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Site specific mutagen reactions leading to the formation of adducts in DNA

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University of Hawai, 1992
SITE SPECIFIC MUTAGEN REACTIONS LEADING TO THE
FORMATION OF ADDUCTS IN DNA

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BY

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ABSTRACT

Several groups of chemicals are known to react with DNA. Some of these are known mutagens while others are potential carcinogens. In this work, an attempt was made to observe if such reactions were random or if indeed there are preferred bases / sequences where adduct formation occurs. A simple model was used for this work in which the DNA chain synthesis is terminated when the Sequenase™ enzyme reaches an adduct. The DNA used was M13mp18 (7.30 kilobases). This DNA is available from commercial sources in either the single or the double stranded circular form. The reactions were carried out with chloroacetaldehyde, or with the methyl, ethyl and propyl derivatives of N'-nitro-N-nitrosoguanidine, or with 2,4-dinitrophenylhydrazine, phenyl hydrazine, hydroxylamine hydrochloride, hydrazine hydrate or with methyl methanesulfonate. Position 81 (thymidine) was found to be a common fall off point with all these chemicals. The other positions of fall off had some overlap but each chemical class had a unique adduct pattern. The methyl, ethyl and propyl derivatives of N'-nitro- N-nitrosoguanidine had the same positions of chain termination.

Varying the salt concentration had a significant effect on the number of adducts formed when M13mp18 DNA was reacted with chloroacetaldehyde. A large number of termination points were observed in the presence of 100mM sodium acetate.
Fluorescence spectroscopy was used to determine if in fact the chloroacetaldehyde was reacting with the DNA or if the mutagen merely prevented the enzyme from acting in the labelling step. It was found that the chloroacetaldehyde reacted with the DNA both in the single and the double stranded forms. Both the absorption and the fluorescence spectra indicated this and that nearly every base was adducted in most of the experiments reported here.

Chloroacetaldehyde was also found to affect the Sequenase™ enzyme causing additional points of chain termination when it was added in the labelling step. Agarose gel electrophoresis showed that chloroacetaldehyde did not alter the migration pattern of either M13mp18 single or double stranded DNA or of the Hind III linear double stranded mixture. No evidence of hydrolysis was obtained. The ratio of supercoiled / relaxed forms of the double stranded M13mp18 DNA was not altered by the chloroacetaldehyde treatment. The adducted DNA sequenced in the same way as the non reacted but there were some 'blank' reactions in the sequencing lanes of the former.
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<th>Description</th>
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<tr>
<td>AMV</td>
<td>Avian Myeloblastosis Virus</td>
</tr>
<tr>
<td>APS</td>
<td>Ammonium Persulfate</td>
</tr>
<tr>
<td>BPB</td>
<td>Bromo Phenol Blue</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine Serum Albumin</td>
</tr>
<tr>
<td>°C</td>
<td>Centigrade</td>
</tr>
<tr>
<td>CAA</td>
<td>2-Chloroacetaldehyde</td>
</tr>
<tr>
<td>dATP</td>
<td>Deoxy Adenosine Triphosphate</td>
</tr>
<tr>
<td>dCTP</td>
<td>Deoxy Cytidine Triphosphate</td>
</tr>
<tr>
<td>ddATP</td>
<td>Dideoxy Adenosine Triphosphate</td>
</tr>
<tr>
<td>ddCTP</td>
<td>Dideoxy Cytidine Triphosphate</td>
</tr>
<tr>
<td>ddGTP</td>
<td>Dideoxy Guanidine Triphosphate</td>
</tr>
<tr>
<td>ddNTP</td>
<td>Dideoxy Nucleotide Triphosphate</td>
</tr>
<tr>
<td>ddTTP</td>
<td>Dideoxy Thymidine Triphosphate</td>
</tr>
<tr>
<td>dGTP</td>
<td>Deoxy Guanidine Triphosphate</td>
</tr>
<tr>
<td>dITP</td>
<td>Deoxy Inosine Triphosphate</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribo Nucleic Acid</td>
</tr>
<tr>
<td>DTT</td>
<td>Dithiothretool</td>
</tr>
<tr>
<td>dTTP</td>
<td>Deoxy Thymidine Triphosphate</td>
</tr>
<tr>
<td>eAMP</td>
<td>1,N{6}-Ethenoadenosine-5'-Monophosphate</td>
</tr>
<tr>
<td>eCMP</td>
<td>3,N{4}-Ethenocytidine-5'-Monophosphate</td>
</tr>
<tr>
<td>ETBR</td>
<td>Ethidium Bromide</td>
</tr>
<tr>
<td>IPC</td>
<td>Integrated Plate/Chamber</td>
</tr>
<tr>
<td>KAc</td>
<td>Potassium Acetate</td>
</tr>
<tr>
<td>Kb</td>
<td>Kilobase</td>
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</tbody>
</table>
MgCl$_2$  Magnesium Chloride  
mM  Milli Mole  
MMS  Methyl Methanesulfonate  
Mn$^{++}$  Manganese Ions  
MNNG  N-methyl-N'\-nitro-N-nitrosoguanidine  
MOPS  3-[N-Morpholino]propanesulfonic Acid  
NaAc  Sodium Acetate  
ng  Nano Gram  
O.D.  Optical Density  
pmol  Picomole  
PP$_i$  Inorganic Pyrophosphate  
RNA  Ribo Nucleic Acid  
SDS  Sodium Dodecyl Sulfate  
SSB Protein  Single Strand Binding Protein  
TBE  Tris-Borate-Ethelenediamine Tetraacetic Acid  
TE  Tris-Ethelenediamine Tetraacetic Acid  
TEMED  N,N,N',N', Tetramethylethelenediamine  
µCi  Micro Curie  
µg  Micro Gram  
µl  Micro Liter  
µM  Micro Mole  
UV  Ultra Violet  
XP  Xeroderma Pigmentosum
CHAPTER 1

INTRODUCTION

It has been suggested that the initial event in chemical carcinogenesis is the reaction of nuclear DNA with various chemicals (Miller and Miller, 1981; Farber, 1984). However, it is not clear whether these reactions occur at specific sites in chromatin or at random locations on the DNA. It appears that the binding of carcinogens to the chromosomal DNA is not random (Oleson et al., 1979). In other words, the particular carcinogen has an affinity to particular bases or indeed particular base sequences. For example, the linker regions of the nucleosomal structure (a basic structure of chromosomal DNA) is a better target for chemical carcinogens than the core regions (Seidman, Slor and Bustin, 1983). A non-random distribution of carcinogens bound to repetitive sequences in vivo has been reported (Gupta, 1984). Further, experiments where benzo[a]pyrene and aflatoxin B1 were used suggest that transcriptionally active chromatin is a more reactive target for these chemical carcinogens than the inactive chromatin (Yu, 1983; Obi, Ryan and Billet, 1986). These results mimic the difference in sensitivity of active and inactive chromatin to digestion by DNase I and other endonucleases (Eisenberg et al., 1985). Enhanced nuclease sensitivity and chemical reactivity in active chromatin is possibly due to its "open" or more easily accessible structure compared with the inactive chromatin.
DNA consists of four bases two of which are purines (adenine and guanine) while the other two are pyrimidines. These bases offer a variety of functional groups to an attacking chemical agent. However, this process is far from random. Two important factors to bear in mind are:
(a) the chemical environment of the bases and
(b) its charge status (neutral or anionic)

Alkylation is a common reaction whereby mutations are formed. The reactions of free bases with alkylating agents such as alkyl halides, sulfates and sulfonates present the simplest situation in which DNA adducts are formed.

Reaction of chloroacetaldehyde with DNA

2-chloroacetaldehyde is a reactive mutagenic chemical that can easily react in this manner with adenine or cytosine, but not with guanine or thymine (Barrio et al., 1972). The effects of this compound on the synthesis of DNA has been investigated (Saffill and Hall, 1986; Kandala et al., 1990). 2-chloroacetaldehyde is a metabolite of vinyl chloride (Jacobsen, 1989) and may be an important human carcinogen. 2-chloroacetaldehyde is believed to be formed in vivo from vinyl chloride via chloroethylene oxide which can spontaneously rearrange to chloroacetaldehyde. Figure 1.1 shows the formation of an adduct by the reaction of DNA with chloroacetaldehyde (Kusmierek and Singer, 1982).
It has been shown that absorbance and fluorescence spectroscopy (Ward et al., 1969; Beardsley and Cantor, 1970; Wintermeyer and Zachau, 1971) coupled with chemical modifications (Gauss et al., 1971) are extremely useful in gaining information on the tertiary structure of transfer RNA. Light absorption of the derivatized compounds gives information as to which base is principally involved and its quantitation. Absorption measurements are more reliable quantitatively compared with fluorescence techniques which are highly sensitive to environmental influences.

The two techniques can be applied to the reaction of both adenosine and cytidine with chloroacetaldehyde which yields highly fluorescent compounds (Secrist III et al., 1971). In addition to absorption, these products are easily distinguishable by their fluorescence emission spectra. It should be possible to follow these reactions by monitoring the increase in the fluorescence emission. The fluorescence lifetimes observed for the adenosine and cytidine products are close to 20 and 7 nanoseconds respectively. The significant difference in lifetimes suggests that time-resolved
fluorescence can be used to distinguish the various events that may be occurring (Jameson, 1984; Jameson and Hazlett, 1991). Further, the fluorescence intensity is sufficient to use techniques such as fluorescence polarization (Weber, 1970) and polarized decay (Stryer, 1968) providing further valuable information such as the size of the molecules and perhaps the shape of it. If energy transfer could be demonstrated, it may be possible to determine the distance between the groups (molecular ruler).

It has been found that near pH 3.5, the cytidine moiety reacts faster with chloroacetaldehyde, while 4.5 is the optimum pH for the reaction with adenosine. It has been shown that (Barrio, Secrist III and Leonard, 1972) the extremely intense emission of 1 upon excitation at 275 nm allows ready detection of the modified adenosine at very low concentrations.

Both 2-chloroacetaldehyde and its diethyl acetal drastically inhibit the synthesis of DNA in *Escherichia coli* at 10-20 uM (Jacobson, 1989). This inhibitory effect was on the synthesis of the DNA rather than on the uptake of thymidine or the formation of nucleotides. Residual DNA made in the presence of chloroacetaldehyde had an average chain length of 300 base pairs compared with several thousands in its absence. Synchronisation experiments indicate that this inhibitory effect of 2-chloroacetaldehyde is reversible if it is removed within 2 hours but not after longer exposures in *in vivo* situations. In *in vitro* experiments, it was also found that there was an increased level of non-complementary nucleotides being incorporated (Hall et al., 1981). If a poly(dA-dT) template was used, 1 dGMP residue was
incorporated for every 60 etheno-adenine residues present where as no increase in the misincorporation of dCMP was observed. With the poly(dC-dG) templates, one misincorporation of dAMP or dTMP occurred in the presence of 30 and 80 etheno-cytosine residues respectively. A nearest neighbor base frequency analysis showed that with the modified poly(dC-dG) templates, the majority of the errors were incorporated opposite the cytosine (or modified cytosine) bases.

![Figure 1.2: Structure of Etheno-cytosine (Oesch and Doerjer, 1982)](image)

Similar reactions were done using 2-bromoacetaldehyde (Kohwi-Shigematsu, Gelinas and Weintraub, 1983). It was shown that this compound reacted selectively at the N-1 and N-6 positions of unpaired adenine and at the N-3 and N-4 positions of unpaired cytosine residues. Bromoacetaldehyde also reacted with chromosomal DNA in intact cells at probable regulatory sequences near active genes. The unstable nature of this compound prevents its selection over the stable chloroacetaldehyde.
It has been reported (Spengler and Singer, 1988) that chloroacetaldehyde forms interstrand crosslinks in vitro in salmon sperm DNA and in the alternating copolymer, poly(dA-dT). The formation of these cross links depended on the time of reaction and the concentration of chloroacetaldehyde. This observation was initially made by noting the changes in the renaturation hysteresis (Singer, Abbott and Spengler, 1984). Although it was reactive with unpaired adenine and cytosine residues, this was not so in reactions with Z-DNA where adenines reacted in their 'syn' conformation but not the cytosines in their 'anti' conformation (Vogt et al., 1988).

Recently the effects of in vitro adduction of 2-chloroacetaldehyde to the lacZ.alpha gene of the phage M13AB28 on in vivo mutagenesis in SOS (Ultra violet)-induced Escherichia coli were examined (Jacobson and Zafri, 1990). The effect on DNA replication by Escherichia coli DNA polymerase I (Klenow fragment) was examined. This data did not indicate a strong SOS dependence for mutagenesis at cytosine lesions. Further, SOS induction does not appear to significantly alter the specificity of base changes at cytosines and adenines. The high efficiency of mutagenesis opposite cytosine lesions without the aid of induced levels of SOS functions suggests that all these DNA lesions lacking normal Watson-Crick base pairing ability nevertheless do not block replication. Further, the relatively non-mutagenic bypass of adenine lesions focuses attention on the need to understand mechanisms of error avoidance in the absence or normal base-pairing information.
Mutations in the nucleophosphoprotein, p53

Recent work has shed considerable light on the mutational hot spots and their relationship to the development of specific cancers. It appears that mutations in the nucleophosphoprotein p53 are emerging as the commonest genetic change in human cancers (Vogelstein, 1990; Harris, 1991). It was even more of a surprise when Hsu et al., (1991) and Bressac et al., (1991) reported that the p53 mutations from one type of cancer seem to be clustered in the same codon. These two groups looked at hepatocellular carcinomas in patients from China and southern Africa respectively. p53 was originally discovered in extracts of transformed cells, reacting with anti-serum from animals with tumors induced by simian virus-SV40 (Linzer and Levine, 1979; Lane and Crawford, 1979). Table 1.1 shows the common mutations in the p53 gene in hepatocellular carcinomas from southern Africa.

Table 1.1: Common mutations in the p53 gene in hepatocellular carcinomas from southern Africa.

<table>
<thead>
<tr>
<th>Codon</th>
<th>Nucleotide</th>
<th>Amino acid change</th>
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<tbody>
<tr>
<td>249</td>
<td>AGG to AGT</td>
<td>Arginine to Serine</td>
</tr>
<tr>
<td>286</td>
<td>8-bp deletion</td>
<td>Frame shift</td>
</tr>
<tr>
<td>157</td>
<td>GTC to TTC</td>
<td>Val to Phenyl Alanine</td>
</tr>
</tbody>
</table>
The p53 gene has been implicated in many inherited and sporadic forms of malignancies in humans (Maehle et al., 1992; Cheng et al., 1992). This gene was present in nearly every case of hepatocellular carcinoma in the two studies mentioned earlier. Over 50% of breast (Bartek et al., 1990), lung (Iggo et al., 1990), colon (Baker et al., 1990) and bladder tumors have been found to carry a p53 mutation (Baker et al., 1989; Nigro et al., 1989). These mutations were clustered in four highly conserved regions (Figure 1.3) among vertebrates (Soussi et al., 1990). Further, 75-80% of colon carcinomas also showed a loss of both p53 alleles, one through deletion and the other through a point mutation (Levine et al., 1991).

