-SH capable of forming a triple-helix with a duplex DNA target molecule should provide information on the presence or otherwise of Cu(II) in the major groove of DNA. An attempt should be made to footprint a well-documented protein:DNA complex such as the $\lambda$ repressor or cro protein complexes with operator DNA (Tullius and Dombroski, 1986) using the Cu(II):reductant system in order to clarify the ability or otherwise of this system to footprint molecules that bind in the major groove.

Also degradation of RNA by Cu(II):reductant systems requires investigation. Although Cu(II) was amongst many other metals used to cleave polyribonucleotides under conditions of high temperature and extended incubation times (section 1.3.2.1 and references therein) it was one of the least effective. Reaction of the Cu(II):reductant system with

polyribonucleotides and especially specific RNA species such as tRNA merits further investigation. Fe(II):EDTA has already found use as an RNA footprinting reagent (Wang and Padgett, 1989) and provided information on protected regions of pre-mRNA splicing complexes. Cleavage of tRNA may offer information on the folding of tRNA by providing cleavage at sites related to positions of bound Cu(II) ions which may link base positions widely spaced in the tRNA sequence (e.g. similar to experiments using $Pb^{2+}$ and other heavy metal ions, section 1.3.2.1 and references therein).

Determination of modifications to DNA bases caused by Cu(II):thiol is needed to provide more evidence for the role of $HO^{\bullet}$ in the DNA cleavage mechanism. $HO^{\bullet}$ modification of DNA produces a characteristic pattern of types of base modification and quantity of each modification which can be determined by HPLC procedures (Hutchinson, 1985). HPLC analysis of DNA degraded by Cu(II):thiol may also reveal any metastable degradation products that are degraded in the procedures involved in preparation of samples for electrophoresis (e.g. incubation in formamide, and heating at 90°C), as was the case for Cu(I):phenanthroline cleavage (reviewed by Sigman and Chen, 1989). Thus, it may be possible to further determine the predominant mechanism of the Cu(II):thiol DNA cleavage reaction.

Finally, it is envisaged that so-called artificial restriction endonucleases will almost certainly be produced using systems similar to known enzymic DNA cleavage methods rather than the free-radical based metal-chelate systems at present used mainly for footprinting. The free radical chemistry of the metal-chelate systems makes DNA cleavage relatively inaccurate, with scissions and modifications occurring at the target site (usually a single base) and neighbouring sites with a variety of products formed, unlike the enzymic

systems where this does not occur under the correct solution conditions. Such future systems, therefore, may be hybrids of sequence-specific DNA-binding molecules and of hydrogen donor-acceptor residues (similar to those found in proteases or DNAseI; see section 1.3.1.2) suitably arranged so as to activate a water molecule to attack the phosphodiester linkage at a specific distance from the sequence defined by the DNA-binding moiety of the hybrid molecule.

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# TIGHT BINDING OF A COPPER (II) PHTHALOCYANINE (CUPROLINIC BLUE) TO DNA

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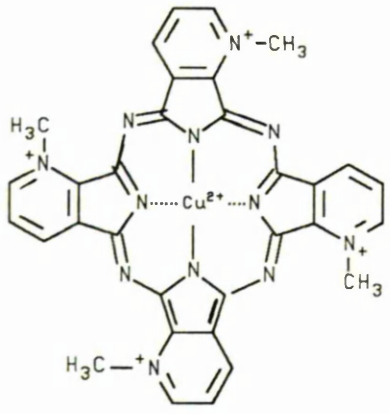
**Summary:** The binding of a cationic phthalocyanine (Cuprolinic Blue) to calf thymus DNA, indicated by the increase in the DNA melting temperature and spectroscopic titration ($K_{aff} > 10^7 M^{-1}$), was characterised by at least two distinct DNA-bound ligand forms possibly arising from intercalated and externally bound species each with $K_{aff}$ values in the region $10^7$ $M^{-1}$. Evidence of strong intercalation was provided by gel electrophoresis of plasmid DNA in the presence of Cuprolinic Blue. The anionic phthalocyanine (copper phthalocyanine 3,4',4'', 4'''-tetrasulfonic acid) does not bind to DNA by spectral criteria, reflecting electrostatic contributions to binding.



In the absence of axial groups, cationic porphyrins such as $H_2$T4MPyP, (tetrakis(4-N-methylpyridylporphine), $Cu^{II}$ T4MPyP and $Ni^{II}$ T4MPyP, interact with GC regions of DNA (the $Zn^{II}$, $Fe^{III}$, $Mn^{III}$ and $Co^{III}$ analogues do not) (1) and it has been proposed that this is true intercalation. In contrast, binding of porphyrins to AT regions of DNA appears to be 'external', using coulombic interactions, possibly involving the minor groove. Footprinting (2-5) and NMR (6) studies have indicated preferred binding at both CpG and TpA sites. Molecular modelling suggests that full intercalation is only possible with CpG sites, because of steric hindrance by the thymine methyl group at the TpA sites (7).

Some phthalocyanines, grossly similar to porphyrins, have been synthesised for use as histochemical reagents (8). Two neighbouring iso-indoline rings of the Cuprolinic Blue chromophore (Scheme 1) have dimensions close to those of a purine:

0006-291X/89 $1.50



Scheme 1. Cuprolinic Blue.

pyrimidine base pair (9) and it was partly on this basis that Cuprolinic Blue was designed as a nucleic acid-specific stain. Further, (metallo) sulphonato-phthalocyanines have been used as photosensitisers in the photodynamic therapy of tumours (10), with associated DNA-strand breakage for the irradiated gallium complex (11). In view of the DNA binding and strand cleavage (2) properties of porphyrins, we studied the interaction of Cuprolinic Blue with DNA.

## MATERIALS AND METHODS

Cuprolinic Blue $^{R}$ was from BDH Ltd (Poole, Dorset). Plasmid DNA (pSP64) was isolated from E. coli HW87 by standard procedures (12). Calf thymus DNA (type I, highly-polymerised), from Sigma Chemical Co (Poole, Dorset), was allowed to hydrate for 1-2 days (4°C) in the required buffer. Vacuum-desiccated plasmid DNA was dissolved in 20mM sodium phosphate, pH 6.7, 90mM NaCl and diluted to a working concentration of 60-70$\mu$M in base-pairs. DNA concentrations were based on $\epsilon_{260} = 13{,}100 M^{-1}cm^{-1}$ (1). DNA samples were electrophoresed for 2hrs. in horizontal submarine agarose gels (0.6% w/v, 20cm x 8cm x 0.6cm) at a constant 90V. Gel and reservoir buffer was TBE buffer (90mM Tris pH 8.2, 90mM boric acid, 2mM EDTA). On completion of electrophoresis gels were ethidium bromide stained and the DNA bands visualised by fluorescence. The relative proportions of the plasmid forms were calculated from the peak areas by LKB microdensitometric scanning, with a correction figure (x1.22) applied for the supercoiled form (13).

Manipulations of phthalocyanine-containing solutions were carried out in minimal light. Incubations (10 min, room temperature) of Cuprolinic Blue (20mM sodium phosphate, pH 6.7, 90mM NaCl) and plasmid DNA (5$\mu$l of each) in polypropylene vials were performed in a ligand:DNA concentration range of R = 0.1 to 1.0 where R is the molar ratio of ligand monomer to DNA base pairs. Samples were then either treated to remove the ligand and recover the DNA (see

below) or prepared for electrophoresis by the addition of concentrated loading buffer and loaded (10μl aliquots) into submarine gel sample wells.

Ligands were extracted from phthalocyanine:DNA mixtures by a procedure verified to remove the free dye from aqueous solution, namely, extraction with a 25:24:1 mixture of phenol:chloroform:butan-2-ol (4 x 50μl) with the resulting aqueous extract retained for DNA precipitation by ethanol. Dried reisolated DNA samples were redissolved in buffer (10μl, 20mM sodium phosphate, pH 6.7, 90mM NaCl), electrophoresed and stained as described above.