Not all base changes will lead to effective mutations. Firstly, the degeneracy of the genetic code may compensate for single base changes. Secondly, an alteration of a base giving an amino acid not very different to the first is unlikely to have a telling effect.
In the case of the China study, point mutations at codon 249 position 3 were shown. These were guanine to thymine and guanine to cytosine. Some of these mutations were consistent with those caused by Aflatoxin B1. Similar specific changes were observed in the ras genes (H-ras, K-ras and N-ras) which were activated by a somatic point mutation leading to the substitution of a single amino acid, usually in positions 12 or 61 (Barbacid, 1987; Bos, 1988; Valencia et al., 1991). It would be of interest to examine similar sequences in other systems in vitro to look into their susceptibility to known carcinogens. At present, not enough is known of the frequency of p53 mutations in humans. The nature and position of a p53 mutation appears to be influenced by the cell or tissue type (Table 1.2). This mutation inactivates the gene coding for the tumor
suppressor. It may be involved in transcriptional regulation and DNA replication. Comparing table 1.2 with table 1.1, it appears that different carcinogens cause different sites of mutation.

Table 1.2: Comparison of p53 mutations in cancers.

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Number of hot spot mutations at amino acid residue</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>175</td>
</tr>
<tr>
<td>Colon (n=35)</td>
<td>8 (23%)</td>
</tr>
<tr>
<td>Lung (n=29)</td>
<td>0 (0%)</td>
</tr>
<tr>
<td>Total tumors (n=94)</td>
<td>11 (12%)</td>
</tr>
</tbody>
</table>

Recently, the p53 protein has been identified as a sequence-specific DNA binding protein. The G residues of a 5 base pair repeat within these sequences appear to be critical for this interaction. The proteins encoded by p53 mutants found in human tumor cells lose this specific DNA binding ability (Kern et al., 1991).

Another important phenomenon showing the formation of adducts in a non-random fashion was seen in G:C to A:T transitions in ultra-violet light treated PZ189 plasmids replicated in xeroderma pigmentosum (XP) or DNA repair proficient human lymphoblasts (Seetharam et al., 1990; Seetharam et al., 1991). There was a restricted spectrum of mutations found with the XP fibroblasts in comparison to the normal. The observation of such ultra-violet
mutational spectra is further proof that adduct formation indeed has a non random fashion which may be decided by a variety of factors both local and general. Locally such parameters as pH, temperature, length of incubation and the concentration of the mutagen are important while general factors such as the constitution of the DNA may be important in the susceptibility of particular areas of the DNA to reaction. Some of the base substitutions and their locations are given in table 1.3.

Table 1.3: Types of single or tandem-base substitution mutations in UV treated PZ189 replicated in xeroderma pigmentosum or DNA repair proficient human lymphoblasts.

<table>
<thead>
<tr>
<th>Number of base changes (%)</th>
<th>Xeroderma pigmentosum</th>
<th>Repair proficient</th>
</tr>
</thead>
<tbody>
<tr>
<td>Transitions</td>
<td></td>
<td></td>
</tr>
<tr>
<td>G:C to A:T</td>
<td>92</td>
<td>60</td>
</tr>
<tr>
<td>A:T to G:C</td>
<td>4</td>
<td>10</td>
</tr>
<tr>
<td>Transversions</td>
<td>12</td>
<td>38</td>
</tr>
<tr>
<td>G:C to A:T</td>
<td>2</td>
<td>16</td>
</tr>
<tr>
<td>G:C to C:G</td>
<td>2</td>
<td>6</td>
</tr>
<tr>
<td>A:T to T:A</td>
<td>8</td>
<td>10</td>
</tr>
<tr>
<td>A:T to C:G</td>
<td>0</td>
<td>6</td>
</tr>
<tr>
<td>Total</td>
<td>108</td>
<td>108</td>
</tr>
</tbody>
</table>
Principles of DNA sequencing methodology

The DNA sequencing principles used involves the synthesis of a DNA strand by a DNA polymerase in vitro (Sanger, Miklen and Coulson, 1977). Synthesis is initiated at only a single site where the appropriate primer anneals to the template DNA. The chain grows with the incorporation of nucleotides at the 3' end. It is terminated by the incorporation of a 2', 3'-dideoxynucleotide (ddNTP) that will not support continued synthesis of DNA. The enzyme initiates synthesis only at the 3' end of a primer already annealed to a template. Generally, the primer is a short synthetic oligonucleotide of around 30 bases that is complementary to the template at a unique position adjacent to the region to be sequenced.

It thus follows that different strands of the newly synthesized DNA will be terminated at different positions depending on the incorporation of the dideoxy nucleotides. This process is entirely random under normal circumstances. Under such conditions when the samples are run on an acrylamide electrophoresis gel, and subjected to autoradiography a series of bands depicting the various bases are observed (Tabor and Richardson, 1987). The use of Mn++ ions and pyrophosphorolysis in the sequencing process have greatly improved the reading of sequences nearer the primer (Tabor and Richardson, 1989 and 1990). Band intensities are very uniform in the presence of manganese ions with a variation in band intensity of only 20% (Fuller, 1989; Ruan et al., 1990).

In the case of adduct formation, it is assumed that the adduct is large enough to be not recognised by the enzyme as a regular base thereby causing chain termination. Termination may not always
occur, so that in some strands DNA synthesis may continue beyond the point where adduct formation has occurred. It follows that only one such adduct is necessary for chain termination and the fall off may occur when the enzyme meets the first adduct in the chain irrespective of the number of adducts that may be present later on. This technique is a novel way of detecting the positions of adducts. The control M13 DNA sequence will ensure that the system will support complete synthesis of nucleotides.

Several factors can limit the ability to determine long and accurate sequences by the dideoxy chain termination method. Until recently, the resolution of autoradiography was limited by the use of high-energy $^{32}\text{P}$ which gives diffuse bands on a film. The use of lower energy $^{35}\text{S}$ labeled nucleotides has greatly improved autoradiographic resolution (Biggin, Gibson and Hong, 1983).

Reactions to form adducts also depend on a variety of variables such as temperature, pH, length of incubation and salt concentration. It is known that (Messing et al., 1977) M13mp18 DNA which is circular and single stranded has several double stranded regions. Since the adduct formation usually occurs at areas of single strands, such parameters as salt concentration become important. Nitroso compounds also are candidates for adduct formation and may indeed have a different affinity to that of chloroacetaldehyde.

N-methyl-N'-nitro-N-nitrosoguanidine (MNNG) acts as a powerful mutagen (Mandell and Greenberg, 1960). Its irreversible binding capacity to cells has been established (Adelberg et al., 1965). It is also an effective carcinogen along with its ethyl
derivative (Schoental, 1966) both of which can induce stomach, oesophageal, intestinal and lung tumors with one or a few doses (Schoental, 1963 and 1965). The propyl analog of this compound may act in a similar fashion (Figure 1.4).

![Chemical structure](image)

Figure 1.4: Structures of N-methyl-N'-nitro-N-nitrosoguanidine and its ethyl and propyl analogs

Experiments done on rats using N-methyl-N'-nitro-N-nitrosoguanidine and its ethyl derivative showed that only the rats treated with the methyl nitroso analog developed tumors suggesting that the carcinogenic action of the N-alkyl-N'-nitroso compounds is related to their alkyl nitroso group. The ethyl and the propyl analogs however maybe more attractive to the formation of stable adducts with the DNA due to their larger size compared with the methyl group.

It is of value to compare the sites of adduct formation with some of these mutagens to see if they react at the same or at different locations. Compounds with bulky side groups would be particularly suitable for this experiment as these would be better able to cause fall off of the polymerase.
CHAPTER 2
MATERIALS

Frequently used buffers

a. **ECoR I restriction enzyme assay buffer**
10X stock solution was purchased from the United States Biochemical Corporation. Working solution, pH7.5. 100mM Sodium Chloride, 50mM Tris.HCl and 10mM Magnesium Chloride. Assayed at 37°C. Buffer stored at -20°C.

b. **Agarose gel loading buffer**
5X stock solution. 50% Glycerol, 100mM EDTA pH 8.0, 1% SDS, 0.1% Bromophenol Blue and 0.1% Xylene cyanol. Stored at 4°C.

c. **Tris-Borate-EDTA (TBE) buffer**
10X stock solution. 108 grams Tris.HCl, 55 grams Borax and 8.3 grams EDTA in a total volume of 1 liter of distilled water. pH of this solution should be 8.3 without adjustment. Stored at 4°C.

d. **Tris-EDTA (TE) buffer**
10mM Tris.HCl and 1mM EDTA. pH 7.4. Stored at 4°C.

e. **Sequenase reaction buffer™**
5X stock solution. 200mM Tris.HCl pH7.50, 100mM Magnesium Chloride and 250mM Sodium Chloride. Stored at -20°C.
f. **Sequenase enzyme dilution buffer™**

10mM Tris.HCl, pH7.5. 5mM Dithiothreitol (DTT) and Bovine serum albumin (BSA) at 0.5mg / ml.

g. **Manganese buffer (for dGTP)**

0.15M Sodium Isocitrate and 0.1M Manganese Chloride

**Frequently used mixtures and solutions**

a. **Bacterial culture medium**

5 grams Bactotryptone (Difco Laboratories, Detroit)

2.5 grams Bactoyeast (Difco Laboratories, Detroit)

2.5 grams Sodium Chloride in a total volume of 500ml water.

b. **Ampicillin stock solution**

25 milligrams of Ampicillin per ml of double distilled water. The mixture was sterilised prior to use.

c. **Loading solution for agarose gels**

0.1% Bromophenol Blue (BPB), 50% Glycerol, 1% SDS, 0.1% Xylene Cyanol and 100mM EDTA, pH8.0.

d. **Ethidium Bromide solution**

0.5 milli grams of ethidium bromide (ETBR) per ml of distilled water. Filtered and stored in a dark brown bottle to protect it from the light. Stored at 4°C in the cold room. Care should be taken in handling this potential mutagen.
e. **5% Acrylamide stock solution**
2.85 grams ultrapure Acrylamide (USBC) and 0.15 grams ultrapure N',N'-methelene-bis-Acrylamide in 10ml of deionised water made fresh.

f. **25% Ammonium persulfate (APS) solution**
0.25 grams Ammonium persulfate in 1ml of deionised water made up fresh in a microfuge tube.

g. **N.N,N',N'-Tetramethylethylenediamine (TEMED)**
washed purchased from Biorad laboratories. Stored at 4°C.

h. **Casting gel solution**
21 grams urea, 5ml TBE stock solution, 10ml 5% Acrylamide-Bisacrylamide solution. Add water to a total volume of 50ml.

i. **Sodium acetate solution**
**Stock solution**
3M Sodium acetate, pH 5.2. Stored at 4°C.

j. **Labelling mixture (dGTP)**
5X stock solution. 7.5µM each of dGTP, dTTP and dCTP. Stored at 4°C.
k. **Dideoxy termination mixtures**

80μM each of dGTP, dCTP, dTTP and dATP. 8μM of the appropriate dideoxy nucleotide. 50mM Sodium Chloride. Stored at -20°C.

l. **Sequenase extended mix™ (for dGTP)**

Stock solution

180μM each of dGTP, dATP, dCTP, dTTP. 50mM Sodium Chloride

Frequently used enzymes, primers and other molecular biology products

a. **pZ189 plasmid/MBM7070 host strain**

pZ189  amp Aat II  RI  Sup F

--------------------------------------------------------------- 200  pBR327 origin

The plasmid and the host strain were kindly supplied by Dr. Michael Seidman, Chief Scientist (Molecular Biology), Otsuka Pharmaceuticals Company Limited., Maryland Research Laboratories, 9900, Medical Center Drive, Rockville, Maryland 20850.

b. **ECoR I restriction enzyme** (100 units / μl) was purchased from New England Biolabs (NEB), 32, Tozer Road, Beverly, MA 01915. and Boehringer Mannheim Biochemicals (BMB), P.O.Box 50816, Indianapolis, IN 46250.
ECoR I digestion occurs at the following positions:

5' G AATT C 3'
3' C TTAA G 5'

c. **ECo 105I restriction endonuclease** and **AMV reverse transcriptase** were purchased from United States Biochemical Corporation, P. O. Box 22400, Cleveland, Ohio 44122.
Recognition sequence of the restriction enzyme: TAC GTA.
Concentration of 18,000 units/ml

d. **Nuclease S1** specific for single stranded DNA and its assay buffer were kindly supplied by Dr Bruce McConnell.

e. **Sequenase™ enzyme** was purchased from United States Biochemical Corporation (USBC), P.O.Box 22400, Cleveland, Ohio 44122.

f. **pBR322 I ECoR I primer** was purchased from New England Biolabs (NEB),
32, Tozer Road, Beverly,
MA 01915-9990.
5' - GTATCACGAGGCCCT - 3'
Restriction cleavage site and primer annealing region of pBR322:

4330 primer 10

5' - GTATCACGAGGCCCT - 3'
3' - ATCCGCATAGTGCTCCGGGAAAGCAGAAGTTCTTAAGAGTA- 5'

ECoRI

This primer is functional in Sanger dideoxy sequencing reactions using alkali-denatured (Chen and Seeburg, 1985) pBR322 as template.

Storage buffer
10mM Tris.HCl, pH7.4, 5mM Sodium Chloride and 0.1mM EDTA

g. M13mp18 primer was purchased from United States Biochemical Corporation (USBC), P.O.Box 22400, Cleveland, Ohio 44122.
0.5 pmol/μl (3ng/μl)
Primer (-40)
5' - GTTTTCCCAGTCACGAC - 3'
Stored at -20°C.

h. Lambda DNA/Hind III fragments was purchased from Bethesda Research Laboratories (BRL), P.O.Box 6009, Gaithersburg, MD 20877.
0.477μg/μl. Stored at -20°C.
Storage buffer
10mM Tris.HCl, pH7.4, 5mM Sodium Chloride and 0.1mM EDTA.

Table 2.1: Fragments generated by lambda DNA (Sanger et al., 1982).

<table>
<thead>
<tr>
<th>DNA fragment #</th>
<th>Kilobase pairs</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>23.130</td>
</tr>
<tr>
<td>2</td>
<td>9.416</td>
</tr>
<tr>
<td>3</td>
<td>6.557</td>
</tr>
<tr>
<td>4</td>
<td>4.361</td>
</tr>
<tr>
<td>5</td>
<td>2.322</td>
</tr>
<tr>
<td>6</td>
<td>2.027</td>
</tr>
<tr>
<td>7</td>
<td>0.564</td>
</tr>
<tr>
<td>8*</td>
<td>0.125</td>
</tr>
</tbody>
</table>

* The migration of this fragment may vary.