Spectral measurements were made with a Pye-Unicam SP8800 double-beam spectrophotometer. Melting profiles for DNA samples were determined at 260nm on this instrument fitted with a temperature-programmed (0.5°C/min, 50-100°C) cell-block with output to a Phillips PM8271 X-Y plotter.

## RESULTS

Increasing the amount of Cuprolinic Blue relative to calf thymus DNA base pairs increased $T_m$ (the temperature of the midpoint of the DNA melting transition detected at 260nm) by 4.5°C at a Cuprolinic Blue:base pair ratio of 0.17 in 10mM Tris, pH 7.0 1mM EDTA buffer.

Addition of small volumes of a calf thymus DNA solution (0.23mM base pairs) to a fixed volume of Cuprolinic Blue (8.2μM in 10mM Tris pH 7.0, 1mM EDTA buffer at 25°C) led to a progressive, marked reduction in intensity of the major visible absorption band (624-632nm region) with lesser but similar effects for the lower intensity bands at lower wavelengths. The band shifted from the 624-632nm double peak to an apparently single peak at 642nm. There was an apparent isosbestic point at 406nm for spectra at different ligand:base-pair ratios, but isobestic behaviour was not tight indicating complex equilibria.

The data from a similar titration, at lower Cuprolinic Blue concentration, gave a non-linear Scatchard plot (Figure 1) indicating more than one class of binding site. At high values of $r(=[\text{ligand}]_{\text{bound}}/[\text{DNA}]_{\text{total}})$ linear behaviour was observed, with binding constant $8.15 \pm 0.01 \times 10^6 M^{-1}$ (0.81 ligand binding sites per base-pair). A region of stronger binding ($K \approx 5.05 \pm 1.31 \times 10^7\ M^{-1}$) but lower occupancy (0.51 ligands/base pair) exists at lower r.

The effect of Cuprolinic Blue concentration on plasmid DNA electophoretic mobility was studied in agarose gels, Figure 2. The following effects were observed: (a) at the lowest R values the mobilities of both supercoiled (SC) and nicked circular (NC) DNA bands were reduced compared to

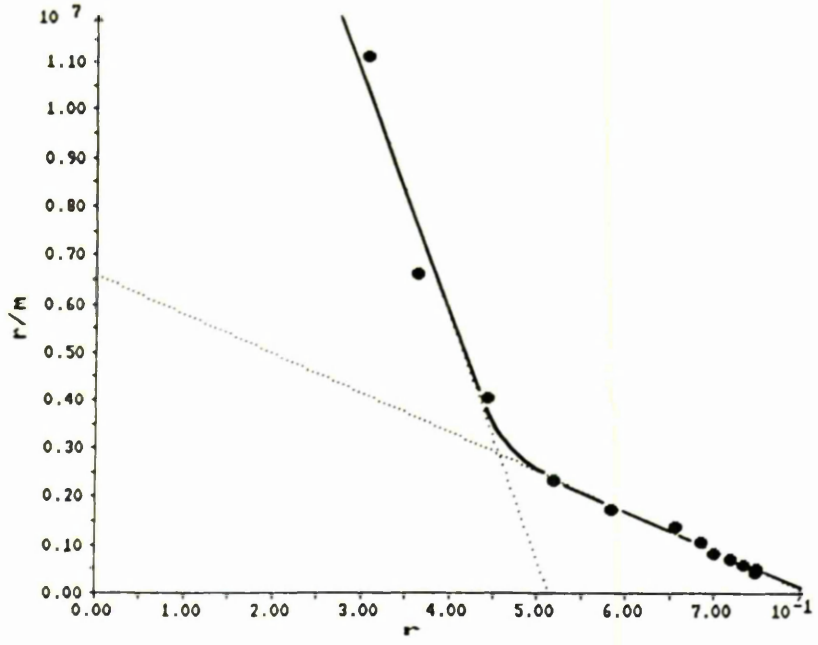



Figure 1. Scatchard plot of Cuprolinic Blue/Calf thymus DNA spectral titration data obtained in 10mM Tris, pH 7.0, 1mM EDTA at 25°C.

controls of DNA alone (lanes 2 to 5). This reduction in electrophoretic mobility increased as R increased. A comparatively greater effect was observed for the SC than the NC band (the former eventually became a diffuse smear, the latter remaining sharp); (b) at a critical R value ($R_C$) the smeared, retarded SC band appeared to co-migrate (lane 6) with the retarded NC band; (c) at $R>R_C$ (lanes 7 to 14) an

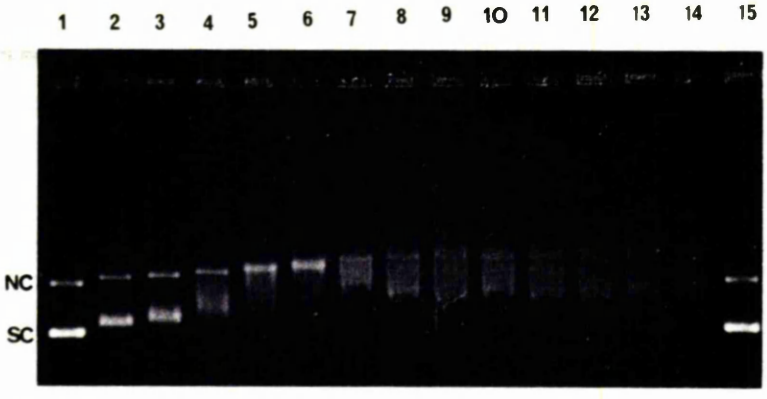



Figure 2. Electrophoretic migration of supercoiled (SC) and nicked/relaxed circular (NC) pSP64 DNA in 0.6% agarose gels in the presence of Cuprolinic Blue. Lanes 1 and 15 contain DNA only, Lanes 2 to 14 contain DNA and Cuprolinic Blue at R = 0.11, 0.15, 0.19, 0.22, 0.26, 0.30, 0.34, 0.37, 0.41, 0.45, 0.49, 0.52 and 0.60, respectively.

increasingly mobile smear/band appeared to progress back down the gel towards the position of the original (control) SC band; (d) vastly increased times in ethidium bromide staining solution were required to visualise bands/smears at high values of R relative to lower R values or control DNA. Ligand extraction (by phenol/chloroform/butanol) of plasmid DNA samples incubated with Cuprolinic Blue, indicated that the original electrophoretic pattern of SC and NC bands was restored by removal of ligand from the DNA.

## DISCUSSION

The melting curve data and spectroscopic titration of calf thymus DNA by Cuprolinic Blue clearly indicate binding of this phthalocyanine to DNA. The apparent binding constant at higher r for Cuprolinic Blue to calf thymus DNA at pH 7.0 in 10mM tris buffer at 25°C is ~$10^7$ $M^{-1}$, with an even stronger association at lower r. In comparison, the binding of T4MPyP to calf thymus DNA (in 6mM $Na_2HPO_4$/2mM $NaH_2PO_4$, 11mM EDTA, 179mM NaCl, pH 6.8 $\mu$ = 0.196 has a binding constant of 1.1 x $10^7$ $M^{-1}$ with an additional weaker binding mode (14). However, values of binding constants for species such as porphyrins and phthalocyanines are complicated by multiple binding modes and dimerisation/stacking phenomena for the free or bound ligand. Similar titrations performed using the anionic phthalocyanine, copper phthalocyanine 3, 4', 4'', 4'''-tetrasulfonic acid, produced no observable phthalocyanine spectral changes, presumably reflecting the importance of electrostatic contributions to the binding of Cuprolinic Blue to DNA.

The plasmid DNA extraction experiments indicated that the interaction is non-covalent (or readily reversed), and that there is no significant DNA cleavage. Thus, the electrophoretic patterns of Figure 2 are explicable in terms of binding. The plasmid DNA:phthalocyanine complexes are extremely stable, surviving electrophoresis for several hours. Furthry.er evidence of complex stability is the time required to obtain a good ethidium bromide stain of Cuprolinic Blue-treated plasmid DNA: this staining time is greater the greater the amount of phthalocyanine bound.