The presence of DNA fragments 1-7 was verified by agarose gel electrophoresis prior to use. The A_{260}/A_{280} ration was routinely observed to be in the range of 1.8-2.0.

Other frequently used materials

a. Qiagen plasmid preparation kit™ for the PZ189 plasmid was obtained from Qiagen Inc., 9259, Eton Avenue, Chatsworth, California 91311.
b. **The Sequenase™ and Taqunase™ kits** were purchased from United States Biochemical Corporation, P.O.Box 22400, Cleveland, Ohio 44122.

c. **Deoxyadenosine 5'-[alpha-Thio] Triphosphate.** $[^{35}S]$ was purchased from DuPont Company, Biotechnology systems, Barley Mill Plaza P-24, Wilmington, Delaware 19898.

12.5mCi/ml (11.05nmol/ml)

**Packaging buffer**

10mM Tricine-NaOH buffer (Good et al., 1966; Rigby et al., 1977), 1mM Dithiothreitol. pH 7.6. Shipped on dry ice. Stored at -70°C.

![Figure 2.1: Structure of $[^{35}S]$ labelled Deoxyadenosine 5'-[alpha-Thio]Triphosphate.](image-url)
d. **Ampicillin (anhydrous)** was purchased from Sigma Chemical Company, P.O.Box 14508, St. Louis, Missouri 63178.

e. **Filters for sterilisation of the Ampicillin solution**
Acrodisc filters (0.2um) were purchased from Gelman Sciences, 600, South Wagner Road, Ann Arbor, Michigan 48106.

f. **Chloroacetaldehyde ~50 weight % solution in water (F.W.78.50)** was purchased from Aldrich Chemical Company Inc., Milwaukee, Wisconsin 53233.

g. **N-Methyl-N'-Nitro-N-Nitrosoguanidine, N-Ethyl-N'-Nitro-N-Nitrosoguanidine and N-Propyl-N'-Nitro-N-Nitrosoguanidine** (Sax, 1979) were purchased from the Sigma Chemical Company, P.O.Box 14508, St. Louis, Missouri 63178.

h. **Hydroxylamine hydrochloride** was purchased from Matheson-Coleman and Bell, Manufacturing Chemists, Norwood, Ohio 45212.

i. **Hydrazine Hydrate (99%)** was purchased from Mallinckrodt Incorporated, St. Louis, Missouri 63147.
j. Phenylhydrazine (100%) was purchased from Scientific Products, 17111, Red Hill Avenue, Irvine, California 92705.

k. 2,4-dinitrophenylhydrazine (10% water) was purchased from J.T.Baker Chemical Company, Phillisburg, New Jersey 08865.

l. Methyl methanesulfonate (97%) was purchased from Aldrich Chemical Company Inc., Milwaukee, Wisconsin 53233.

m. 1,N(6)-Ethenoadenosine-5'-Monophosphate was purchased from Molecular Probes Inc., P. O. Box 22010, 4849, Pitchford Avenue, Eugene, Oregon 97402. Stored at 0°C in a dessicator.

n. 3,N(4)-Ethenocytidine-5'-Monophosphate was purchased from Sigma Chemical Company, P. O. Box 14508, St. Louis, Missouri 63178. Stored at 0°C in a dessicator.

o. Polaroid type 55 films for photographing of agarose gels These were purchased from City Art Works, 1133, Nuuanu Avenue, Honolulu, Hawaii 96817.
Frequently used instruments

a. *Sequi-Gen nucleic acid sequencing cell* was purchased from Biorad Laboratories, 1414, Harbor Way South, Richmond, California 94804.

b. *Mini-sub DNA cell* was purchased from Biorad Laboratories, 1414, Harbor Way South, Richmond, California 94804.

c. *EC-103 Power Apparatus* manufactured by the EC Apparatus Corporation was purchased from Fischer Scientific, 2170, Martin Avenue, Santa Clara, California 95050.

d. *Fischer-Biotech gel drying system* was supplied by Dr Hans Knoll.

e. *Cold Finger* was supplied by Dr Bruce McConnell.

f. *The pump* used to dry the gels was supplied by Dr Richard John Guillory.

g. *X-ray films*
35 X 43cm X-ray films to expose sequencing gels were purchased from Fuji Photo Film Limited, Honolulu, Hawaii.
h. **Film cassettes**
These were supplied gratis by Fuji Photo Film Limited.

i. **Developer/Fixer solutions**
The developer/replenisher and the fixer/replenisher solutions were purchased from Eastman Kodak Company, Rochester, New York 14650.

j. **SLM 8000C Spectrofluorometer**
purchased from SLM Instruments, Inc., 810, West Anthony Drive, Urbana, IL 61801 was made available for fluorescence studies courtesy of Dr David M. Jameson.

k. **Perkin-Elmer Spectrophotometer** purchased from Perkin-Elmer Instrument Sales, 411, Clyde Avenue, Mountain View, CA 94043 was made available for spectrophotometric studies courtesy of Dr David M. Jameson.
CHAPTER 3

METHODS

Bacterial cultures
The medium consisting of Bactotryptone (5 grams), Bactoyeast (2.5 grams) and Sodium chloride (2.5 grams) was dissolved in 500 milliliters of double distilled water. It was divided into five equal volumes and transferred into erlenmeyer flasks and autoclaved for thirty (30) minutes. While still hot, the prepared ampicillin solution was added at a final concentration of 50µg per ml of culture. The flasks were allowed to cool to room temperature. This can take up to 45 minutes. Under sterile conditions, the culture in each of these flasks was inoculated with a single drop of PZ189 plasmid. The flask tops were left loose to allow the bacteria to breathe. The cultures were shaken overnight at 2,000 rpm at 37°C. The flasks were well secured prior to switching on the shaker.

Ampicillin stock solution
25 milligrams of the Ampicillin powder per milliliter of distilled water was used. This mixture was vortexed in an eppendorf tube and the resulting solution was sterilized by injecting it through a millipore (0.2µm) filter. It was aliquoted and stored at -20°C and thawed prior to each use.
**Bacterial stock (containing the PZ189) storage**

The bacterial growth in the overnight culture can be estimated by the extent of the frothiness of the mixture. To one of the flasks, 35ml of 100% glycerol was added and mixed well. This solution was aliquoted into autoclaved eppendorf tubes and stored at -70°C.

**Preparation of the pure PZ189 plasmid**

500ml of the *Escherichia coli* culture was used for the 'maxi' preparation. The culture was spun down at 0°C and the following procedure was adopted:

a. Resuspended the pellet in buffer P1 (50mM Tris.HCl, 10mM EDTA, pH8).

b. Added 10ml of buffer P2 (0.2M NaOH, 1% SDS), mixed gently and incubated at room temperature for 5 minutes.

c. Added 10ml of buffer P3 (2.55M KAc, pH4.8) and the contents were mixed immediately but gently and centrifuged at 4°C for 30 minutes (>20,000g). The supernatant was removed promptly.

d. Centrifuged the supernatant at 4°C for 10 minutes (>20,000g) and obtained a non turbid lysate.

e. A Qiagen-tip 500 was equilibrated with 10ml of buffer QBT (750mM NaCl, 50mM MOPS, 15% ethanol, 0.15% Triton, pH7) and the liquid was allowed to empty by gravity flow.

f. The supernatant from step d was applied to this column and allowed to flow by gravity. Then the column was washed with 3X10ml of buffer QC (1,000mM NaCl, 50mM MOPS, 15% ethanol, pH7).
The DNA was eluted with 15ml of buffer OF (1,250mM NaCl, 50mM MOPS, 15% ethanol, pH8.2) and precipitated with 0.7 volumes of isopropanol and centrifuged at 4°C for 30 minutes.

The DNA was subsequently washed with 70% ethanol and redissolved in a suitable volume of double distilled water.

**Determining the purity and yield of DNA**

An aliquot of the DNA was diluted in 10mM Tris-HCl, pH7.5 and the optical density of it was measured at 260nm and 280nm. The ratio of \( \text{OD}_{260}/\text{OD}_{280} \) was determined. If the ratio is between 1.8 and 2.0 the DNA is pure enough for sequencing. Based on the information obtained a range of concentrations of the DNA was run on a 0.8% agarose gel to determine the percentages of chromosomal, nicked and supercoiled DNA. A plasmid standard of known concentration and size should be run on the same gel whenever possible.

**Calculation of the molecular weight of the DNA**

This is important to know in sequencing reactions. A larger quantity of DNA is required for double stranded templates (atleast 3\( \mu \)g) compared to single stranded DNA (1-2\( \mu \)g).

a. Determine the size of the DNA by comparison with stanards on agarose. Sizes are usually given in base pairs for double stranded DNA or nucleotides for single stranded. It is more accurate to first do a restriction digest on double stranded supercoiled DNA.

b. For double stranded DNA, \( \text{MW} \) (g/mole) = \# bases \times 660 (ds DNA)

c. For single stranded DNA, \( \text{MW} \) (g/mole) = \# bases \times 330 (ss DNA)
Preparation and running of agarose horizontal gels

This type of electrophoresis is used to determine the quantity and quality of DNA samples. The DNA can be restriction digested and run with a ladder to determine the concentration, molecular weight and molecular form (i.e. supercoiled, linear etc.). At pH7, the DNA is negatively charged mainly due to the phosphodiesters in its backbone. Therefore, it will migrate to the anode. Since the charge: mass ratio is the same for all DNA molecules, the rate of migration is primarily determined by the size and the conformation of the DNA. The rate of migration will be dependent on the concentration of agarose, voltage used, electrophoresis buffers and the conformation of the DNA (see table 3.1).

Table 3.1: Agarose gel conditions for DNAs of various sizes

<table>
<thead>
<tr>
<th>DNA Size Range (Kb)</th>
<th>Optimum Agarose Concentration (%)</th>
<th>Voltage Gradient (Volts/cm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>100 - 20</td>
<td>0.3</td>
<td>5</td>
</tr>
<tr>
<td>60 - 5.0</td>
<td>0.3</td>
<td>10</td>
</tr>
<tr>
<td>20 - 1.0</td>
<td>0.6</td>
<td>20</td>
</tr>
<tr>
<td>10 - 0.8</td>
<td>0.7</td>
<td>20</td>
</tr>
<tr>
<td>7 - 0.5</td>
<td>0.9</td>
<td>25</td>
</tr>
<tr>
<td>6 - 0.4</td>
<td>1.2</td>
<td>25</td>
</tr>
<tr>
<td>4 - 0.2</td>
<td>1.5</td>
<td>25</td>
</tr>
<tr>
<td>3 - 0.1</td>
<td>2.0</td>
<td>25</td>
</tr>
</tbody>
</table>
0.3 grams of agarose was added to 30ml of electrophoresis buffer and microwaved for 30 seconds or until the agarose was completely dissolved. Then it was reweighed and double distilled water added to correct the deficit. This solution was allowed to cool to about 50°C. Cooling the mixture will aid pouring and also avoids warping the casting tray. The appropriate comb was placed at one end and the gel allowed to set in the horizontal position for one hour. Carefully remove air bubbles near the comb as soon as the gel is poured. Once set, the comb was carefully removed and the samples loaded.

(a) Sample DNA solution 3μl
(b) Loading solution 5μl
(c) Electrophoresis buffer 15μl

The voltage used was set at 70 milli volts. The gel was run until the bromophenol blue dye had migrated most of the length of the gel. This took approximately 90 minutes for a 5% agarose gel. Then it was stained in ethidium bromide for 30 minutes and destained for 15 minutes in distilled water. Next, the gel was viewed under ultraviolet (UV) light and a photograph taken. The protocol for picture taking is included in addendum 1.

ECoR I restriction digest of DNA

The mixture for this consisted of ECoR I enzyme (2μl), 10X assay buffer (2.5μl), DNA (2μl) and double distilled water (18.5μl). Samples were well mixed and incubated for 2 hours at 37°C. At this point it was precipitated in 100% ethyl alcohol and redissolved in the electrophoretic buffer and loaded onto the agarose gel.
Incubation of M13mp18 ss DNA with chloroacetaldehyde (CAA)

\[
\begin{align*}
\text{(A)} & : \ce{H-C-C-H} \\
\text{(B)} & : \ce{Cl-C-C-H}
\end{align*}
\]

Figure 3.1: Structures of acetaldehyde (A) and its chloro analog (B).

M13mp18 single stranded DNA (0.2μg/μl) was incubated with chloroacetaldehyde in the presence of 50mM sodium acetate, pH5.2 for 60 minutes at 37°C. The amounts of DNA and the carcinogen were varied to get the best combination. Table 3.2 illustrates the reactions of M13mp18 single stranded DNA with chloroacetaldehyde.

Table 3.2: Incubation of M13mp18 single stranded DNA with chloroacetaldehyde for running on agarose.

<table>
<thead>
<tr>
<th></th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
</tr>
</thead>
<tbody>
<tr>
<td>M13 DNA</td>
<td>10μl</td>
<td>10μl</td>
<td>10μl</td>
<td>10μl</td>
<td>None</td>
</tr>
<tr>
<td>CAA (1:10 mix)</td>
<td>None</td>
<td>5μl</td>
<td>1μl</td>
<td>None</td>
<td>1μl</td>
</tr>
<tr>
<td>NaAc, pH5.2</td>
<td>2μl</td>
<td>2μl</td>
<td>2μl</td>
<td>2μl</td>
<td>2μl</td>
</tr>
<tr>
<td>DD Water</td>
<td>88μl</td>
<td>83μl</td>
<td>87μl</td>
<td>88μl</td>
<td>97μl</td>
</tr>
</tbody>
</table>

100μl  100μl  100μl  100μl  100μl
Precipitation of the DNA and following incubation with the carcinogen.

Add 4 volumes of 100% ethanol to the incubation mixture

Leave at -70°C for 30 minutes to precipitate the DNA

Centrifuge at 14,000 rpm for 15-20 minutes at 0°C

Discard the supernatant

Wash the pellet with 70% ethanol

Centrifuge at top speed for 1 minute

Invert and dry for 1 hour

Dissolve the DNA in an appropriate volume to run it on agarose.

The drying of the tubes must be complete. Ethanol residues may interfere with sample migration in agarose gels. It also interferes with sequencing reactions.
Sequencing of chloroacetaldehyde reacted M13mp18 DNA

The procedure for sequencing is essentially the same as for M13mp18 control DNA. However, no dideoxy compounds are used to terminate the reactions. Instead, the extended dGTP mix contained in the Sequenase™ kit containing all the regular nucleotides in the same concentrations is used. Thus, any bands seen may indicate 'hot spots' due to chloroacetaldehyde. The abundance of the regular nucleotides ensures that chain termination does not take place due to a lack of regular nucleotides.

Table 3.3: Incubation of M13mp18 single stranded DNA with CAA

<table>
<thead>
<tr>
<th></th>
<th>1</th>
<th>2</th>
<th>3</th>
</tr>
</thead>
<tbody>
<tr>
<td>M13mp18 DNA</td>
<td>20μl</td>
<td>20μl</td>
<td>20μl</td>
</tr>
<tr>
<td>CAA</td>
<td>1μl (1:100)</td>
<td>1μl (1:10)</td>
<td>None</td>
</tr>
<tr>
<td>3M NaAc, pH5.2</td>
<td>4μl</td>
<td>4μl</td>
<td>4μl</td>
</tr>
<tr>
<td>DD Water</td>
<td>175μl</td>
<td>175μl</td>
<td>176μl</td>
</tr>
<tr>
<td></td>
<td>200μl</td>
<td>200μl</td>
<td>200μl</td>
</tr>
</tbody>
</table>

These mixtures were incubated for 2 hours at 37°C. Incubations were also done using the dideoxy ATP and the dideoxy GTP mixtures along with the carcinogen.
Effect of varying the concentration of salt on the ability to form adducts

This experiment was done along the same lines as the previous one but with variations in the concentration of salt (Table 3.4). The volume of the carcinogen was held at constant at 10μl of a 1:100 dilution mixture of chloroacetaldehyde. The total volume in each tube was brought up to 200μl using double distilled water.

Table 3.4: Incubation of M13mp18 DNA with varying amounts of salt

<table>
<thead>
<tr>
<th></th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
</tr>
</thead>
<tbody>
<tr>
<td>M13 DNA</td>
<td>20μl</td>
<td>20μl</td>
<td>20μl</td>
<td>20μl</td>
<td>20μl</td>
</tr>
<tr>
<td>CAA (1:100 mix)</td>
<td>10μl</td>
<td>10μl</td>
<td>10μl</td>
<td>10μl</td>
<td>None</td>
</tr>
<tr>
<td>NaAc, pH5.2</td>
<td>4μl</td>
<td>7μl</td>
<td>67μl</td>
<td>None</td>
<td>4μl</td>
</tr>
<tr>
<td>(50mM)</td>
<td>(100mM)</td>
<td>(1M)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DD Water</td>
<td>166μl</td>
<td>163μl</td>
<td>103μl</td>
<td>170μl</td>
<td>176μl</td>
</tr>
<tr>
<td></td>
<td>200μl</td>
<td>200μl</td>
<td>200μl</td>
<td>200μl</td>
<td>200μl</td>
</tr>
</tbody>
</table>

Post incubation treatment was the same as before.

Sequencing of M13mp18 DNA reacted with nitroso compounds

Methyl, ethyl and the propyl derivatives of N'-Nitro-N-Nitrosoguanidine were used in this experiment. These potent carcinogens were reacted with M13 DNA in various concentrations at
37°C. The period of incubation was 2 hours. The resulting mixture was subjected to scheme 1 and the DNA was precipitated. It was then sequenced using the extended dGTP mix as described previously. All three compounds are not very soluble in distilled water, and vigorous mixing was necessary to make stock solutions of 1mg/ml. Table 3.5 shows the different incubations done.

Table 3.5: Incubation of M13mp18 single stranded DNA (0.2 µg/µl) with the nitroso compounds for sequencing. All volumes are in micro liters.

<table>
<thead>
<tr>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
<th>8</th>
<th>9</th>
</tr>
</thead>
<tbody>
<tr>
<td>M13 DNA</td>
<td>20</td>
<td>20</td>
<td>20</td>
<td>20</td>
<td>20</td>
<td>20</td>
<td>20</td>
<td>20</td>
</tr>
<tr>
<td>Nitroso compound</td>
<td>2*</td>
<td>10*</td>
<td>20*</td>
<td>2#</td>
<td>10#</td>
<td>20#</td>
<td>2^</td>
<td>10^</td>
</tr>
<tr>
<td>NaAc, pH5.2</td>
<td>4</td>
<td>4</td>
<td>4</td>
<td>4</td>
<td>4</td>
<td>4</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td>DD Water</td>
<td>174</td>
<td>166</td>
<td>156</td>
<td>174</td>
<td>166</td>
<td>156</td>
<td>174</td>
<td>166</td>
</tr>
<tr>
<td>200</td>
<td>200</td>
<td>200</td>
<td>200</td>
<td>200</td>
<td>200</td>
<td>200</td>
<td>200</td>
<td>200</td>
</tr>
</tbody>
</table>

* N-Methyl-N'-Nitro-N-Nitrosoguanidine
# N-Ethyl-N'-Nitro-N-Nitrosoguanidine
^ N-Propyl-N'-Nitro-N-Nitrosoguanidine
Sequencing of M13mp18 DNA reacted with hydroxylamine hydrochloride and hydrazine hydrate

This incubation was done along the same lines as that with the nitroso compounds. The stock solutions used were 5 mg/ml (hydroxylamine hydrochloride) and 1 mg/ml (hydrazine hydrate). Both compounds went into solution rapidly. These substances were incubated with M13 DNA in various concentrations at 37°C for 2 hours (Table 3.6). The resulting mixtures were subjected to scheme 1 as in previous experiments.

\[
\begin{align*}
\text{NH}_2 - \text{OH.HCl} & & \text{NH}_2 - \text{NH}_2\text{H}_2\text{O} \\
\text{(A)} & & \text{(B)}
\end{align*}
\]

Figure 3.2: Structures of hydroxylamine hydrochloride (A) and hydrazine hydrate (B).
Table 3.6: Incubation of M13mp18 single stranded DNA with hydroxylamine hydrochloride and hydrazine hydrate for sequencing. All volumes are in micro liters.

<table>
<thead>
<tr>
<th></th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
</tr>
</thead>
<tbody>
<tr>
<td>M13mp18 ss DNA</td>
<td>20</td>
<td>20</td>
<td>20</td>
<td>20</td>
<td>20</td>
<td>20</td>
</tr>
<tr>
<td>Mutagen</td>
<td>1*</td>
<td>5*</td>
<td>10*</td>
<td>2#</td>
<td>10#</td>
<td>20#</td>
</tr>
<tr>
<td>NaAc, pH5.2</td>
<td>4</td>
<td>4</td>
<td>4</td>
<td>4</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td>DD Water</td>
<td>175</td>
<td>171</td>
<td>166</td>
<td>174</td>
<td>166</td>
<td>156</td>
</tr>
</tbody>
</table>

200 200 200 200 200 200

* Hydroxylamine hydrochloride

# Hydrazine hydrate

Sequencing of M13mp18 DNA reacted with phenylhydrazine and 2,4 dinitrophenylhydrazine

Figure 3.3: Structures of phenylhydrazine (A) and 2,4-dinitro phenyl hydrazine (B).
Stock solutions of 2,4-dinitrophenylhydrazine and phenyl hydrazine at 1mg/ml were prepared. The remainder of the protocol is identical to the previous experiment.

Table 3.7: Incubation of M13mp18 single stranded DNA with phenylhydrazine and 2,4 dinitrophenylhydrazine for sequencing. All volumes are in micro liters.

<table>
<thead>
<tr>
<th></th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
</tr>
</thead>
<tbody>
<tr>
<td>M13 DNA</td>
<td>20</td>
<td>20</td>
<td>20</td>
<td>20</td>
<td>20</td>
<td>20</td>
</tr>
<tr>
<td>Phenyl Hydrazine</td>
<td>2</td>
<td>10</td>
<td>20</td>
<td>2*</td>
<td>10*</td>
<td>20*</td>
</tr>
<tr>
<td>NaAc, pH5.2</td>
<td>4</td>
<td>4</td>
<td>4</td>
<td>4</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td>DD Water</td>
<td>174</td>
<td>166</td>
<td>156</td>
<td>174</td>
<td>166</td>
<td>156</td>
</tr>
</tbody>
</table>

200 200 200 200 200 200

* 2,4-dinitrophenylhydrazine
Sequencing of M13mp18 DNA reacted with methyl methanesulfonate

A 1/100 dilution of the methyl methanesulfonate (MMS) was used for the incubation with the M13mp18 single stranded DNA (0.2 μg/μl). The concentration of the stock solution was 1 mg%.

Table 3.8: Incubation of MMS (1/100) with M13mp18 ss DNA. All volumes are in micro liters.

<table>
<thead>
<tr>
<th></th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
</tr>
</thead>
<tbody>
<tr>
<td>M13 DNA</td>
<td>20</td>
<td>20</td>
<td>20</td>
<td>20</td>
</tr>
<tr>
<td>MMS (1:100)</td>
<td>1</td>
<td>2</td>
<td>5</td>
<td>10</td>
</tr>
<tr>
<td>NaAc, pH5.2</td>
<td>4</td>
<td>4</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td>DD Water</td>
<td>175</td>
<td>174</td>
<td>171</td>
<td>166</td>
</tr>
<tr>
<td></td>
<td>200</td>
<td>200</td>
<td>200</td>
<td>200</td>
</tr>
</tbody>
</table>

Effect of Chloroacetaldehyde on the Sequenase enzyme™

In previous experiments, the aim was to see the effect of the various mutagens on the single stranded DNA. Following the reaction of the DNA with the chemical the reacted portion is extracted using 100% methanol. It is possible that the extraction may not get rid of all of the chemical and thereby become available for interaction with the enzyme. Thus it is necessary to do a control experiment where the chemical is added in the labelling step. This was done according to table 3.9.
Table 3.9: Sequencing M13mp18 single stranded DNA in the presence of chloroacetaldehyde in the labelling step. All tubes contain the annealed DNA mixture.

<table>
<thead>
<tr>
<th>Lane</th>
<th>Treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lane 1</td>
<td>Dideoxy ATP control</td>
</tr>
<tr>
<td>Lane 2</td>
<td>Dideoxy TTP control</td>
</tr>
<tr>
<td>Lane 3</td>
<td>Dideoxy CTP control</td>
</tr>
<tr>
<td>Lane 4</td>
<td>Dideoxy GTP control</td>
</tr>
<tr>
<td>Lane 5</td>
<td>No dideoxy nucleotides. 1µl of 0.0001% CAA</td>
</tr>
<tr>
<td>Lane 6</td>
<td>1µl of 0.0001% CAA in the presence of 1µl ext GTP</td>
</tr>
<tr>
<td>Lane 7</td>
<td>1µl of 0.0001% CAA in presence of ddATP</td>
</tr>
<tr>
<td>Lane 8</td>
<td>1µl of 0.0001% CAA in presence of ddGTP</td>
</tr>
<tr>
<td>Lane 9</td>
<td>Same as lane 5 except 1µl of 0.001% CAA</td>
</tr>
<tr>
<td>Lane 10</td>
<td>Same as lane 6 except 1µl of 0.001% CAA</td>
</tr>
<tr>
<td>Lane 11</td>
<td>Same as lane 7 except 1µl of 0.001% CAA</td>
</tr>
<tr>
<td>Lane 12</td>
<td>Same as lane 8 except 1µl of 0.001% CAA</td>
</tr>
</tbody>
</table>

Sequencing of chloroacetaldehyde reacted PZ189 plasmid DNA

The protocol is identical to that with the M13 control DNA. Since the plasmid is double stranded, any reactions with the chloroacetaldehyde would largely depend on single stranded regions of the plasmid either due to 'breathing' or to local conditions. Table 3.10 shows the incubations carried out. The DNA was denatured prior to sequencing it.
Table 3.10: Reactions of PZ189 plasmid with CAA

<table>
<thead>
<tr>
<th></th>
<th>1</th>
<th>2</th>
</tr>
</thead>
<tbody>
<tr>
<td>PZ189 plasmid DNA</td>
<td>36μl</td>
<td>36μl</td>
</tr>
<tr>
<td>CAA (1:100)</td>
<td>1μl</td>
<td>5μl</td>
</tr>
<tr>
<td>NaAc, pH5.2</td>
<td>2μl</td>
<td>2μl</td>
</tr>
<tr>
<td>DD Water</td>
<td>61μl</td>
<td>57μl</td>
</tr>
<tr>
<td></td>
<td>100μl</td>
<td>100μl</td>
</tr>
</tbody>
</table>

Restriction digestion of M13mp18 replicated form with ECo 105I enzyme for running on agarose

The replicated form of M13mp18 DNA has a single restriction site for ECo 105I. This enables the production of linear double stranded DNA having the same number of base pairs as the circular form. Table 3.11 shows the contents of the mixture that was loaded on agarose to determine the nature of the restriction digest.

Table 3.11: Restriction digestion of M13mp18 RF form with ECo 105I

<p>| | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>M13mp18 RF form</td>
<td>5μl</td>
</tr>
<tr>
<td>ECo 105I</td>
<td>1μl</td>
</tr>
<tr>
<td>Assay buffer (10X)</td>
<td>4μl</td>
</tr>
<tr>
<td>Distilled Water</td>
<td>30μl</td>
</tr>
</tbody>
</table>
The incubation of the above mixture was at 37°C for 1 hour. It was then loaded on the agarose gel along with the samples as follows.

Table 3.12: Samples loaded on agarose for identifying the restriction digest

<table>
<thead>
<tr>
<th></th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ladder DNA</td>
<td>2 μl</td>
<td>None</td>
<td>None</td>
<td>None</td>
</tr>
<tr>
<td>M13 ss DNA</td>
<td>None</td>
<td>5 μl</td>
<td>None</td>
<td>None</td>
</tr>
<tr>
<td>M13 RF DNA</td>
<td>None</td>
<td>None</td>
<td>5 μl</td>
<td>None</td>
</tr>
<tr>
<td>Cut M13 RF</td>
<td>None</td>
<td>None</td>
<td>None</td>
<td>5 μl</td>
</tr>
<tr>
<td>Buffer</td>
<td>13 μl</td>
<td>10 μl</td>
<td>10 μl</td>
<td>None</td>
</tr>
<tr>
<td>Stop Solution</td>
<td>5 μl</td>
<td>5 μl</td>
<td>5 μl</td>
<td>5 μl</td>
</tr>
</tbody>
</table>

20 μl 20 μl 20 μl 20 μl
Sequencing of M13mp18 replicated form of DNA treated with chloroacetaldehyde

The DNA (0.2 μg/μl) was reacted with the carcinogen as shown below:

Table 3.13: Incubation of M13mp18 RF DNA with chloroacetaldehyde

<table>
<thead>
<tr>
<th></th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
</tr>
</thead>
<tbody>
<tr>
<td>M13mp18 DNA</td>
<td>50</td>
<td>50</td>
<td>50</td>
<td>50</td>
</tr>
<tr>
<td>CAA</td>
<td>5 (1/100)</td>
<td>1 (1/10)</td>
<td>5(1/10)</td>
<td>0</td>
</tr>
<tr>
<td>NaAC, pH5.2</td>
<td>4</td>
<td>4</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td>DD Water</td>
<td>141</td>
<td>145</td>
<td>141</td>
<td>146</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td>200</td>
<td>200</td>
<td>200</td>
<td>200</td>
</tr>
</tbody>
</table>

At the end of the 1 hour incubation at 37°C, The DNA was precipitated using alcohol as described previously. It was then denatured using the alkaline denaturation method and sequenced as described previously.
Sequencing of single stranded M13mp18 DNA treated with chloroacetaldehyde and then subjected to alkaline denaturation

The purpose of this experiment was to evaluate the strength and stability of the adducts following alkaline treatment. The DNA was reacted as shown in table 3.14.