Binding of cationic ligands to plasmid DNA reduces DNA electrophoretic mobility relative to native DNA (15, 16). Hence, liganded DNA will be retarded relative to non-liganded

DNA, an effect seen at low R values. This retardation effect has been reported for several compounds (17) including porphyrins (15,16). However, the results for Cuprolinic Blue show both an initial reduction in mobility and an apparent reversal of retardation occurring as R increases past Rc (Figure 2). This second effect cannot be readily explained by an external ligand binding mode alone, as increasing ligand concentration would be expected to produce a continuous decrease in the DNA mobility, up to a point where the DNA was saturated with ligand. In the absence of any DNA cleavage to produce linear molecules (which would migrate at a position between that of the SC and NC bands), the origin of the increasingly mobile DNA, appearing as R increases above Rc, must be from increasingly liganded NC or SC DNA molecules. As NC DNA becomes increasingly liganded by intercalation and/or electrostatic binding of cationic phthalocyanines it can only become more slowly migratory. However, intercalation of ligand into supercoiled DNA produces unwinding of the double helix, leading to a reduction in the number of negative superhelical turns present thereby reducing the electrophoretic mobility of the DNA as it becomes less rigid and rod-like (15,16,18,19). At $R_c$ there is sufficient ligand intercalated to remove all the superhelical turns originally present in the DNA and a single band appears on electrophoresis. Above Rc the DNA is further unwound and becomes positively supercoiled; the molecule becomes more compact and thus increases in electrophoretic mobility. This biphasic effect has been reported for a diacridine bis intercalator (17) and an intercalating porphyrin, $H_2T4MPyP$ (2).

In summary, direct electrophoresis, optical spectroscopy and $T_m$ data indicate that Cuprolinic Blue binds strongly to DNA in at least two ways without causing chemical cleavage under the present conditions. The strong interaction causing an apparent reduction and reversal of plasmid DNA supercoiling as seen by electrophoresis may be caused by intercalation, either complete or partial (8,9) as described for porphyrins (7).

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# APPARENT SEQUENCE PREFERENCE IN CLEAVAGE OF LINEAR B-DNA BY THE Cu(II):THIOL SYSTEM

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Summary: Cleavage of a linear tyr T fragment of duplex DNA by Cu(II):thiol combinations occurs with apparent sequence preference. The sequence selectivity was unaffected by the size, chirality or substitution pattern of the monothiol partner, presumably indicating that the Cu(II):RSH system is probing some local conformational/dynamic aspect of the DNA.


The anticancer drug bleomycin offers several molecular features worthy of emulation. It binds to DNA (preferentially at a two-base pair step, 5'-GT-3') and as its Fe(II) complex, which is believed to produce oxygen-derived free radicals, causes strand scission(1). Based on this concept, synthetic mimics of bleomycin action have been prepared and tested.

Various molecules have been used for DNA recognition, e.g. methidium(2,3), distamycin(4,5), oligonucleotides(6,7), and various intercalators(8). The DNA cleavage mechanism used predominantly to-date has been iron-based using the Fe(II):EDTA system(2-7) although Fe(II) porphyrins have also worked(8,9). However, in addition, a number of metal complexes can cause DNA strand cleavage,e.g. B-DNA is cleaved by Cu(II):phenanthroline complexes plus thiols(10). Octahedral Ru-phenanthroline complexes bind to DNA(11) and the Co(III) analogues bind

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preferentially to Z-DNA (left-handed helix) or B-DNA (right-handed), according to their chirality, and can be photoactivated to produce strand nicks(12). Methidium-EDTA:Fe(II) has been used for the footprinting of small ligands on DNA restriction fragments and sequence-specific analogues have been used for DNA affinity-cleavage. Both techniques allow direct study of ligand binding sites on native DNA(4).

It is of considerable importance for both molecular biologists and, in the longer term, clinicians to find alternative free radical generators capable of cleaving DNA at preselected sequences. The origin and chemical history of a free radical can have a major bearing on its subsequent chemical activities. Thus, the development of new DNA cleavage mechanisms is important as new cleavage (as well as binding) patterns are to be expected.