Table 3.14 Incubation of M13mp18 DNA with chloroacetaldehyde

<table>
<thead>
<tr>
<th></th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
</tr>
</thead>
<tbody>
<tr>
<td>M13mp18 DNA</td>
<td>20</td>
<td>20</td>
<td>20</td>
<td>20</td>
<td>20</td>
</tr>
<tr>
<td>CAA</td>
<td>1 (1/100)</td>
<td>5 (1/100)</td>
<td>1 (1/10)</td>
<td>5 (1/10)</td>
<td>0</td>
</tr>
<tr>
<td>NaAC, pH 5.2</td>
<td>4</td>
<td>4</td>
<td>4</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td>DD Water</td>
<td>175</td>
<td>171</td>
<td>175</td>
<td>171</td>
<td>176</td>
</tr>
<tr>
<td></td>
<td>200</td>
<td>200</td>
<td>200</td>
<td>200</td>
<td>200</td>
</tr>
</tbody>
</table>

At the end of the incubation the DNA was treated with the alkali mixture prior to sequencing it.

Sequencing of single stranded M13mp18 DNA using AMV reverse transcriptase in lieu of Sequenase™ version 2.0

This procedure was carried out to compare the sequences with those obtained with the sequenase enzyme in the presence of chloroacetaldehyde. This was a control experiment to look for the effects of different enzymes on chloroacetaldehyde. The protocol adopted for this experiment appears below.
(1) **Annealing / Reaction Buffer (5X)**

250mM Tris.HCl, pH 8.3, 40mM MgCl₂
250mM NaCl, 5mM DTT

(2) **Labelling Mix (this is a 1:5 dilution of Sequenase kit mix)**

1.5μM dGTP, 1.5μM dTTP and 1.5μM dCTP

(3) **Termination Mixes (μM).**

2.5 μl in each tube

<table>
<thead>
<tr>
<th>dGTP Mixes</th>
<th>Mix</th>
<th>G</th>
<th>A</th>
<th>T</th>
<th>C</th>
</tr>
</thead>
<tbody>
<tr>
<td>dGTP</td>
<td>800</td>
<td>800</td>
<td>800</td>
<td>800</td>
<td>800</td>
</tr>
<tr>
<td>dATP</td>
<td>800</td>
<td>800</td>
<td>800</td>
<td>800</td>
<td>800</td>
</tr>
<tr>
<td>dCTP</td>
<td>800</td>
<td>800</td>
<td>800</td>
<td>800</td>
<td>800</td>
</tr>
<tr>
<td>ddGTP</td>
<td>80</td>
<td>None</td>
<td>None</td>
<td>None</td>
<td>None</td>
</tr>
<tr>
<td>ddATP</td>
<td>None</td>
<td>160</td>
<td>None</td>
<td>None</td>
<td>None</td>
</tr>
<tr>
<td>ddTTP</td>
<td>None</td>
<td>None</td>
<td>160</td>
<td>None</td>
<td>None</td>
</tr>
<tr>
<td>ddCTP</td>
<td>None</td>
<td>None</td>
<td>None</td>
<td>80</td>
<td>None</td>
</tr>
</tbody>
</table>

**Procedure**

**Annealing**

Mix 7μl (0.5 pmol) M13mp18 ss DNA, 1μl 0.5 μM primer and 2μl 5X buffer. Heat to 65°C and cool slowly to < 37°C (~30 mins). Then chill until use.
Labelling
Add 0.5μl $^{35}$S dATP (5μCi), 2μl labelling mix and 1μl AMV reverse transcriptase (~30 units/μl). Mix and incubate at 42°C, for 15 minutes.

Termination
Transfer 3.3μl aliquots to termination tubes containing 3μl termination mixes. Incubate for 15 minutes at 42°C. Stop the reaction by adding 4μl of the stop solution (from the sequenase™ kit).

Sequencing of single stranded M13mp18 DNA using TaqQuenase™ version 2.0 or the Klenow fragment in lieu of Sequenase™ version 2.0
The sequencing procedure was very similar to that used with the sequenase enzyme. The manufactures protocol was followed to sequense the DNA using these two enzymes.

Choosing a primer
Ideally the primer should contain about 50% G/C content (range of 40-60%). This is important to have effective annealing. A primer with a low G/C content may have a low melting temperature. A primer length of 18 nucleotides is satisfactory. A computer search of the known portion of the sequence will help in checking for other regions where priming might occur. This step will help plan for the optimal primer sequence to eliminate multiple priming.
Denaturation of double stranded DNA

The alkaline denaturation method which is more suitable for supercoiled double stranded DNA was used (Hattori and Sakaki, 1986).

(a) 18μl of DNA (at least 3μg) was mixed with 2μl of sodium hydroxide and incubated for 5 minutes at room temperature.

(b) To this add 8μl of ammonium acetate (pH7.5) and 100μl of 95% cold ethanol. The mixture was incubated for thirty (30) mins at -20°C.

(c) The sample was centrifuged for 30 mins at 14,000 rpm at 4°C. The supernatant was carefully removed.

(d) The pellet was washed by adding 500μl of cold 70% ethanol, inverting the tube once and centrifuging for one minute. Draw off the ethanol taking care not to disturb the pellet.

(e) Dry the pellet and resuspend it in 7μl of sterile distilled water and proceed with the sequencing reactions.

Annealing of the template to the primer

1-2μg of DNA (at least 3μg of double stranded DNA) in 7μl of sterile distilled water is mixed with 1μl of the appropriate primer (0.5pmoles/μl) and 2μl of the sequenase reaction buffer. The total volume is 10μl. It is important to maintain a 1:1 ratio of primer to template. Mix the contents well by centrifuging briefly. Place on a float in a beaker of water in a 65°C water bath and incubate for 2 minutes. At the end of this time period, the beaker was removed from the water bath and allowed to cool slowly to 30°C. This took
approximately 40 minutes. At the end of this time period, the annealed templates were placed on ice.

**Labelling and termination reactions**

While the annealing mixture was cooling, the termination tubes were prepared by labelling 4 tubes, one each for the four bases for each template being sequenced. 2.5μl of the dideoxy termination mixtures were added to the appropriate tubes (ddGTP to the G tube, ddATP to the A tube and so on). These tubes were kept on ice. Next, the labelling mixture was diluted 1:5 in sterile distilled water. This is a good dilution for reading sequences from 30 to 200 bases. To the cooled annealing reaction, the following were added:

- 2μl of the diluted labelling mix
- 1μl Dithiothreotol (DTT) solution
- 0.5μl [35S] dATP (5μCi)

The mixture was briefly centrifuged to collect the contents at the bottom and kept on ice. Sequenase enzyme was diluted 1:8 in ice cold enzyme dilution buffer. 2μl of this is required for each template. Once the enzyme was added to the annealing mixture, it was incubated for 2-5 minutes. At this time, the termination tubes were transferred to a 37°C water bath to pre-heat. When the labelling reaction incubation was completed, 3.5μl was immediately transferred to the termination tubes (4 tubes for each template). After 5 minutes, 4μl of the stop solution was added to each of these. With the 35S label, the samples are stable for up to 1 week.
Typical gel conditions (7M urea and 50°C) denature adenine/thymine secondary structure. However, it does not do so to structures containing 3 or more guanine/cytosine pairs in succession. Thus, these structures remain folded and run faster through the gel matrix than the equivalent unfolded DNA and run together with small pieces of DNA in the sequence. This can be a major problem in reading sequences accurately.

To eliminate such band compressions inosine (dITP) can replace guanine. The resultant cytosine/inosine interactions are much weaker and are easily denatured (Figure 3.4). Although this modification appears to eliminate the problem, the T7 DNA polymerase enzyme will sometimes pause at regions of exceptional secondary structure when dITP is used. Thus, it is best to run dGTP reactions in parallel with the dITP reactions. Another approach to eliminate compressions is the use of gels containing formamide. This compound increases the denaturing capacity of the gel.
Figure 3.4: Inosine / Cytosine base pair. I / C base pair with two hydrogen bonds is a weaker interaction than the G / C base pair (3 hydrogen bonds) and as a result the DNA is more readily denatured in the gel.

Template and secondary structure problems

Template and secondary structure problems appear on the sequencing gel as bands at the same position in all four lanes. This can be due to the blocking of the synthesis of DNA by a loop structure or by contaminants in the template. Strong hairpin structures (inverted repeats) can impede the enzyme and cause "pauses" at these regions in the template. If the pausing is due to strong secondary structure and not due to an unclean template the addition of 0.5μg of SSB protein during the labelling reaction can be of benefit. SSB binds to the template and removes secondary structures that will otherwise impede the polymerase. SSB protein can be used on both single and double stranded templates. In order
to inactivate this protein, 0.1μg proteinase K must be added to the samples after the addition of the stop solution and the samples incubated for 20 minutes at 65°C. The samples must be heated for 2 minutes in a boiling water bath prior to loading onto the gel.

The use of pyrophosphatase for sequencing

It has been reported (Tabor and Richardson, 1987) that under certain situations, the intensities of some of the bands on the sequencing gel can be weak. This observation is particularly apparent when the reactions are run for over 30 minutes and when dITP is used. These weak bands are the result of pyrophosphorolysis. This is the reverse reaction of the reaction catalysed by DNA polymerase. This can be prevented by using the inorganic pyrophosphatase (Tabor and Richardson, 1990; Ruan et al., 1990). All known DNA polymerases catalise the template-dependent incorporation of deoxynucleotide onto the 3' hydroxyl terminus of a primer, with the release of inorganic pyrophosphate (PPi). Catalysis requires the presence of a divalent cation, preferably Mn++. Under the normal conditions of DNA synthesis, the forward reaction is greatly favored over the reverse reaction (Pyrophosphorolysis). Many DNA polymerases also catalyze the hydrolysis of nucleotides from the 3' terminus of DNA through a separate exonuclease activity. This reaction is unlike pyrophosphorolysis because the substrate is water and the product is a nucleoside monophosphate.
The polymerisation, pyrophosphorolysis and exonuclease reactions are summarised as follows:

\[
\begin{align*}
&M^{2+} \\
\text{DNA}_n + dNTP &\rightleftharpoons \text{DNA}_{n+1} + PP_i \quad \text{Polymerase} \\
&M^{2+} \\
\text{DNA}_n + dNTP &\rightleftharpoons \text{DNA}_{n+1} + PP_i \quad \text{Pyrophosphorylase} \\
&M^{2+} \\
\text{DNA}_n + H_2O &\rightleftharpoons \text{DNA}_{n-1} + dNMP \quad \text{Exonuclease}
\end{align*}
\]

(M\(^{2+} = Mg^{2+}\) or Mn\(^{2+}\))

Although pyrophosphorolysis is much slower as compared with polymerisation, it can affect the DNA sequencing reactions. Inorganic pyrophosphatase catalyzes the hydrolysis of pyrophosphate to two molecules of orthophosphate.

\[
P_2O_7^{4-} + H_2O \rightleftharpoons 2 \text{HPO}_4^{2-}
\]

The addition of this enzyme completely stabilizes all band intensities, even when incubating the reactions for 60 minutes and when using dITP in place of dGTP.

**Sequencing gel electrophoresis**

Polyacrylamide gels used for this purpose are prepared by the co-polymerisation of acrylamide and a cross-linker, N',N'-methylene-bis-acrylamide. A ratio of 19:1 acrylamide to bis-acrylamide was used. This ratio and the total concentration of acrylamide in the gel determine the pore size of the matrix and its sieving effect on the DNA molecules as they pass through it. The urea at a 8M concentration was used to help keep the DNA denatured. The addition of formamide to the gel (upto 40%) increases its
denaturing capacity. This can eliminate the compressions seen in G/C rich regions.

The Sequi-Gen cell (Figure 3.5) used in the experiments contained an upper buffer chamber (Integral Plate/Chamber). The IPC is a glass plate and buffer bonded together with a permanent adhesive at the side and bottom edges of the plate. This forms a thin, 0.5cm buffer chamber that extends over the entire area of the gel plate. A platinum electrode wire, strung across the bottom of the IPC, functions as the cathode. The spacers used were 0.4mm in thickness. The gel was 38cm in length. Saw tooth combs consisting of 16-24 wells were used in the runs. Electrophoresis was done at a voltage ranging from 1,100-1,500 volts so as to maintain a running temperature of 45-50°C. The time of the run varied from 3-6 hours. These extremely thin gels were very difficult to handle and required careful preparation and attention to detail as does every experiment.

The glass plates were cleaned thoroughly first in soap and water and then in isopropanol. The plates must be completely free of dust. Repeated wiping with isopropanol was done to achieve this. Clean plates and spacers are very important in order to pour gels without bubbles or leaks. The cleaned glass plates were kept horizontally on the bench and the clean dry spacers were rested on the long edges of the glass plate. Align the outer plates and spacers with the bottom edge of the inner glass plate so that the two plates are flush at the bottom.

It is important to have perfectly clean spacers and glass to make a water tight seal. They must be wiped just prior to assembly. Dust/adhesives under the spacers can be seen by rubbing the spacer
Figure 3.5: The sequi-Gen Cell used for DNA sequencing
Figure 3.6: The gel drying apparatus
onto the plate and looking for "wetting" patterns in the reflected light. The clamps are then slid over the plate assembly, one clamp at a time. The clamping should begin at one end of the plate, either the top or the bottom. When fully clamped a snapping sound can be heard. The fit must be reasonably tight to prevent any leakage. The casting gel solution was next made up fresh (see Chapter 2). The casting tray must be clean and dry. A cushion and a sealing strip were placed at the bottom of it with the paper on top. The casting tray sealing gel was prepared as follows:

- 5% Acrylamide gel solution: 10mL
- 25% Ammonium Persulfate: 70μL
- TEMED: 50μL

The sealing gel will polymerise in about 30 seconds, depending on the ambient temperature and the dissolved oxygen concentration. Thus, speed is important. Immediately, this solution was poured onto the entire sealing strip evenly. This should saturate it so as to prevent any leaks when the gel is being poured. As the gel is polymerising on the paper strip, the IPC assembly is placed firmly and quickly on it and the screws are tightened. Now the casting tray is firmly attached to the clamps. Now the gel solution can be seen to have moved in-between the two gel plates by capillary action across the full width of the gel. It will be allowed to set for about 2 minutes. At this time it must be verified that the sealing gel has polymerised by tipping the IPC assembly to both corners. If polymerised, it will not flow either way. All potential leaks around the bottom of the gel plates and the spacers are sealed in this way,
similar to a plug gel. The apparatus is now left in a almost horizontal plane to prevent it falling over.

The remaining 40ml of the casting gel mix is used to prepare the sequencing gel. To this, 52µl of the freshly prepared ammonium persulfate (1.3µl of APS per ml of gel) and 40µl of TEMED (1µl of TEMED per ml of gel) will be added. The mixture will take approximately 20 minutes to polymerise. The gel will be poured from the top. A 50ml syringe was used to do this holding the gel at an angle. The syringe will be placed so that the liquid enters the gel plates near one spacer. Tilt the gel assembly upwards and to the side so that the gel solution flows into the plate sandwich along the length of one spacer. This reduces the formation of air bubbles during filling. If bubbles are found at the end of pouring, the glass plate should be tapped gently to dislodge them. Once completed, a saw toothed comb is placed up side down 1cm into the short plate. This would leave a flat surface when the gel is set enabling the creation of wells by turning the comb the right side up.

Care must be taken to not spill the gel solution into the chamber during pouring mainly by controlling the rate of addition from the syringe. This can be acheived by placing paper towels in the chamber space during pouring. Once completed, the gel is left at a 5° inclination or less for atleast 3 hours before running. It must however be used before 20 hours from the time of pouring.

Once the gel is polymerised and ready to run, the casting tray is pulled out and the bottom of the gel cleaned. The comb is removed and the flat surface must first be washed in double distilled water to remove any unpolymerised acrylamide. The gel is now lowered
into the universal base between the alignment blocks and the stabiliser bar is inserted. The upper buffer chamber is now filled with 1X TBE (the electrode buffer) using the flared portion of the panel as a fill spout. The level of the buffer should be about 1/4" from the top of the fill spout at all times during the run. The lower chamber is filled with 300-350ml of the same 1X TBE buffer. The levelling screws must now be adjusted to ensure that the apparatus is horizontal.

The gel should be pre run at 1500 volts for approximately one hour without the comb at which time the flat upper surface should be washed with buffer to remove any urea prior to placing the comb (Adly Yakoub, Personal Communication). This is an added precaution to ensure that the samples will enter the gel evenly and will not be obstructed by the urea. Pre-running the gel at 45-50°C often results in better resolution. It is important to keep an eye on the buffer level in the upper chamber while the gel is heating up. The gel should never be allowed to heat up > 65°C which can damage the sequencing apparatus.

The sequenced samples must be denatured by heating in a boiling water bath for 2 minutes prior to loading. Longer times may cause a higher background. To prevent most nucleic acid secondary structure from reannealing, the samples are quench-cooled on ice soon after the high temperature incubation. The power supply is turned off and the upper safety cover is removed. The wells are once again rinsed using a pasteur pipette to remove any dissolved urea before applying the samples to the pre-heated gel. Usually 3-4μl of
any sample is sufficient. Once completed, the upper safety cover is reconnected and the power supply is turned on.

Power requirements for DNA sequencing gels are usually indicated by the gel running temperature. It is best to run at 50°C for the best resolution. The gel temperature indicator is used to monitor the temperature at all times. Periodically, the upper buffer level was checked to see that it is above the minimum level. The running time will depend mainly on the % of acrylamide and the number of bases that need to be read. Table 3.15 gives the running times for the various % of total acrylamide and other factors.

Table 3.15: Running times for denaturing polyacrylamide gels (Kambara et al., 1988).
Approximate hours to run to bottom of 45cm gel

<table>
<thead>
<tr>
<th>Bases</th>
<th>% Total Acrylamide</th>
<th>4%</th>
<th>5%</th>
<th>6%</th>
<th>8%</th>
<th>10%</th>
</tr>
</thead>
<tbody>
<tr>
<td>50</td>
<td></td>
<td>1.2</td>
<td>1.4</td>
<td>1.7</td>
<td>2.3</td>
<td>3.1</td>
</tr>
<tr>
<td>100</td>
<td></td>
<td>1.7</td>
<td>2.2</td>
<td>2.8</td>
<td>4.0</td>
<td>5.5</td>
</tr>
<tr>
<td>150</td>
<td></td>
<td>2.3</td>
<td>3.0</td>
<td>3.8</td>
<td>5.7</td>
<td>8.0</td>
</tr>
<tr>
<td>200</td>
<td></td>
<td>2.9</td>
<td>3.8</td>
<td>5.7</td>
<td>8.0</td>
<td>10.4</td>
</tr>
<tr>
<td>250</td>
<td></td>
<td>3.4</td>
<td>4.6</td>
<td>6.0</td>
<td>9.1</td>
<td>12.8</td>
</tr>
<tr>
<td>300</td>
<td></td>
<td>4.0</td>
<td>5.4</td>
<td>7.1</td>
<td>10.9</td>
<td>15.3</td>
</tr>
<tr>
<td>400</td>
<td></td>
<td>5.1</td>
<td>7.0</td>
<td>9.2</td>
<td>14.3</td>
<td>20.2</td>
</tr>
</tbody>
</table>

60
When the run is completed due to the dye front running off the gel or when it has travelled a sufficient distance the power supply is turned off. The upper buffer is poured into a collecting bottle, the clamps are removed and the outer glass plate is removed by prying gently at the top of the gel. The gel should stick to the siliconised plate only. The remaining plate and spacers are thoroughly cleaned with soap and water and drip dried.

Recently, the use of salt gradient gels have optimised the number of bases that can be read from a single-loading on a DNA sequencing gel (Sheen and Seed, 1988; Brummet, 1991). The difference here is in the constitution of the running buffers. The gel is set up for electrophoresis with 0.5X TBE in the upper chamber and 1X TBE in the lower chamber. At some time during the run the lower buffer is made 1M in sodium acetate by the addition of 1/2 volume of 3M sodium acetate, pH5. Electrophoresis now continues under these conditions. The higher electrolyte concentration in the lower reservoir causes a decrease in the voltage gradient in the lower portion of the gel resulting in a slower migration of the DNA molecules through the gel. This causes a compaction of the bands in the lower portion of the gel. The final result is a more even distribution of bands. The higher molecular weight bands are allowed to migrate further in to the gel increasing their resolution, without the loss of the lower molecular weight bands by migration out of the bottom of the gel.

With this method, 250-300 bases can be read. The time of electrophoresis prior to the addition of electrolyte determines the position of the band compaction in terms of distance from the
sequence priming site. Typically, under these conditions the gel is run for about an hour at 60 watts prior to the addition of the sodium acetate.

**Removal of hygroscopic urea from the sequencing gel**

The gel plate is transferred onto a tray and the urea removal solution (Methanol 100ml; Acetic acid 100ml in a total volume of 1 liter) is added to it. The gel is soaked in it for 20-30 minutes.

**Gel drying and autoradiography**

The gel is transferred to a sheet of whatmann 3mm paper pre-sprayed with distilled water. Lay the filter paper on top of the gel and gently rub it flat. Air bubbles must be got rid of. Now the gel can be picked up by carefully lifting the filter paper from one end. The gel is covered with a saran wrap and air bubbles are smoothed out by rubbing with a paper towel. The edges of the gel are trimmed out to fit the dryer (Figure 3.6). An hour of drying at 80°C is usually sufficient to dry a thin gel using a good vaccum. The dried gel is transferred to a cassette and exposed using a high speed x-ray film. Since 35S label is used, it is important to remove the saran wrap prior to exposure. Cassettes with intensifying screens are preferred.
Developing of x-ray films

Usually, overnight exposure is sufficient. Sometimes, it may be necessary to expose for longer periods of time to get better resolution. The film is immersed in a developer bath (under the red light) and rinsed for 5-7 minutes. Then it is carefully removed and inserted in the stop solution which is a 2% solution of acetic acid. Finally, it is rinsed in the fixer solution for a further 6-7 minutes. The film is now washed and the room lights can be switched on. The gel can now be read under a scanner or a viewing machine.

Incubation of M13mp18 single and double stranded DNA in serial dilutions in the presence and absence of chloroacetaldehyde

This experiment was designed to detect extremely low concentrations of DNA and to look for any enhancement of band intensity by the addition of chloroacetaldehyde (Tables 3.16 and 3.17). The temperature of incubation was 37°C. The time of incubation was 2 hours. At the end of the incubation, 5μl of the tracker dye was added to each tube and run on a 0.7% agarose gel.
Table 3.16: Incubation of single stranded M13mp18 DNA in the presence and absence of chloroacetaldehyde

<table>
<thead>
<tr>
<th></th>
<th>A</th>
<th>B</th>
<th>C</th>
<th>D</th>
<th>E</th>
</tr>
</thead>
<tbody>
<tr>
<td>M13 ss DNA (µl)</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>(µg weight)</td>
<td>(0.2)</td>
<td>(0.1)</td>
<td>(0.04)</td>
<td>(0.02)</td>
<td>(0.01)</td>
</tr>
<tr>
<td>NaAc, pH 4.9 (µl)</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>CAA (1:10) (µl)</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>DD Water (µl)</td>
<td>12*</td>
<td>12*</td>
<td>12*</td>
<td>12*</td>
<td>12*</td>
</tr>
<tr>
<td></td>
<td>15</td>
<td>15</td>
<td>15</td>
<td>15</td>
<td>15</td>
</tr>
</tbody>
</table>

* In the absence of CAA the volume of double distilled water is raised to 13µl to bring the total up to 15µl.
Table 3.17: Incubation of double stranded M13mp18 DNA in the presence and absence of chloroacetaldehyde

<table>
<thead>
<tr>
<th></th>
<th>A</th>
<th>B</th>
<th>C</th>
<th>D</th>
<th>E</th>
</tr>
</thead>
<tbody>
<tr>
<td>M13 ds DNA (µl)</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>(µg weight)</td>
<td>0.2</td>
<td>0.2</td>
<td>0.1</td>
<td>0.01</td>
<td>0.004</td>
</tr>
<tr>
<td>NaAc, pH 4.9 (µl)</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>CAA (1:10) (µl)</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>DD Water (µl)</td>
<td>12^</td>
<td>12^</td>
<td>12^</td>
<td>12^</td>
<td>12^</td>
</tr>
</tbody>
</table>

15 15 15 15 15

^ In the absence of CAA, the volume of double distilled water is raised to 13µl to bring the total upto 15µl.

Incubation of Hind III double stranded linear DNA in the presence and absence of chloroacetaldehyde

The previous two experiments were done using circular DNA. In this experiment, linear DNA was reacted with chloroacetaldehyde. The incubation was carried out at 55°C. Table 3.18 gives the composition of the incubation mixtures.
Table 3.18: Incubation of Hind III DNA in the presence and absence of chloroacetaldehyde

<table>
<thead>
<tr>
<th></th>
<th>A</th>
<th>B</th>
<th>C</th>
<th>D</th>
<th>E</th>
<th>F</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hind III DNA (μl)</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>(μg weight)</td>
<td>(0.5)</td>
<td>(0.25)</td>
<td>(0.05)</td>
<td>(0.025)</td>
<td>(0.001)</td>
<td>(0.0005)</td>
</tr>
<tr>
<td>NaAc, pH4.9</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>CAA (1:10) (μl)</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>DD Water (μl)</td>
<td>12*</td>
<td>12*</td>
<td>12*</td>
<td>12*</td>
<td>12*</td>
<td>12*</td>
</tr>
</tbody>
</table>

*In the absence of chloroacetaldehyde, the volume of double distilled water is raised to 13μl to bring the total up to 15μl.

Absorbance Spectroscopy
Using the Perkin-Elmer spectrophotometer, the absorbance of ethidium bromide was measured in both Tris-HCl buffer, pH 8 and distilled water. Further, the absorbance of chloroacetaldehyde (CAA) treated single stranded and double stranded DNA were measured and compared with the untreated DNA. Freshly prepared samples of etheno-AMP and etheno-CMP were also subjected to absorbance measurements and these in turn were compared with earlier spectra. The extinction coefficients for etheno AMP and etheno CMP at the different pH values are shown in table 3.19.
<table>
<thead>
<tr>
<th>Compound</th>
<th>pH</th>
<th>Wavelength (nm)</th>
<th>$e \times 10^{-3}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>etheno AMP</td>
<td>Neutral</td>
<td>300</td>
<td>2.6</td>
</tr>
<tr>
<td></td>
<td></td>
<td>275</td>
<td>6.2</td>
</tr>
<tr>
<td></td>
<td></td>
<td>265</td>
<td>5.9</td>
</tr>
<tr>
<td></td>
<td></td>
<td>258</td>
<td>4.7</td>
</tr>
<tr>
<td></td>
<td>.05N HCl</td>
<td>274</td>
<td>10.3</td>
</tr>
<tr>
<td>.05N NaOH</td>
<td>300</td>
<td>275</td>
<td>6.3</td>
</tr>
<tr>
<td></td>
<td></td>
<td>265</td>
<td>6.5</td>
</tr>
<tr>
<td></td>
<td></td>
<td>258</td>
<td>5.7</td>
</tr>
<tr>
<td>etheno CMP</td>
<td>Neutral</td>
<td>292</td>
<td>6.7</td>
</tr>
<tr>
<td></td>
<td></td>
<td>281</td>
<td>11.1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>272</td>
<td>11.7</td>
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<td></td>
<td>.05N HCl</td>
<td>302</td>
<td>7.0</td>
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<td></td>
<td></td>
<td>288</td>
<td>12.3</td>
</tr>
<tr>
<td></td>
<td></td>
<td>248</td>
<td>4.4</td>
</tr>
<tr>
<td>.05N NaOH</td>
<td>292</td>
<td>281</td>
<td>10.9</td>
</tr>
<tr>
<td></td>
<td></td>
<td>272</td>
<td>12.0</td>
</tr>
</tbody>
</table>
Fluorescence Spectroscopy

The appeal of this technique lies in its sensitivity. Further, fluorescence can be compared to a molecular stopwatch whereby molecular events can be monitored. Excitation and emission spectra of the following compounds or mixtures of compounds were measured using the SLM 8000C spectrofluorometer.

1. Etheno-AMP with / without ethidium bromide
2. Etheno-CMP with / without ethidium bromide
3. M13mp18 etheno single stranded DNA with / without ethidium bromide
4. M13mp18 single stranded DNA with / without ethidium bromide
5. M13mp18 etheno double stranded DNA with / without ethidium bromide
CHAPTER 4

RESULTS AND DISCUSSION

Calculation of the molecular weight of the DNA

Knowing the molecular weight of the DNA to be used in any particular experiment is of importance especially if that piece of DNA is to be sequenced. Running on an agarose gel with a ladder of known fragments is sufficient for this purpose. Figure 4.1 shows the gel that was used for this purpose and Figure 4.2 shows the graph that was used to calculate the molecular weight.
Figure 4.1: 0.7% Agarose horizontal gel containing DNA of unknown molecular weight.