Studies from our laboratory have shown recently that, in the presence of $Cu^{2+}$ ions, thiols can cause single-strand breaks in supercoiled plasmid DNA at micromolar concentration(13). We now report on the reaction of the Cu(II):RSH cleavage system with tyr T DNA, a fragment of linear B-DNA of defined sequence.

## MATERIALS AND METHODS

The restriction enzymes RsaI, AvaI and the Klenow fragment of DNA polymerase I were obtained from Boehringer Mannheim, EcoRI restriction endonuclease and calf thymus DNA (Type I, highly polymerised) from Sigma and [$\alpha$-$^{32}$P]dATP from Amersham. Analar-grade sodium phosphate and copper sulphate were from BDH. Cysteine (L- and D- forms) and 3-mercaptopropionic acid were from Aldrich. Glutathione and dithiothreitol were from Sigma. Freshly prepared thiol solutions were assayed immediately before use using Ellman's reagent (5,5'-dithio bis(nitrobenzoic acid)) to determine free thiol concentrations(14).

Plasmid pKM$\Delta$98 was prepared in predominantly supercoiled form by standard procedures(15) from E. coli strain CBI. The end-labeled, 160 bp tyr T fragment of pKM$\Delta$98 was produced by

EcoR1 digestion, 3'-end labelling at the EcoR1 site with [$\alpha^{32}$-P]dATP using the Klenow fragment of DNA polymerase I, followed by digestion with AvaI. The labelled, low molecular weight restriction fragment was purified by non-denaturing polyacrylamide gel electrophoresis on 8% gels. Fragments were eluted from gel slices with 1mM EDTA, pH 8.0 buffer. After ethanol precipitation, the fragments were washed with 70% ethanol (x3), redissolved in distilled water and stored at -20°C. The guanine-specific dimethylsulphate/piperidine treatment of the tyr T fragment was carried out according to Maxam and Gilbert(16).

Copper(II):thiol-mediated scission of the labelled DNA fragments was initiated by adding an aliquot (5$\mu$l) of thiol solution (0.5mM in SH groups) to 20$\mu$l of sodium phosphate (12.5mM, pH 8.2) buffer containing sonicated calf-thymus DNA (50$\mu$M bps), 3'-end labelled fragment (~75 CPS, <7 pmoles) and $CuSO_4$ (0.25mM). Reactions (15 min, 37°C) were terminated by precipitation with ethanol. Samples were resuspended in 90% formamide loading buffer, heated for 2 minutes at 90°C, plunged into ice-water and loaded onto 0.2mm thick, 40cm long, 8% polyacrylamide, 1:20 cross-linked sequencing gels containing 7M urea. After electrophoresis (90 min., 1400V) gels were fixed in 10% acetic acid, rinsed with water and dried down onto glass plates at 90°C. Dried gels were exposed for 10-20 hours to preflashed(17) Fuji RX-100 X-ray film at -80°C with an intensifying screen. Bands in the digestion patterns were assigned to particular nucleotides of the fragment by comparison with a control lane of the fragment cleaved specifically at guanine residues (G-track) and with reference to the known nucleotide sequence of the fragment(18).

## RESULTS AND DISCUSSION

Figure 1 clearly shows that incubation of the 160 bp tyr T fragment of linear, duplex DNA with the $Cu^{2+}$:RSH system led to marked DNA damage. There was no appreciable DNA damage when the tyr T DNA fragment was incubated with $Cu^{2+}$ or with thiol, in the absence of the other partner of the $Cu^{2+}$:RSH pair. In contrast, there was a 'ladder' of DNA fragments produced when the tyr T DNA was incubated in the presence of both $Cu^{2+}$ ($2.0 \times 10^{-4}$M) and thiol ($1.0 \times 10^{-4}$M) (compare lanes 4-7 with lanes 2 and 9, Figure 1). The ladder arises from nicking of the tyr T DNA to produce fragments differing in length by single nucleotide units. Such a result is similar to the effects observed for footprinting agents (e.g. Fe:EDTA, DNase I) on free tyr T or other linear DNA fragments (19).It is apparent from