Lane 1: Hind III Marker DNA (Ladder)
Lane 2: Uncut PS189 plasmid
Lane 3: Uncut PZ189 plasmid
Figure 4.2: Plot of the logarithm of the molecular weight versus the distance of migration of Hind III marker DNA. This plot can be used to estimate the molecular weight of an unknown piece of DNA.
From the above two figures, the molecular weight of the PZ189 plasmid was estimated to be 5.5 kilo bases. Reliability of such results depends on the accuracy of measurements and other statistical errors. However, it is fairly accurate for calculating the amount of primer to be used when the DNA is to be sequenced.

Identification of the various different forms of DNA

Figure 4.1 clearly shows the presence of various forms of the same piece of DNA. Double stranded plasmids such as PZ189 can exist in the supercoiled form when it migrates the greatest compared with the regular double stranded monomer (lane 3). The supercoiling enables the molecules to move farther due to their compactness. This explains the difference in migration of molecules with the same molecular weight. Often, these molecules are in the form of dimers and trimers which migrate much less due to their higher molecular weights. In the case of single stranded DNA, this phenomenon is not prominent due to the absence of supercoiling. In the experiments that follow, it was seen that the M13 control DNA migrated as a single band.

Restriction digest of PZ189 plasmid DNA

This was done to confirm that the various bands seen were indeed PZ189 and not an artefact. Whatever the form of the plasmid, it will get digested by ECoR I and as seen in figure 4.3 a single bright band replaces all the bands seen in the previous figure.
Figure 4.3: An ECoR I restriction digest of PZ189 plasmid DNA.

Lane 1: Hind III Marker DNA (Ladder)
Lane 2: PZ189 plasmid digested with ECoR I restriction enzyme
Lane 3: Uncut PZ189 plasmid
Figure 4.4: Incubation of M13mp18 single stranded DNA with chloroacetaldehyde for running on 0.7% agarose

Lane 1: Hind III Marker DNA (Ladder)
Lane 2: M13mp18 control DNA alone
Lane 3: M13mp18 control DNA reacted with 5μl CAA (1:100)
Lane 4: M13 control DNA reacted with 1μl CAA (1:100)
Lane 5: M13mp18 control DNA not reacted with any CAA
Lane 6: Same as lane 4 but without any M13mp18 control DNA
The reaction of DNA with bromoacetaldehyde in any concentration occurs selectively at N-1 and N-6 positions of unpaired adenines and at N-3 and N-4 positions of unpaired cytosines. The less reactive chloroacetaldehyde is expected to do the same. The post reaction M13 DNA is found to have a migration identical to the control DNA. This observation is extremely important for subsequent experiments involving sequencing procedures. If the DNA was altered or denatured to any extent it would not be sequenceable and thus it would not be possible to see any adducts.

Sequencing of M13mp18 single stranded DNA incubated with chloroacetaldehyde

The previous experiment paved the way for sequencing reactions which were done using different concentrations of the carcinogen. Three (3) clear bands were seen in the lanes treated with the chloroacetaldehyde which were absent in the lane containing M13mp18 DNA not treated with chloroacetaldehyde (Figure 4.5). The base sequences and the sites of reaction of these three 'hot spots' are shown below:

<table>
<thead>
<tr>
<th>Hot spot 1:</th>
<th>70  80  90  100</th>
</tr>
</thead>
<tbody>
<tr>
<td>TCA_GTC_CAT</td>
<td>AGC_GTT_TTC_C</td>
</tr>
<tr>
<td>TGT_GTA_AT</td>
<td>TG_TAC_GTT_GC</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Hot spot 2:</th>
<th>160  170  180  190</th>
</tr>
</thead>
<tbody>
<tr>
<td>GGT_GCT_TA_AT</td>
<td>G_ACGT_AGCT_A</td>
</tr>
<tr>
<td>ACT_CAC_ATTA</td>
<td>ATT_GCG_TTGC</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Hot spot 3:</th>
<th>210  220  230  240</th>
</tr>
</thead>
<tbody>
<tr>
<td>CG_CTT_TTC_AG</td>
<td>TCG_GAA_AAC_C</td>
</tr>
<tr>
<td>TGT_GTC_GCA</td>
<td>GCT_GCA_TAA</td>
</tr>
</tbody>
</table>
Figure 4.5: Part of a DNA sequencing gel showing the three (3) hot spots whose positions are shown previously when M13 DNA was reacted with chloroacetaldehyde.

<table>
<thead>
<tr>
<th>Lane Symbol</th>
<th>Contents</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>M13mp18 ss DNA + ddA</td>
</tr>
<tr>
<td>T</td>
<td>M13mp18 ss DNA + ddT</td>
</tr>
<tr>
<td>C</td>
<td>M13mp18 ss DNA + ddC</td>
</tr>
<tr>
<td>G</td>
<td>M13mp18 ss DNA + ddG</td>
</tr>
<tr>
<td>ddA</td>
<td>Same as lane 'A' + CAA (1:100 or 1:10)</td>
</tr>
<tr>
<td>ddG</td>
<td>Same as lane 'G' + CAA (1:100 or 1:10)</td>
</tr>
<tr>
<td>CAA</td>
<td>Only CAA (1:100 or 1:10). No dideoxy nucleotides</td>
</tr>
<tr>
<td>Water</td>
<td>No nucleotides, dideoxy nucleotides or CAA</td>
</tr>
</tbody>
</table>
In addition, several other bands are seen upstream which may be read accurately by running the gel for longer periods of time. Since heavier fragments travel far less compared to the lighter ones, they tend to be clustered together and difficult to define. The three bases involved in stopping the synthesis of DNA were thymine (81), guanine (165) and adenine (217). Perhaps, it is the local DNA structure and other conditions that determine the eligibility of the adenines and the cytosines to react under these conditions.

The three hot spots are close to each other spanning a length of 140 bases in a 7.3 kilobase molecule. Since this gel was readable to within 10 bases of the primer, it appears that these three positions are the principal places vulnerable to chloroacetaldehyde in this region after a two hour incubation. Larger fragments may light up on the gel due to these same three spots appearing in them. Several runs of this reaction indicated that these three positions were not random.

Effects of varying the concentration of salt on the ability to form adducts

In this experiment the identical hot spots seen in the previous experiment are seen in all lanes in varying intensities, except the last lane which had no chloroacetaldehyde. The differences were clearly seen in the sample containing 100mM sodium acetate, pH5.2. This sample had several other points of termination over and above the three regular ones at positions 82, 83, 84, 148 and 202. Figure 4.6 illustrates the effect of varying the salt concentration.
Of the unique stopping points, lane 2 has a high intensity band at a guanine base at position 148 which is not seen in any of the other lanes. From these data it appears that both purines and pyrimidines were responsible for chain termination.

**Incubation of nitroso compounds with M13 control DNA**

The methyl, ethyl and the propyl analogs of the N'-nitro-N-nitrosoguanidine reacted at the identical positions of the M13 DNA (Figure 4.7).
Figure 4.6: Part of a DNA sequencing gel showing the positions of reaction when M13mp18 ss DNA was reacted with CAA (1:100) in the presence of varying salt concentrations.

Lane 1: 50mM NaAc, pH 5.2
Lane 2: 100mM NaAc, pH 5.2
Lane 3: 1000mM NaAc, pH 5.2
Lane 4: Water only. No salt.
Lane 5: 50mM NaAc, pH 5.2 but no CAA
Figure 4.7: Part of a DNA sequencing gel showing the positions of reaction when M13mp18 single stranded DNA was reacted with the methyl, ethyl and the propyl analogs of N'-nitro-N-nitrosoguanidine

Lanes 1, 2 and 3: N-methyl-N'-nitro-N-nitrosoguanidine (Table 3.5)
Lanes 4, 5 and 6: N-ethyl-N'-nitro-N-nitrosoguanidine
Lanes 7, 8 and 9: N-propyl-N'-nitro-N-nitrosoguanidine
The three analogs reacted similarly with the M13 and the regions of reactivity are shown below:

Region 1

<table>
<thead>
<tr>
<th></th>
<th>20</th>
<th>30</th>
<th>40</th>
<th>50</th>
<th>60</th>
<th>70</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>OCTGCGGTC</td>
<td>GACTCTAGAG</td>
<td>GATCCGCGCC</td>
<td>TACGCCGCTC</td>
<td>GAATCGTAA</td>
<td>TCATGGTCAT</td>
</tr>
</tbody>
</table>

Region 2

<table>
<thead>
<tr>
<th></th>
<th>80</th>
<th>90</th>
<th>100</th>
<th>110</th>
<th>120</th>
<th>130</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>AGCTGTTCG</td>
<td>TGTTGAAAT</td>
<td>TGGATCCG</td>
<td>TCACAATTCC</td>
<td>ACAACACATA</td>
<td>CGAGCGGAA</td>
</tr>
</tbody>
</table>

Region 3

<table>
<thead>
<tr>
<th></th>
<th>140</th>
<th>150</th>
<th>160</th>
<th>170</th>
<th>180</th>
<th>190</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>GCATAAAGTG</td>
<td>TAAAGCCCTGG</td>
<td>GGTGCTAAT</td>
<td>GAGTGACCTA</td>
<td>ACTGACATTA</td>
<td>ATTCGGTTGC</td>
</tr>
</tbody>
</table>

It is seen that guanines and cytosines react more frequently with the nitroso compounds as compared with the other two bases. The fact that all three analogs react in the same positions indicate that it is the nitroso ring and not the side group that determines where adducts are formed. Further, regions rich in cytosines and guanines are more susceptible to adduct formation.

Incubation of M13 DNA with hydroxylamine hydrochloride and hydrazine hydrate

This incubation was done according to table 3.6. The bases at which adducts were formed are seen in figure 4.8.
Figure 4.8: Part of a sequencing gel showing the positions of adduct formation when M13mp18 single stranded DNA was reacted with hydroxylamine hydrochloride and hydrazine hydrate.

Lanes 1, 2 and 3: Hydroxylamine hydrochloride (Table 3.6)
Lanes 4, 5 and 6: Hydrazine hydrate (Table 3.6)
The regions of reaction of these two compounds were the same in both position and intensity and were as follows:

```
20  30  40  50  60  70
CCTGAGGTC GACGCTAGAG GATCCTGGGG TACGGAGCTC GAATTCGTAA TCATGGTCAT

80  90 100 110 120 130
AGCTGTTCCT GTGTTGAAAT TGTTATGCCC TCACAATTTCC ACACAAACATA CGACCCGCAA
```

**Incubation of M13 DNA with phenyl hydrazine and 2,4 dinitro phenyl hydrazine**

It is seen that the 2,4 dinitro phenyl hydrazine reacted with the DNA in three key sequences which were nearly identical to the those with the nitroso compounds.

```
80  90 100 110 120 130
AGCTGTTCCT GTGTTGAAAT TGTTATGCCC TCACAATTTCC ACACAAACATA CGACCCGCAA
```

**Incubation of M13 DNA with methyl methane sulfonate**

Figure 4.9 shows part of the sequencing gel indicating the points of chain termination in this incubation which was done according to table 3.8.
Figure 4.9: Part of a sequencing gel showing the reactions of M13 mp18 single stranded DNA with varying concentrations of methyl methanesulfonate (1mg %)

Lane 1: 1µl of 1:100 MMS solution (Stock concentration: 1mg %)
Lane 2: 2µl of 1:100 MMS solution
Lane 3: 5µl of 1:100 MMS solution
Lane 4: 10µl of 1:100 MMS solution
Reaction of methyl methane sulfonate produced two clear bands at position 81 (thymine) and position 97 (cytosine).

<table>
<thead>
<tr>
<th>80</th>
<th>90</th>
<th>100</th>
<th>110</th>
<th>120</th>
<th>130</th>
</tr>
</thead>
<tbody>
<tr>
<td>AGCTGTTC</td>
<td>TGTGTAAT</td>
<td>TGTTAGGC</td>
<td>TCACAATTC</td>
<td>ACAAACATA</td>
<td>CGAGCGGAA</td>
</tr>
</tbody>
</table>

**Incubation of chloroacetaldehyde with PZ189 plasmid DNA**

Reactions of single stranded DNA have an advantage over identical reactions with double stranded DNA in that most of the DNA in the former would be in the single stranded form making the bases available to attack by chemicals. It follows that provided the salt concentration and other factors are appropriate reactions may be seen where as with the PZ189 plasmid this was not the case. The incubations were done as per table 3.9.

Under the conditions used, no hot spots were observed. This can be due to a variety of causes.

(a). If the DNA was mostly in the double stranded form, it makes it difficult for chemicals to react with it and if the DNA is highly super coiled, this may indeed be the case.

(b). If the salt concentration is extremely high, this would neutralise most if not all of the negative charges in the DNA making the two strands come together. This would result in principally double stranded DNA.

(c). If the DNA being reacted is rich in G:C content, this would also affect the separation of the strands.
(d). The breathing pattern of the DNA and the time of exposure to the chemical are both important as in the process of breathing the DNA is single stranded and would be vulnerable to chemical provided the reaction is allowed to go on for a long enough period.

(e). The temperature of reaction too must be taken into consideration as higher temperatures usually increase the rate of reaction and can also cause the denaturation of DNA making it single stranded.

(f). According to the multi-stage model of carcinogenesis, each chemical recognises different bases/sequences for adduction and if these are present in the DNA being reacted an adduct may be formed provided the other conditions are favorable. It remains to be proved that this plasmid indeed has such bases/sequences for this chemical or any other.

From the data given here, it is clear that particular groups of a compound are important in adduct formation. Both bromo and chloroacetaldehyde hit the same bases, while the three nitroso compounds react at identical positions too.

Perhaps the local structure of the DNA at these vulnerable points is of importance in addition to the base sequences. This need not be a consequence of DNA breathing as shown by the M13 work. Salt concentration no doubt plays a key role here. DNA is normally negatively charged and repels other strands. With the addition of a salt which has positively charged ions there is neutralisation of these negative forces causing attraction of opposite strands and the hiding of potential hot spots. Further work needs to be done with
variations in temperature and increased concentrations of chemical to see what effect these have on adduct formation.

**Effect of chloroacetaldehyde on the sequenase™ version 2.0 enzyme**

When the experiment was carried out according to table 3.9, it was evident that the chloroacetaldehyde had an effect on the enzyme causing additional fall-offs. Since no such effect is seen in figure 4.5, it appears that all of the CAA was removed by the alcohol precipitation prior to sequencing.

**Restriction digestion of RF M13mp18 DNA with ECo 105I**

Figure 4.10 shows the presence of a single band in lane 4 indicating that the restriction enzyme cuts the DNA at only one site. The experiment was carried out according to table 3.12.
Figure 4.10: Restriction digest of M13 RF DNA using ECo 105I

Lane 1: Hind III Marker DNA (Ladder)
Lane 2: M13mp18 single stranded DNA
Lane 3: M13mp18 double stranded DNA
Lane 4: Restriction digested M13 RF DNA
Sequencing of M13mp18 RF DNA treated with Chloroacetaldehyde

This experiment was designed as a comparison to its single stranded counterpart. Here, a far larger number of bands were seen suggesting that the chloroacetaldehyde may be having a much larger effect of inhibiting the enzyme in the double stranded DNA (Figure 4.11). It is also possible that the secondary structure is greater here which may contribute to chain termination.
Figure 4.11: Sequencing gel of M13mp18 double stranded DNA reacted with chloroacetaldehyde

Lane A: M13mp18 RF DNA + ddA
Lane T: M13mp18 RF DNA + ddT
Lane C: M13mp18 RF DNA + ddC
Lane G: M13mp18 RF DNA + ddG
Lane 1: M13mp18 RF DNA + 5μl CAA (1:100)
Lane 2: M13mp18 RF DNA + 1μl CAA (1:10)
Lane 3: M13mp18 RF DNA + 5μl CAA (1:10)
Lane 4: M13mp18 RF DNA + Water. No CAA
Incubation of serial dilutions of M13mp18 single stranded DNA treated with chloroacetaldehyde for running on agarose

Figure 4.12 (A) shows the appearance and intensity of the bands when serial dilutions of DNA were treated with a constant amount of CAA (1µl of 1:100). Figure 4.13 (B) shows the control experiment where identical dilutions of the DNA were run in the absence of CAA. It is seen that the treating of the single stranded DNA with chloroacetaldehyde does not affect the intensity of the bands. Perhaps the nature of this DNA prevents any enhancement of the fluorescence of ethidium bromide when the CAA is added.
Figure 4.12 (A): Gel of Serial dilutions of DNA treated with CAA

Lane 1: 0.2μg M13mp18 ss DNA + 1μl of 1:10 CAA
Lane 2: 0.1μg M13mp18 ss DNA + 1μl of 1:10 CAA
Lane 3: 0.04μg M13mp18 ss DNA + 1μl of 1:10 CAA
Lane 4: 0.02μg M13mp18 ss DNA + 1μl of 1:10 CAA
Lane 5: 0.01μg M13mp18 ss DNA + 1μl of 1:10 CAA

Figure 4.13 (B): Control Experiment

The lanes have the corresponding amounts of DNA as in figure 4.12. No CAA was added.
Incubation of serial dilutions of M13mp18 double stranded DNA with chloroacetaldehyde for running on agarose

The concentrations of the DNA used here are the same in the presence and absence of chloroacetaldehyde. Figures 4.14 and 4.15 illustrate the results. It is clear that a 50-fold increase in the sensitivity is seen when the double stranded DNA is treated with chloroacetaldehyde. This finding is significant as the single stranded DNA does not show this amplification when treated with CAA. This may be a novel way of widening the difference between the single and the double stranded DNA. Further, it allows the detection of extremely low concentrations of DNA which would not have been possible earlier. It is possible that the chloroacetaldehyde enhances the fluorescence displayed by ethidium bromide. The mechanism of this is not clear. The fact that the single stranded DNA does not show this in fact points towards an energy transfer phenomenon. However, it was not possible to demonstrate this in the present group of experiments.
Figure 4.14 (A): Incubation of serial dilutions of double stranded M13mp18 DNA with CAA (1\(\mu\)l of 1:100)

Lane 1: 0.2\(\mu\)g M13mp18 ds DNA without any CAA
Lane 2: 0.2\(\mu\)g M13mp18 ds DNA + 1\(\mu\)l of 1:10 CAA
Lane 3: 0.1\(\mu\)g M13mp18 ds DNA + 1\(\mu\)l of 1:10 CAA
Lane 4: 0.01\(\mu\)g M13mp18 ds DNA + 1\(\mu\)l of 1:10 CAA
Lane 5: 0.004\(\mu\)g M13mp18 ds DNA + 1\(\mu\)l of 1:10 CAA

Figure 4.15 (B): Control experiment to figure 4.14 in the absence of chloroacetaldehyde (CAA)
Incubation of Hind III double stranded DNA treated with chloroacetaldehyde

The previous experiments led the way to the reaction between a piece of linear double stranded DNA with CAA. Here too there is an increase in the intensity of the fluorescence in the presence of chloroacetaldehyde (Figures 4.16 and 4.17). However, the enhancement seen is much less than with the M13 DNA. Perhaps the linearity of the DNA may make it difficult for energy transfer to take place. There may be secondary structure effects contributing to the problem. Changes in the conditions of the incubation such as raising the temperature, incubating for a longer period of time and lowering the salt concentration may give further enhancement. Further, using a more sensitive dye in lieu of ethidium bromide may be of use.
Figure 4.17 (B): Incubation of serial dilutions of Hind III DNA with CAA (1μl of 1:10 CAA)

Lane 1: 0.5μg Hind III DNA + 1μl of 1:10 CAA  
Lane 2: 0.25μg Hind III DNA + 1μl of 1:10 CAA  
Lane 3: 0.05μg Hind III DNA + 1μl of 1:10 CAA  
Lane 4: 0.025μg Hind III DNA + 1μl of 1:10 CAA  
Lane 5: 0.001μg Hind III DNA + 1μl of 1:10 CAA  
Lane 6: 0.0005μg Hind III DNA + 1μl of 1:10 CAA

Figure 4.16 (A): Control experiment to figure 4.17 in the absence of chloroacetaldehyde (CAA)
Effect of increasing the concentration of chloroacetaldehyde on the intensity of the ethidium bromide when identical amounts of DNA are used

The previous experiments indicated that the double stranded DNA when reacted with chloroacetaldehyde (CAA) gives enhanced fluorescence. In this experiment, the reverse was done where the concentration of DNA was kept constant while using increased amounts of chloroacetaldehyde. It was seen that this had a very profound effect on the illumination of the bands as seen in figure 4.18. This result can be compared with figure 4.4 where single stranded DNA was reacted with increasing concentrations of chloroacetaldehyde. In that experiment, no enhancement was seen in the fluorescence confirming the belief that this method may be useful to enhance the difference between single and double stranded DNA.

Treatment of M13mp18 double stranded DNA with S1 nuclease followed by ECo 105I digestion

This experiment was performed to examine the mobility of the single stranded regions of the double stranded form of M13mp18 DNA. As figure 4.19 shows, lane 4 contains a smear indicating that the single stranded region moves from place to place within the molecule. Since S1 nuclease only cuts at single stranded regions, it can do so virtually anywhere on the molecule since the said region is mobile. This explains the smearing pattern seen in lane 4.
Figure 4.18: Incubation of M13mp18 double stranded DNA with increasing concentrations of chloroacetaldehyde for running on agarose

Lane 1: 0.4μg M13mp18 ds DNA. No CAA
Lane 2: 0.05μg M13mp18 ds DNA + 1μl of 1:10 CAA
Lane 3: 0.05μg M13mp18 ds DNA + 5μl of 1:10 CAA
Lane 4: 0.05μg M13mp18 ds DNA + 10μl of 1:10 CAA

Figure 4.19: Treatment of M13mp18 double stranded DNA with S1 nuclease followed by ECo 105I restriction enzyme

Lane 1: Hind III Marker DNA (Ladder)
Lane 2: M13mp18 double stranded DNA
Lane 3: M13mp18 double stranded DNA treated with S1 nuclease
Lane 4: M13mp18 double stranded DNA treated with S1 nuclease followed by ECo 105I restriction enzyme

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Absorbance Spectroscopy

This is a useful method of studying the properties of biological molecules, particularly DNA whose single and double stranded populations have markedly differing absorption properties. It is seen that (Figure 4.20) single stranded DNA absorbs more light at every wavelength in the range in which it was measured. This is explained by the exposed nature of the single strands. By contrast, the bases in double stranded DNA are shielded accounting for the lower absorption. The rise in the absorption after adduction by CAA in both single and double stranded DNA clearly suggests that a chemical reaction has occurred between the two entities. Further, the more exposed nature of the single strands enables more reaction to take place with CAA accounting for the higher absorption after reaction.

Fluorescence Spectroscopy

The emission spectra of etheno-AMP (analogous to the compound formed when the adenines in the DNA react with chloroacetaldehyde) and that of DNA adducted with CAA are shown in figure 4.21. There is a clear overlap of the peaks indicative of the fact that the DNA reacts chemically with CAA to form etheno derivatives. In figure 4.22, it is seen that the emission spectrum is markedly higher throughout the measuring range of wavelengths with the reacted DNA giving further proof that there is a chemical reaction between the DNA and chloroacetaldehyde.
Figure 4.20: Absorption spectra of single and double stranded M13mp18 DNA before and after a 24 hour incubation with chloroacetaldehyde

$A_{300}^{ss} = 0.345$ (e ss DNA) and $0.201$ (e ds DNA)
Figure 4.21: Comparison of the emission fluorescence spectra of etheno-AMP and single stranded DNA adducted with chloroacetaldehyde (CAA)
Emission Spectra of etheno-AMP and Chloroacetaldehyde - Reacted DNA
Exciting at 300 nm
Figure 4.22: Emission spectra of chloroacetaldehyde reacted single stranded M13mp18 DNA compared with Chloroacetaldehyde and the single stranded DNA on their own
Emission Spectra of DNA, Chloroacetaldehyde, and Chloroacetaldehyde - Reacted DNA
Exciting at 300 nm

3.000E+03

Emission

0.000E+00

Labellable DNA

Chloroacetaldehyde

DNA

EM WL (tice 50/10 nm)
Monitoring of the reaction between chloroacetaldehyde and M13mp18 single stranded DNA using absorption spectroscopy

This reaction was followed at various time intervals and it was seen that at time $= 0$, the DNA had a typical spectrum which changed upon the addition of CAA (Figures 4.23 and 4.24). With time, the absorption at 320nm increased steadily for over 120 hours (Figures 4.25 through 4.29). This is further proof that the CAA reacts with DNA chemically.

The increase in absorbance observed was plotted against time and it was seen that after 160 hours of incubation, the absorbance had reached a plateau and was seen to level off (Figure 4.30).
Figures 4.23: Absorption spectrum of M13mp18 single stranded DNA
Figure 4.24: Absorption spectrum of M13mp18 single stranded DNA immediately after the addition of CAA
SPEC 2

M1Jmp18 SS DNA w/ CHLOROACETALDEHYDE

T = 0 HOURS

Absorbance (AU)

Wavelength (nm)
Figures 4.25 through 4.27:

Absorption spectra of M13mp18 single stranded DNA in the presence of chloroacetaldehyde at 24, 48 and 120 hours of incubation
Figure 4.28: Plot of absorbance versus length of incubation when M13mp18 single stranded DNA was reacted with chloroacetaldehyde
CHAPTER 5

CONCLUSIONS

The work described has answered some questions while raising many others. It is appropriate to discuss the results in this context.

The purpose of this study was to develop an assay where by sites of adduct formation could be identified directly by modifications in the DNA sequencing methodology described by Sanger. In this work using the viral M13mp18 DNA (as a representative DNA of known sequence), the sequencing was done following two hour incubations of the DNA with the appropriate chemical in the presence of 50mM sodium acetate, pH5.2. At that point, a handful of sites were identified when the first 300 bases were sequenced. Later work involving absorbance and fluorescence spectroscopy showed that the absorbance steadily increased with time prior to reaching a plateau state. From this data, it appeared that no more than 3% of the total adductable sites were in fact adducted in the first 2 hours of incubation. Further, while the positions of adducts had some overlap there appeared to be a unique pattern of adduction for each of the different classes of compounds used to react with the M13mp18 DNA.

These observations indicate that a chemical reaction occurs between chloroacetaldehyde and the DNA and that absorbance and fluorescence spectroscopy can be used to monitor these reactions. The reaction is not random and specific sites of reaction principally involving adenine and cytosine were observed.
The difference in the fluorescence exhibited by single and double stranded M13mp18 DNA with chloroacetaldehyde may be used as a means of enhancing the difference between the two populations of DNA. The fluorescence of the ethidium bromide is enhanced when double stranded DNA is reacted with chloroacetaldehyde while the single stranded DNA produces the same weak signal in the presence and the absence of the chemical. This finding may have commercial applications where the double stranded DNA can be identified by enhanced fluorescence in the presence of polynucleotides which do not show such enhancement.
APPENDIX 1

PHOTOGRAPHING AN AGAROSE GEL UNDER ULTRA-VIOLET (UV) LIGHT

**Loading the film (with room lights on)**

1. The handle on the film cassette should be on the "loading (L)" position.
2. Slip film into the cassette by placing fingers on the edge of the film and pushing the film until a click is heard. The film used is the Polaroid type 55.
3. Slip the film cassette into the setup by slipping it in between and under the two black side rods.

**Setting up the camera (with room lights off, safe light on)**

1. Focus on the plastic ruler by turning the left hand knob. Use the spotlight to assist viewing. Check that the camera is set at f stop 4.5, B setting.
2. Place gel on the UV illuminator glass plate and remove air bubbles. Arrange gel so that it is in the middle of the field.
3. Move camera set by pushing the grey button and cassette across to the left so that the film is now over the focuser and it clips into place.
To take the picture

1. Pull out the film strip tab until the film stops.
2. Press the shutter button and hold for 20 seconds.
3. Push the tab at the end of the film packet back into the cassette.

Processing of the negative and print (room lights on)

1. Push the grey button (on left) to slide the camera set up to the original position. Remove the film cassette and push the silver handle to "P" for processing.
2. Pull out the film in one smooth motion. Wait for 20 seconds and then open the packet by peeling away from the negative and print.
3. Develop the negative in a solution of 30% sodium thiosulfate solution until it is clear. Next, rinse the negative in water. Store the used solution in a dark brown bottle after the negative has been developed.
4. To preserve the print, wipe with sponge provided by Kodak.
APPENDIX 2

PARTIAL SEQUENCE OF M13MP18
SINGLE STRANDED DNA (7.3 KB)

The partial sequence beginning at the priming site is given below (Yanisch-Perron, Vieira and Messing, 1985).

<p>| | | | | | | | | | | | | | | |</p>
<table>
<thead>
<tr>
<th></th>
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<th></th>
<th></th>
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APPENDIX 3

THE USE OF INTENSIFYING SCREENS FOR VISUALIZING RADIOACTIVE MOLECULES RESOLVED BY GEL ELECTROPHORESIS

There are two principal problems encountered in this type of work as follows:

(a) Low energy beta particles emitted by $^{35}\text{S}$ are quenched within the gels. Conversion of the emitted energy to visible light by an organic scintillator (PPO) infused into the gel increases the penetration range and thus increases the proportion of the emitted energy that is absorbed by the adjacent X-ray film.

(b) High energy beta particles emitted by $^{32}\text{P}$ pass through and the film in the form of visible light by placing a high density fluorescent "intensifying screen" beyond the film.

For maximum efficiency in the detection of the isotopes and for quantitation of the images obtained, it is necessary to bypass the reversible first stage of latent image formation in the film. This is most easily achieved by pre-exposing the film to an instantaneous flash of light for $< 1$ millisecond (Laskey, 1980).
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