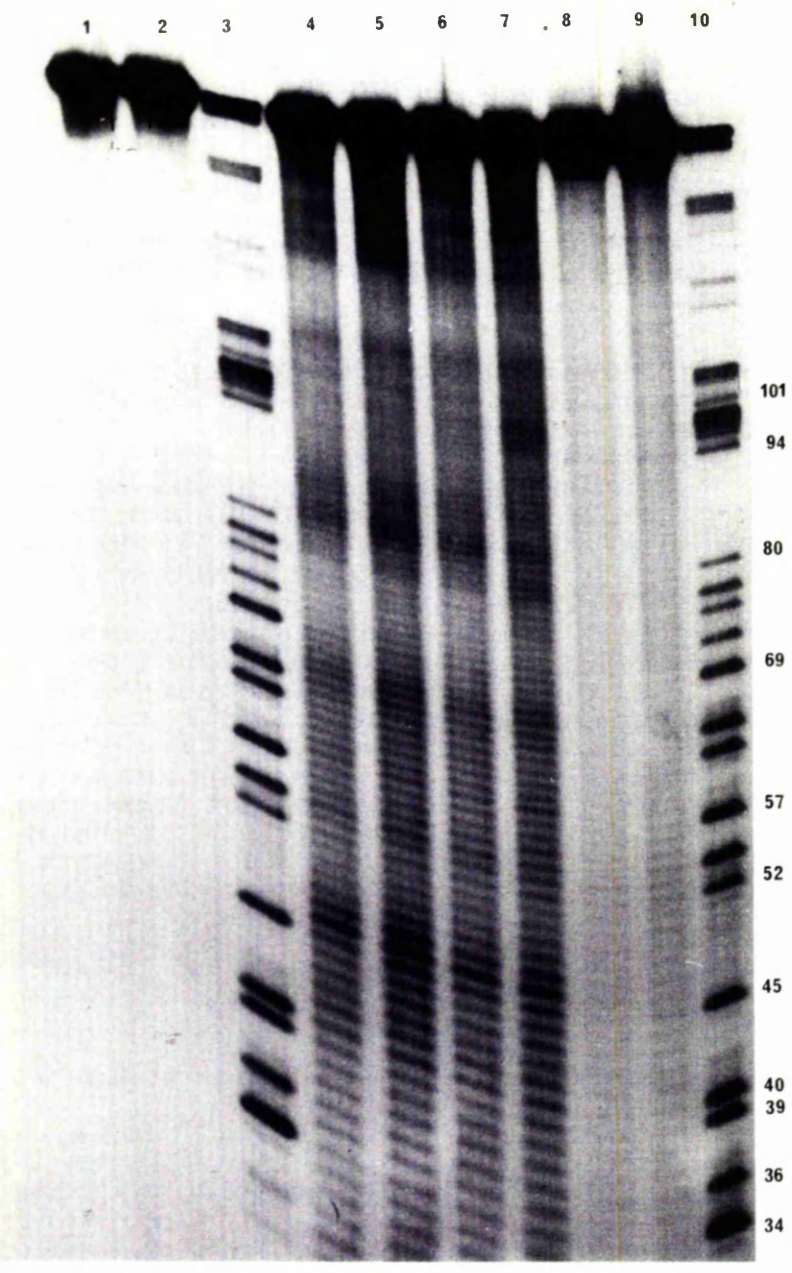


Figure 1.
Cleavage by the $Cu^{2+}$:thiol combination of the tyr T DNA fragment. Lane 1, DNA only; lane 2, DNA + $Cu^{2+}$; lanes 3 and 10, Maxam-Gilbert G-Tracks; lanes 4-8, DNA + $Cu^{2+}$ in the presence of D-cysteine, L-cysteine, glutathione, 3-mercaptopropionate and dithiothreitol, respectively; lane 9, DNA + L-cysteine. Concentrations, incubation media and conditions are given in the text. Down the right hand side the nucleotide positions are numbered based on the G-track and known sequence of the tyr T fragment (see text).

lanes 4 to 7 of Figure 1 that the intensities of the bands, and hence the relative amounts of nicking at each nucleotide position, vary along the DNA sequence.

TABLE 1 SITES OF PREFERRED CLEAVAGE OF THE tyr T DNA FRAGMENT BY $Cu^{2+}$:RSH

| Position No. (Crick Strand) | Sequence (3'-5') |
|---|---|
| 40-45 | GTCAAG |
| 53-58 | AGTTGC |
| 61-66 | TGTGAA |
| 79-84 | AGTAAA |
| 93-98 | CGCGGG |
| consensus | pu-pu-pyr-pu-pu-pu |

Comparison of lanes 4 to 7 shows that the apparent sequence preference by the $Cu^{2+}$:RSH system is grossly unaffected by the nature of the thiol. For example, the same regions of selective cleavage are predominant for cysteine, glutathione (an N-blocked cysteine) and for 3-mercaptopropionic acid (with no $\alpha$-amino-group). The reaction is also independent of the stereochemistry of the thiol as D-and L- cysteine show the same sequence preferences, lanes 4 and 5. Thus, it is clear that the reaction monitored by this procedure is that of a reaction product formed from the $Cu^{2+}$:RSH combination, not a $Cu^{2+}$:RSH complex itself. Likely candidates are oxygen-derived free radicals, e.g. $O_2^{-\cdot}$, $HO^{\cdot}$, $HO_2^{\cdot}$, as postulated for plasmid DNA cleavage by this reagent system (13). Our preliminary studies indicate that the sites of preferred cleavage remain constant under all conditions producing limited cleavage, i.e. variations in the concentrations of the reagents used, and the temperature and length of the incubation period. Oxidative damage at poly-G sequences in DNA has been reported for the copper(II):hydrogen peroxide combination, in the absence of thiols(20).

The enhanced cleavage regions of the tyr T fragment with $Cu^{2+}$:RSH (Figure 1) are summarised in Table 1. These cleavage sites refer only to the 3' end-labelled lower (Crick) strand; the upper (Watson) strand was unlabelled. Cleavage sites on the

upper strand have not yet been determined. The preferred sequence is only described as a consensus at the crude level of pu-pu-pyr-pu-pu-pu (Table 1).

The size of this 'conserved' region (appropriately 6-base pairs), corresponding to just over a half-turn of a B-helix is much larger than any of the thiols studied, which are themselves of varying sizes, stereochemistries and charge distributions. Therefore, it is likely that the $Cu^{2+}$:RSH system is sensitive to the conformation/dynamics of linear DNA. The details of the underlying causes of the apparent sequence selectivity are not yet clear. However, other features such as sequence dependence of groove dimensions(21, 22) and charge density distribution may be important.The copper:phenanthroline footprinting reagent also exhibits sequence specificity(23) but, this reagent is believed to bind directly to DNA(10) and to produce *in situ* a cleaving species other than HO· (which is the species causing Fe:EDTA activity in footprinting).

Supercoiled, plasmid DNA appears to be cleaved(13) at concentrations 100-fold or so less than those required for linear DNA, presumably reflecting the different sensitivities of the two forms of DNA to the reagent. Further, whereas for plasmid DNA some dithiols (depending on disulphide ring size) were efficient partners of $Cu^{2+}$ in the cleavage reaction, for *tyr T* cleavage dithiols were singularly inefficient e.g. dithiothreitol (Figure 1, lane 8) and 1,3-propanedithiol (results not shown).

In conclusion, we have shown that the $Cu^{2+}$:RSH system cleaves linear (*tyr T*) DNA in addition to supercoiled plasmid DNA (as previously reported(13)). Perhaps surprisingly for such a 'simple' reagent and for such a range of thiols there is an apparent sequence selectivity, independent of the nature of the

thiol partner. This presumably reflects some local DNA conformational or dynamic property registered by its chemical susceptibility to some (as yet unidentified) product of the $Cu^{2+}$:RSH system. The $Cu^{2+}$:RSH system may therefore prove useful as a probe of DNA structure or as a new class of specialised footprinting/affinity cleaving reagent. Such aspects are under active investigation in our laboratory.

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